

1. Submitted to Philippine Journal of Science (30-1-2023)
  - Correspondence
  - Document
  - Cover Letter
  - List Reviewer
  - Authorship Statement
2. First Revision: Format Revision Based on Philippine Journal of Science (28-4-2023)
  - Correspondence
  - Document
  - Cover Letter
  - List Reviewer
  - Authorship Statement
3. Status Manuscript: Information about the manuscript (8-7-2023)
  - Correspondence
4. Second Revision: Major Revision (13-10-2023)
  - Correspondence
  - Review Note
  - Document
5. Third Revision: Major Revision (15-1-2024)
  - Correspondence
  - Review Note
  - Document
6. Final Decision (17-4-2024)
  - Correspondence
  - Decision Letter
  - Document
7. Paper Accepted (15-6-2024)
  - Correspondence
  - Copyediting Process
  - Document

1. Submitted to Philippine Journal of Science (30-1-2023)
  - Correspondence
  - Document
  - Cover Letter
  - List Reviewer
  - Authorship Statement



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**(no subject)**

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <philjournsci@gmail.com>

Wed, Apr 26, 2023 at 11:08 AM

Dear Dr. CAESAR A. SALOMA  
Editor-in-Chief in PJS

Sincerely,

I am interested in publishing my manuscript in PJS so that I send my manuscript with the title " Effect of Steeping Temperature and Storage Time on the Bioactive Compounds, Antioxidant and Antidiabetic Activities of *Pluchea Indica* Less Tea" . I also send a cover letter, list reviewer recommendation and form an authorship statement to be considered

Thanks for attention

Regards

Paini Sri Widyawati


---

**4 attachments**

 **COVER LETER.pdf**  
68K

 **[PRO] Form - Authorship Statement3.pdf**  
86K

 **LIST REVIEWER RECOMMENDATION.pdf**  
149K

 **Effect of Brewing Temperature and Storage Time on Antioxidant and Antidiabetic Properties of Pluchea Tea Final.docx**  
92K

## **COVER LETTER**

Indonesia, April 26<sup>th</sup> 2023

Dear the Editorial Board of the PJS

Greetings,

I, the undersigned below:

Name :

1. Dr. Painsi Sri Widyawati, S.Si., M.Si. Email : [painsi@ukwms.ac.id](mailto:painsi@ukwms.ac.id)
2. Yufita Ratnasari Wilianto, M.Farm.Klinis, Apt. Email : [yufitaratnasari@ukwms.ac.id](mailto:yufitaratnasari@ukwms.ac.id)

Institution : 1. Food Technology Department, Agricultural Technology Faculty, Widya Mandala Surabaya Catholic University, Indonesia  
2. Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic University, Indonesia

has submitted a manuscript with the title “**Effect of Steeping Temperature and Storage Time on the Bioactive Compounds, Antioxidant and Antidiabetic Activities of *Pluchea Indica* Less Tea**” in the PJS for publication. This manuscript is the result of collaborative research between lecturers in the Food Technology Study Program, Faculty of Agricultural Technology and Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic University, Indonesia which has the criteria of originality, merit, scientific novelty, and significance. Currently the manuscript in part or whole is not under consideration for publication in other journals.

Thank you for your attention

Sincerely,



Dr. Painsi Sri Widyawati, S.Si., M.Si.

## PJS Authorship Statement



### PHILIPPINE JOURNAL OF SCIENCE

Publisher: Department of Science and Technology

Editorial Office: Science and Technology Information Institute (STII)

DOST Compd., Gen. Santos Ave., Bicutan, Taguig, Metro Manila, Philippines

Email: [philjournsci@gmail.com](mailto:philjournsci@gmail.com) Telefax No. (+63 2) 837-2195, 837-7520

### AUTHORSHIP STATEMENT

#### **Effect of Steeping Temperature and Storage Time on the Bioactive Compounds, Antioxidant and Antidiabetic Activities of *Pluchea Indica* Less Tea**

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Philippine Journal of Science. Each author also agrees that this paper is currently not under consideration in any other journals elsewhere in the world upon submission to PJS. Lastly, each author hereby validates his/her consent regarding the submission and publication (in its final form) of such manuscript bearing his/her full name.

#### **Authorship contributions**

Please indicate the specific contributions made by each author. The name of each author (e.g., J.J. De La Cruz) must appear at least once in each of the three categories below.

#### **Category I**

Conception and design of study: \_Paini Sri Widyawati, Yufita Ratnasari Wilianto

Acquisition of data: Paini Sri Widyawati

Analysis and/or interpretation of data: Paini Sri Widyawati, Yufita Ratnasari Wilianto

#### **Category II**

Drafting the manuscript: Paini Sri Widyawati

Revising the manuscript for significant intellectual content: Paini Sri Widyawati, Yufita Ratnasari Wilianto

#### **Category III**

Approval of the version of the manuscript to be published (the names of all authors must be listed): Paini Sri Widyawati, Yufita Ratnasari Wilianto

## PJS Authorship Statement

### Acknowledgments

All persons who have made substantial contributions to the work reported in the manuscript (e.g., technical help, writing and editing assistance, general support) but who do not meet the criteria for authorship are named in the Acknowledgements and have given their written permission to be named.

This statement is signed by all the authors:

Author's name (typed)

Author's signature

Date

Paini Sri Widyawati



April 26<sup>th</sup> 2023

Yufita Ratsanasari Wilianto



April 26<sup>th</sup> 2023

**LIST OF REVIEWER RECOMMENDATION**

<b>No.</b>	<b>Reviewer's Name</b>	<b>Institution</b>	<b>Email</b>
1.	Derya Arslan	Department of Food Engineering, Faculty of Engineering and Architecture, Necmettin Erbakan University, Koyceğiz Campus, Meram, Konya 42080, Turkey	dears@erbakan.edu.tr
2.	Patricia Carloni	Agricultural, Food and Environmental Sciences – D3A, Università Politecnica delle Marche, Via Brecce Bianche, I-60131 Ancona, Italy	p.carloni@univpm.it
3.	Panagiotis Kafelas	Department of Nutrition–Dietetics, Harokopio University, El Venizelou 70, Kallithea 17671, Athens, Greece.	panos@maich.gr
4.	Kiki Febrianto	Brawijaya Senso-Gastronomy Center, Departemant Agricultural Product Technology, Faculty of Agricultural Technology, Brawijaya University, Malang, Indonesia.	kiki.fibrianto@ub.ac.id
5.	Nikmatul Ikhrom Eka Jayani	Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Surabaya, 60293 Kalirungkut, Surabaya, Indonesia	nikmatul.ikhrom@staff.ubaya.ac.id

1 [ **Effect of Steeping Temperature and Storage Time on the Bioactive Compounds,**  
2 **Antioxidant and Antidiabetic Activities of *Pluchea Indica* Less Tea**

3 Painsi Sri Widyawati<sup>1\*)</sup>, Yufita Ratnasari Wilianto<sup>2)</sup>

4 <sup>1)</sup>Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala  
5 Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

6 <sup>2)</sup>Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic  
7 University, Kalisari Street Number 1, Surabaya 60272, Indonesia

8 Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea*  
9 *indica* Less, storage time

10

11

12

13

14

15

16

17

18

19



## 20 ABSTRACT

21 This study was done to determine effect of steeping temperature and storage time on the  
22 bioactive compounds, antioxidant and antidiabetic activities of *Pluchea indica* Less tea  
23 infusion. The research used a randomized block design with two factors, i.e., steeping  
24 temperature (60, 70, 80, and 95°C) and storage time (0 and 5 years). The steeping  
25 temperature and storage time influenced the bioactive compounds, antioxidant and  
26 antidiabetic activities of samples. Total phenolic content and total tannin content went up  
27 along with increased antioxidant activity. Treatment resulted simple phenolic compounds,  
28 such as gallic acids, (+)-catechins, kaempferols, myricetins, quercetins, 3,4-di-O-  
29 caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, 4,5-di-O-caffeoylquinic acids. Total  
30 flavonoid content was decreased for storage time and significant different at higher  
31 steeping temperatures. The total flavonoid content had graph pattern similar with  $\alpha$ -  
32 amylase and  $\alpha$ -glucosidase inhibition activities. This means, the antidiabetic activity was  
33 largely determined by the total flavonoid content and structure of phenolic compounds. In  
34 order, to get high antioxidant activity, it was chosen pluchea tea stored at high steeping  
35 temperature, but high antidiabetic activity was fresh pluchea tea steeped at a low  
36 temperature.

37

## 38 INTRODUCTION

39 Pluchea tea is a product of pluchea leaf processing introduced by world people (Srisook  
40 et al., 2012; Widyawati et al., 2016) because of the efficacy of the active components in  
41 pluchea leaves, as an herbal plant that has been widely used for traditional medicine and  
42 food (Chan et al., 2022). Pluchea tea is composed many nutrients and bioactive

43 compounds useful to body health. The nutrient compositions in the pluchea tea include  
44 protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates, calcium,  $\beta$ -carotene, and  
45 vitamin C, whereas bioactive compounds is comprised, i.e., chlorogenic acid, caffeic acid,  
46 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 3,4-di-O-  
47 caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, quercetin,  
48 myricetin, kaempferol, total anthocyanin,  $\beta$ -carotene, and total carotenoid (Suriyaphan,  
49 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et al., 2022, Chan et al., 2022).

50 Steeping process of pluchea tea leaves can be performed with fresh or dry leaves  
51 infusion by hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et al.,  
52 2020; Jayani et al., 2022). In Asian area, especially in Indonesian, people usually  
53 consume the pluchea tea with brewing of powdered pluchea leaves in tea bag by hot  
54 water or boiling water. Each tea bag contained 2 g of pluchea leaf powder is steeped with  
55 100 mL hot water or boiling water. Widyawati et al. (2016) claimed that steeping of 2 g  
56 pluchea tea at 95°C for 5 minutes results total phenolic content, total flavonoid content,  
57 the ability to scavenge DPPH free radicals, and the capability of reduce ferric ions 9.3 mg  
58 gallic acid equivalent (GAE)/g samples, 22.0 mg gallic acid equivalent (GAE)/g samples,  
59 27.2 mg gallic acid equivalent (GAE)/g samples, and 10.2 mg gallic acid equivalent  
60 (GAE)/g samples, respectively. Werdani and Widyawati (2018) reported that drinking of  
61 pluchea tea in the morning and evening regularly (2 g/100 mL) can decline blood sugar  
62 levels.

63 Steeping pluchea tea with hot water at 95°C for 5 minutes certainly determines the  
64 stability and amount of extracted bioactive compounds, that influences the biological  
65 activity, especially antioxidant and antidiabetic activities. Silva-Ramirez et al. (2020)

66 reported that the infusion process can influence their content and composition of the  
67 bioactive compounds and antioxidant activity of tea. Acar et al. (2022) informed that  
68 infusion quality of herb tea extract depends on several factors, i.e., time and temperature.  
69 Polyphenol profile and antioxidant properties of herb tea infusion decline with an increase  
70 in steeping/brewing and storage temperatures and longer exposure times.

71 Several studies have mentioned the effect of steeping temperature to bioactive  
72 compounds and antioxidant activity, such as some white and green teas are effective with  
73 hot water at 90°C for 7 min (Castiglioni et al., 2015), roseship tea is effectively at infusion  
74 time around 6-8 min at temperatures of 84-86°C (Ilyasoglu and Arpa, 2017), the coffee  
75 brewing temperature influences the caffeine content extracted (Zarwinda and Sartika,  
76 2018), the steeping of dark tea at 92°C for 27 min results the highest total phenol content  
77 and antioxidant activity (Wang et al., 2022). The study of the effect of steeping  
78 temperature to pluchea tea infusion was carried out to afford information about  
79 preparation of pluchea tea most efficiently to get higher the bioactive compounds,  
80 antioxidant and antidiabetic activities.

81 On the other hand, storage time of pluchea tea also affects the levels of the  
82 bioactive compounds and biological activity because this tea usually is stored for a  
83 several months until years (Jayani et al., 2022). Tea or herbal tea is generally stored in  
84 ambient temperature and packed in tea bag or Alu foil standing proud or a combination  
85 of both. Many researchers informed that storage time decreases the bioactive  
86 compounds, antioxidant and antidiabetic activities, i.e., juice from *Momordica charantia* L.  
87 (Lin et al., 2020), dried *Piper betlle* extracts (Ali et al., 2018), white tea (Xu et al., 2019),  
88 kinnow-amlam beverages (Purewal et al., 2022), whole wheat flour (Zhang et al., 2021).

89 Therefore, this research studied effect of steeping temperature and storage time on the  
90 bioactive compounds, antioxidant and antidiabetic properties of pluchea tea. The study  
91 was emphasized to determine total phenolic content, total flavonoid content, total tannin  
92 content, scavenging activity of DPPH free radical, ferric reducing power,  $\alpha$ -amylase and  
93  $\alpha$ -glucosidase inhibition activities, and phenolic compound profile.

94

## 95 MATERIALS AND METHODS

### 96 MATERIALS

97 The pluchea leaves were collected from gardens in Mangrove areas, Wonorejo,  
98 Surabaya, Indonesia. The pluchea plants were included in Asteraceae family with  
99 specification according to the GBIF taxon ID number database:3132728. Then, the  
100 material was treated based on Widyawati et al. (2022) method. The pluchea tea packed  
101 in tea bag (2 g/tea bag) was steeped with hot water temperatures of 60, 70, 80, and 95°C  
102 for 5 min and storage times of 0 (control) and 5 years (stored) with infusion method. Then,  
103 the samples preparation was done based on Widyawati et al. (2016) and Widyawati et al.  
104 (2022) methods.

105

### 106 REAGENTS

107 The compounds used to analyze including 2,2-diphenyl-1-picrylhydrazyl (DPPH),  
108 sodium carbonate, gallic acid,  $\alpha$ -amylase,  $\alpha$ -glucosidase, pNPG (p-nitrophenyl- $\alpha$ -  
109 glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-O-caffeoylquinic  
110 acid, 4,5-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and (+)-catechin were  
111 purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, Folin–Ciocalteu’s Phenol,

112 sodium nitric, aluminum chloride, ferric chloride, sodium dihydrogen phosphate, sodium  
113 phosphate, potassium ferricyanide, starch, acetic acid, and sodium hydroxide were  
114 purchased from Merck (Kenilworth, NJ, USA). Aquadest and aquabidest were purchased  
115 by PT Aqua Surabaya.

116

## 117 METHODOLOGY

118

### 119 TOTAL PHENOLIC CONTENT ANALYSIS

120 Total phenolic content of steeping pluchea tea was conducted by Gao et al. (2019)  
121 method based on spectrophotometric analysis. Total phenolic content assay using redox  
122 analysis between phenolic compounds and phosphomolybdic /phosphotungstic acid  
123 complexes is founded on the electron transfer in an alkaline medium from the phenolic  
124 compounds to result a blue colored solution because of phosphotungstic/  
125 phosphomolybdenum complex formation. Total phenolic content was measured by  
126 Spectrophotometer (spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  760 nm and  
127 a reference standard was a gallic acid. The results were expressed as mg gallic acid  
128 equivalents (GAE)/g samples.

129

### 130 TOTAL FLAVONOID CONTENT ASSAY

131 Total flavonoid content of the samples was determined by the spectrophotometric  
132 method based on the reaction between  $AlCl_3$  and  $NaNO_2$  with an aromatic ring of  
133 flavonoid compounds, especially flavonol and flavon (Shraim et al., 2021). The reaction  
134 between  $AlCl_3$  and flavonoid compounds resulted a yellow solution. Then, the red solution

135 was produced after NaOH solution addition that was measured by a spectrophotometer  
136 (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  510 nm. A (+) catechin was  
137 used as a reference standard compound, and the results were expressed as mg catechin  
138 equivalents (CE)/g samples.

139

#### 140 TOTAL TANNIN CONTENT ANALYSIS

141 Total tannin content of the samples was analyzed by Folin-Ciocalteu method  
142 based on Chandran and Indira (2016). The reaction between the samples and reagents  
143 obtained blue dark color solution that measured by a spectrophotometer  
144 (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  760 nm. This analysis used a  
145 tannic acid as a reference standard and was expressed as mg tannic acid equivalents  
146 (TAE) /g samples.

147

#### 148 DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

149 The DPPH free radical scavenging activity was measured by the  
150 spectrophotometric method (Widyawati et al., 2017) to determine AA of the brewing of  
151 pluchea tea to donor hydrogen atom to nitrogen atom in DPPH resulting DPPH-H  
152 compound with a yellow-colored solution. The reaction between the DPPH in methanol  
153 solution with the samples was measured by a spectrophotometer (Spectrophotometer  
154 UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  517 nm. The reference standard compound was  
155 gallic acid and the results of analysis were expressed as mg gallic acid equivalents  
156 (GAE)/g samples.

157

## 158 FERRIC REDUCING POWER ANALYSIS

159 Ferric reducing power was determined by Widyawati et al. (2014) method. Potency  
160 of the steeping pluchea tea reducing iron (III) to iron (II) ion was analyzed by  
161 spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  700 nm.  
162 The reducing capacity of antioxidant compounds of the steeping pluchea tea increased  
163 related to intensity of blue color solution. The bigger of reducing power, the higher of blue  
164 color intensity. The reference standard used as gallic acid, and the results were  
165 expressed as mg gallic acid equivalent (GAE)/g samples.

166

## 167 $\alpha$ -AMYLASE ENZYME INHIBITION CAPACITY ASSAY

168 In vitro inhibition of  $\alpha$ -amylase enzyme was determined by Widyawati et al. (2020)  
169 method. Samples of steeping pluchea tea at various steeping temperatures and storage  
170 times were analyzed by spectrophotometer UV-Vis (Spectrophotometer UV-Vis-1800,  
171 Shimadzu, Japan) based on reaction between bioactive compounds and  $\alpha$ -amylase  
172 enzyme. Then, the residue enzyme was reacted with starch and the capacity of the  $\alpha$ -  
173 amylase enzyme hydrolyzed the starch to release glucose that could be analyzed based  
174 on absorbance at  $\lambda$  540 nm. The inhibition percentage of  $\alpha$ -amylase was assessed by  
175 the following formula:  $(ACb - ACa) - (As - Ab) / (ACb - ACa) \times 100\%$ . Where, ACb was  
176 absorbance of 100% enzyme activity (only solvent with enzyme), ACa was absorbance  
177 of 0% enzyme activity (only solvent without enzyme), As was absorbance of tested  
178 sample with enzyme, Ab was absorbance of tested sample without enzyme.

179

180

## 181 $\alpha$ -GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

182 The analysis of the  $\alpha$ -glycosidase inhibitor activity was done by Widyawati et al.  
183 (2020) method with slight modification. The samples were reacted with the  $\alpha$ -glycosidase  
184 enzyme, and then the residue of this enzyme hydrolyzed p-nitrophenyl- $\alpha$ -D-  
185 glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The inhibitor activity of  
186 steeping pluchea tea to enzyme was measured by spectrophotometer UV-Vis  
187 (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at  $\lambda$  405 nm. The inhibition  
188 percentage of  $\alpha$ -glycosidase was assessed by the following formula:  $(ACb - ACa) - (As$   
189  $- Ab) / (ACb - ACa) \times 100\%$  Where, ACb was absorbance of 100% enzyme activity (only  
190 solvent with enzyme), ACa was absorbance of 0% enzyme activity (only solvent without  
191 enzyme), As was absorbance of tested sample with enzyme, Ab was absorbance of  
192 tested sample without enzyme.

193

## 194 HPLC ANALYSIS OF PHENOLICS

195 The phenolic compounds of samples were analyzed by HPLC based on  
196 Kongkiatpaiboon et al. (2018) method with modification. HPLC separation was achieved  
197 on LC20AD series (Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence  
198 UFLC LC-20AD pump, SIL-20 AC<sub>HT</sub> autosampler, CTO-10AS VP column oven, CBM-20A  
199 system controller, and SPD-40 detector. The separation was done in a Shim-pack VP-  
200 ODS C18 column (5 $\mu$ m  $\times$  50 mm  $\times$  4.6 mm I.D.) with a GVP-ODS Cartridges (2pcs) guard  
201 column (10 mm  $\times$  4.6 mm I.D.). The mobile phases were (A) 0.5% acetic acid in water  
202 and (B) methanol using gradient elution: 10% B in A to 50% B in A for 40 min; 100% B  
203 for 20 min. This column was re-equilibrated with 10% B in A for 10 min prior to each



204 analysis and the flow rate was set at 1.0 ml/min with the controlled temperature at 40°C.  
205 SPD-40 detector was set at the wavelength of 280 nm and injection volume was 20 µL  
206 for every sample and reference standard.

207

## 208 EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

209 The research design used a randomized block design with two factors, i.e., the  
210 brewing temperature (B) and the storage time (L). The steeping temperature of pluchea  
211 tea consisted of four treatment levels, including 60°C (B1), 70°C (B2), 80°C (B3), and  
212 95°C (B4), and the storage time of pluchea tea was composed two levels, i.e., 0 year  
213 /fresh (L0), and 5 year/stored (L2). Each treatment was repeated six times in order to  
214 obtain 48 experiment units. The HPLC analysis of phenolic was repeated two times. The  
215 data of samples were analyzed by ANOVA at  $p \leq 5\%$ , and continued by DMRT (Duncan  
216 Multiple Range Test) at  $p \leq 5\%$ . Data were expressed as the mean  $\pm$  SD. The analysis  
217 used SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

218

## 219 RESULTS AND DISCUSSIONS

220 Pluchea leaf tea is produced by young pluchea leaf from 1-6 level on each branch  
221 the shoot (Widyawati et al., 2022), that is steeped at 95°C for 5 min, has many biological  
222 activities, such as antioxidant activity (Widyawati et al., 2016), antidiabetic activity  
223 (Werdani and Widyawati, 2018), anti-inflammatory (Srisook et al., 2015). The chemical  
224 constituents in pluchea tea involve alkaloids, flavonoids, phenolics, sterols, cardiac  
225 glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2 g/100 mL  
226 steeping pluchea tea has total phenolic content 9.3 mg gallic acid equivalents (GAE)/g

227 samples, total flavonoid content 22.0 mg catechin equivalents (CE)/g samples, DPPH  
228 free radical scavenging activity 27.2 mg gallic acid equivalents (GAE)/g samples, and  
229 ferric reducing power 10.2 mg gallic acid equivalents (GAE)/g samples (Widyawati et al.,  
230 2016). Previous research has informed related to the composition of phytochemical  
231 compounds in pluchea leaves, such as phenolic acids such as chlorogenic acids, caffeic  
232 acids, 3-O-caffeoylquinic acids, 4-O-caffeoylquinic acids, 5-O-caffeoylquinic acids, 3,4-  
233 di-O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic  
234 acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin;  $\beta$ -  
235 carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al.,  
236 2019; Chan et al., 2022; Widyawati et al., 2022). Presence of phytochemical compounds  
237 in herbal product were influenced by environmental factors, i.e., temperature, light  
238 exposure, oxygen level, pH and moisture. The structure of phytochemical compounds in  
239 herbal tea is very sensitive of the surrounding changes. The effect arising from these  
240 changes causes the structure of the phytochemical molecule to be degraded to produce  
241 smaller size molecules or to combine to produce larger size molecules (Ali et al., 2018;  
242 Jayani et al. 2022, Ramphinwa et al., 2023). Therefore, this study emphasized the effect  
243 of steeping temperature and storage time of pluchea tea on levels of the bioactive  
244 compounds, antioxidant and antidiabetic properties and phenolic compound profile.

245

## 246 BIOACTIVE COMPOUNDS

247 The bioactive compounds are active compounds in plants that are essential to  
248 protect a body health (Nguyen and Chuyen, 2020). These compounds usually have many  
249 biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer,

250 antimicrobial, antibacterial, anti-cholesterol and so on (Suriyaphan, 2014; Acar et al.,  
251 2022). Phenolic compounds are potential bioactive compounds in plants, that have  
252 responsible redox properties to scavenge free radicals as cause with a number of chronic  
253 diseases (Noreen et al., 2017; Arya t al., 2019; Acar et al., 2022).

254         The steeping temperature (60, 70, 80 and 95°C) and storage time (fresh and  
255 stored) determined total phenolic content, with values ranging from 4.39±0.49 to  
256 71.38±4.14 mg GAE/g samples. The total phenolic content of pluchea tea infused at  
257 different temperature and stored at different time that statistical analyzed by ANOVA at  $\alpha$   
258  $\leq 5\%$  shown at Figure 1. The total phenolic content of samples was significantly influenced  
259 by the steeping temperature and storage time. The highest total phenolic content was  
260 detected in the L95 sample infused at 95°C and stored for 5 years (71.38±4.14 mg GAE/g  
261 samples) with was followed by L80 sample infused at 80°C and stored for 5 years  
262 (62.60±2.49 mg GAE/g samples) and L70 sample infused at 70°C and stored for 5 years  
263 (60.68±3.79 mg GAE/g samples) and L60 sample infused at 60°C and stored for 5 years  
264 (46.67±5.38 mg GAE/g samples). The total phenolic contents of steeping fresh pluchea  
265 tea (B60) had a lower total phenolic content (4.39±0.48 mg GAE/g samples) than the  
266 steeping stored pluchea tea for 5 years (48.67±5.38 until 71.38±4.14 mg GAE/g samples).  
267 Fresh pluchea tea had a lower total phenolic content than stored pluchea tea for 5 years,  
268 besides that the higher the steeping temperature also caused the greater the extracted  
269 total phenolic content. The temperature of infusion influenced total phenolic content, it  
270 could relate to migration process of phenolic compounds to the water because of  
271 increasing contact between this compounds and water. The same phenomena also  
272 occurred in Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al. (2022).

273 This occurrence showed that steeping temperature and storage time caused the  
274 process of degradation and oxidation of phenolic compounds. Su et al. (2019) reported  
275 that temperature treatment can stimulate the release of phenolic compounds and  
276 increase antioxidant activity of lychee juice stored at different temperatures of 4 and 45°C  
277 and different storage times (fresh and 72 hours). Hydrogen bonding is affected by  
278 temperature treatment because the hydrogen bond between phenolic compounds and  
279 proteins can be degraded that the measured levels of phenolic compounds are higher.  
280 The phenomena were supported by Ali et al. (2018); Jayani et al. (2022) and Ramphinwa  
281 et al. (2023). Zhang et al. (2021) reported that phenolic compounds present in plants are  
282 not completely stable, but are easily degraded during storage after harvest. Reblova  
283 (2012) claimed that antioxidant compounds can be slowly degraded with increasing  
284 temperature. Besides that, Fibrianto et al. (2021) also stated that the brewing  
285 temperature has an effect on the extracted antioxidant compounds, such as alkaloids,  
286 catechins and tannins. Thus, there is an assumption that the phenolic compounds in  
287 pluchea tea are degraded due to oxidation and hydrolysis because of temperature and  
288 storage time and can be easily extracted during brewing, thus increasing the phenolic  
289 content as the steeping temperature and storage time increase.

290 Based on using of a reference standard could be informed that phenolic  
291 compounds in steeping pluchea tea, including gallic acids, (+)-catechins, myricetins,  
292 quercetins, kaempferols, 3,4-di-O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and  
293 4,5-di-O-caffeoylquinic acids was showed in Table 1. Gallic acids and (+)-catechins were  
294 relative stable phenolic acid because of very small changes at different steeping  
295 temperature and storage time with concentration about  $0.21 \pm 0.00 - 0.24 \pm 0.02$  µg/g

296 samples and  $0.32 \pm 0.02$  –  $0.60 \pm 0.04$   $\mu\text{g/g}$  samples, respectively. However, myricetin,  
297 quercetin and 3,4-di-O-caffeoylquinic acid showed drastic increasing at higher steeping  
298 temperature and longer storage time. It's meant that these compounds tended relatively  
299 labile. Kaempferol, 3,5-di-O-caffeoylquinic acid and 4,5-di-O-caffeoylquinic acid  
300 underwent moderate changes compared to the other two groups of phenolic acids.  
301 Therefore myricetin, quercetin and 3,4-di-O-caffeoylquinic acid were easier to dissolve at  
302 higher steeping temperature and storage time can cause macromolecules of three  
303 phenolic acids in herbal tea convenient degradable to form simple phenolic compounds  
304 for storage, as explained by Su et al. (2019), Ali et al. (2018); Jayani et al. (2022);  
305 Ramphinwa et al. (2023), and Zhang et al. (2021). Degradable polyphenol compounds  
306 have a simple structure and free hydroxyl groups that can react with Folin-Ciocalteu's  
307 Phenol reagent, resulting complex blue solution that can detected as total phenolic  
308 content.

309 Flavonoids are the major phenolic compounds having potential as chemical and  
310 biological activities, especially as radical scavenging and antimicrobial activities (Ayele et  
311 al., 2022; Chandra et al., 2014). These compounds are the bioactive compounds that can  
312 protect the human body from the oxidative stress caused many degenerative diseases,  
313 especially cancer, cardiovascular problems and ageing (Mathur and Vijayvergia, 2017).  
314 Total flavonoid content analysis for pluchea tea at various steeping temperatures and  
315 storage times were showed in Figure 1. The total flavonoids of steeping pluchea tea  
316 decreased with increasing storage time, but increased with increasing brewing  
317 temperature. The highest total flavonoid content was owned by fresh pluchea tea which  
318 was brewed at  $95^\circ\text{C}$  ( $147.42 \pm 14.03$  mg CE/g samples) and the lowest was owned by

319 pluchea tea which had been stored for 5 years at various brewing temperatures (between  
320 24.75±2.47-33.71±3.06 mg CE/g samples). Statistical analysis by ANOVA analysis at  
321  $\alpha \leq 5\%$  proven that brewing temperature and storage time of fresh pluchea tea had a  
322 significant effect on the total flavonoid content, but the stored pluchea tea (L) had no  
323 significant effect. Storage time had a significant effect on the total flavonoid content of  
324 brewing pluchea tea. Ali et al. (2018) reported that the degradation of bioactive  
325 compounds can take place through several stages, such as pre-treatment, processing,  
326 and storage, as is the case with medicinal plants which are dried, extracted and stored in  
327 the long term. Brewing temperature and storage time have an influence on the oxidation  
328 and polymerization processes that are stimulated by light. According to Noree et al.  
329 (2017), that the total flavonoid content test with  $\text{AlCl}_3$  and  $\text{NaNO}_2$  reagents measures  
330 flavone compounds, these compounds have activity due to the presence of a free  
331 hydroxyl functional group at position 4' in the compound. Degradation of flavone  
332 compounds due to temperature and storage causes the breaking of methylation bonds.  
333 Kim et al. (2020) also confirmed, that the total phenolic content and total flavonoid content  
334 of matcha are decreased with increasing brewing temperature and storage time. Xu et al.  
335 (2019) informed, that storage time can give a big impact on chemical composition  
336 changes with trending not the same.

337         The tannins have a various type of compounds are water-soluble polyphenols that  
338 are current in many plant foods and have a number of effects on health (Balaky et al.,  
339 2021). Tannins are bioactive compounds that provide properties, such as astringent, anti-  
340 diarrheal, antibacterial and antioxidant (Malangngi et al., 2012). Data analysis showed,  
341 that the total tannin content of brewing pluchea tea increased with increasing brewing

342 temperature and storage time, as seen in Figure 1. Steeping pluchea tea contained  
343 tannins ranging from  $4.81 \pm 0.58$ - $17.42 \pm 1.04$  (mg TAE/g samples). The tannin content  
344 increased with increasing storage time and brewing temperature. The results of the  
345 ANOVA statistical analysis at  $\alpha \leq 5\%$ , showed a significant increase in tannin content levels  
346 with increasing brewing temperature and storage time. The fresh pluchea tea brewed at  
347  $60^\circ\text{C}$  had the lowest tannin content level, was  $4.81 \pm 0.58$  mg TAE/g samples. The stored  
348 pluchea tea brewed at  $95^\circ\text{C}$  had the highest tannin content level, was  $17.42 \pm 1.04$  mg  
349 TAE/g samples. The results showed, that the higher the brewing temperature and the  
350 longer the storage time caused the tannin compound polymerization process to occur. Ali  
351 et al. (2018) said that pH, storage temperature, chemical structure and concentration,  
352 light, oxygen, enzymes and metal ions affect the presence of bioactive compounds in the  
353 material. Rusita et al. (2019) emphasized that tannins are a polar compound, that is  
354 resistant to heating, as a result the tannin content in pluchea tea increases with increasing  
355 brewing temperature and storage time, this is caused tannins are thermostable complex  
356 compounds.

357

## 358 ANTIOXIDANT ACTIVITY

359 Antioxidant activity is capability of compounds to inhibit the oxidation of  
360 macromolecules from biological target that involve in oxidative chain reactions (Ali et al.,  
361 2005; Oh et al., 2013). In the research, the antioxidant activity assay used was DPPH  
362 Free Radical Scavenging Activity (DPPH) and ferric reducing power (FRAP), Ali et al.  
363 (2005) and Huang et al. (2005) informed that phenolic compounds have antioxidant

364 activity because of their redox properties, such as hydrogen atom donor, electron transfer,  
365 reducing agent, and singlet oxygen quenchers.

366 DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate  
367 antioxidant activity because this method is very simple that is suitable to measure the  
368 donating hydrogen atoms capability of herbal tea. This reaction can cause the purple color  
369 of DPPH reduced to be yellow color (Munteanu and Apetrei, 2021; Baliyan et al., 2022).  
370 The result of DPPH assay in pluchea tea was showed in Figure 2. The DPPH values  
371 accrued with higher steeping temperature and longer storage time. Statistical analysis by  
372 ANOVA at  $\alpha \leq 5\%$  proven that the higher the steeping temperature of fresh pluchea tea  
373 (B60-B95) was consistent the ability to DPPH free radicals scavenging activity, whereas  
374 the stored pluchea tea resulted the higher activity and the values went up as rising of the  
375 infusion temperature. Pluchea tea storage at room temperature for 5 years resulted the  
376 DPPH free radical scavenging activity by more than 100%. The steeping at higher  
377 temperatures could significantly increase the DPPH free radical scavenging activity in  
378 stored pluchea tea around 15-25%. Brewing at 80-95°C in stored pluchea tea  
379 insignificantly affected this antioxidant activity. Scavenging activity of DPPH free radicals  
380 was correlated with total phenolic and tannin levels, but inversely to total flavonoid levels.  
381 The phenomenon of the DPPH values in pluchea tea is contrary with the results of the  
382 study by Lin et al. (2020). However, this study was in accordance with Thanajiruschaya  
383 et al. (2010), claimed that during the storage process it is possible to form complex  
384 phenolic compounds which provide a high ability to scavenge DPPH free radicals. This  
385 research also demonstrated that longer storage time and higher infusion temperature  
386 produced many simple phenolic compounds with free hydroxyl groups capable to donor



387 hydrogen atom to DPPH free radical. Many phenolic acids, such as gallic acids, (+)-  
388 catechins, myricetins, kaempferols, quercetins, 3,5-di-O-caffeoylquinic acids, 3,4-di-O-  
389 caffeoylquinic acids, 4,5-di-O-caffeoylquinic acids have established potential antioxidant  
390 activity (Kumar and Goel, 2019).

391 FRAP is method that identifies the antioxidant capacity of the phytochemical  
392 component through measured absorbance, as a result of the reaction among antioxidant  
393 compounds, potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a  
394 color complex, that can be measured at  $\lambda$  700 nm (Fu et al., 2011; Al-Temimi and  
395 Choudhary, 2013). The principle of testing the ability to reduce iron ions is that  
396 antioxidants can reduce potassium ferrocyanide ( $\text{Fe}^{3+}$ ) to potassium ferrocyanide ( $\text{Fe}^{2+}$ ).  
397 Potassium ferrocyanide reacts with ferric chloride to form a ferric-ferrous complex and  
398 results green color solution (Widyawati et al., 2017).

399 The data showed, that the FRAP of pluchea tea became significantly different with  
400 going up brewing temperature and storage time (Figure 1). The FRAP value increased  
401 with higher steeping temperature and longer storage time, the lowest FRAP value was  
402 owned by pluchea tea which was brewed at 60°C at  $3.95 \pm 0.17$  mg gallic acid equivalents  
403 (GAE)/g samples, and the highest was owned by pluchea tea which was stored for 5  
404 years at  $48.63 \pm 10.83$  mg gallic acid equivalents (GAE)/g samples. FRAP of the pluchea  
405 was significant correlated with the DPPH free radical scavenging activity, total phenolic  
406 and tannin contents. This case was contrast to the antioxidant activity of DPPH and FRAP  
407 on matcha, because the longer storage time reduces the levels of catechin content (Kim  
408 et al. 2020), and also the case of the effect of temperature and storage time in betel (*Piper*  
409 *bettle* L.) extract (Ali et al., 2018). Thanajiruschaya et al. (2010) revealed that the

410 antioxidant activity of rice stored at high temperatures is greater than that stored at low  
411 temperatures. The ferric reducing capability of pluchea tea infusion corresponded to  
412 simple phenolic acid values, presence of them in samples could accrue antioxidant  
413 activity because of ability of the electron transfer from free hydroxyl groups of phenolic  
414 acids.

415

#### 416 ANTIDIABETIC ACTIVITY

417 Antidiabetic activity is potency of phenolic compounds to revise glucose uptake or  
418 keep away blood glucose go up.  $\alpha$ -amylase and  $\alpha$ -glucosidase are digestive enzymes  
419 which involve to control dietary carbohydrate and increase in postprandial blood glucose  
420 in human body (Fu et al., 2017). The phenolic compounds proven having the capability  
421 to bind protein that they can inhibit  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Hardoko et  
422 al., 2019; Martinez-Solis et al., 2022). Previous research of Werdani and Widyawati  
423 (2018), claimed that pluchea tea infusion is potential as antidiabetic agents. This  
424 observation test is based on the breakdown ability of the substrate to produce a colored  
425 product, which is measured at  $\lambda = 540$  nm. The results showed, that the steeping pluchea  
426 tea was able to inhibit the action of the  $\alpha$ -amylase enzymes (Figure 3). The pluchea tea  
427 infusion had very good activity, more than 50% and even almost 100% for fresh pluchea  
428 tea which was brewed at 60, 70 and 80°C and stored pluchea tea which steeped at 60°C.  
429 Whereas fresh pluchea tea brewed at 95°C for 5 minutes had an activity of inhibiting the  
430 alpha amylase enzyme of less than 50%, which was equal to  $40.08 \pm 1.12\%$ . Widyawati et  
431 al. (2017) detected the ability to inhibit the  $\alpha$ -amylase enzyme from fresh pluchea tea  
432 brewed at 95°C for 5 minutes by 28.79%. Increasing the brewing temperature and storage

433 time reduced the ability to inhibit the  $\alpha$ -amylase enzyme. The results of the analysis based  
434 on the ANOVA statistical test at  $\alpha \leq 5\%$  showed, that the brewing temperature and storage  
435 time had a significant effect on the ability to inhibit the  $\alpha$ -amylase enzyme. This ability was  
436 inversely proportional to the levels of total phenolic content, total tannin content, DPPH,  
437 and FRAP. This inhibitory activity was thought to be contributed by other bioactive  
438 compounds, besides phenolics which are sensitive to brewing temperature and storage  
439 time. Li et al. (2018) stated that there are flavonoid compounds that contribute to the  
440 ability to inhibit the  $\alpha$ -amylase enzyme. Flavonoid compounds with a hydroxyl structure  
441 at C-4' in ring B are more effective than C-6 in ring A. Akah et al. (2011) informed that the  
442 phytochemical compounds, such as terpenoids, saponins, flavonoids, glycosides and  
443 carbohydrate, and alkaloids are good antidiabetic metabolites. Sangeetha and Vedaşree  
444 (2012) explained, that the ability to inhibit the  $\alpha$ -amylase enzyme was determined by the  
445 content of the phenolic compound and protein. The  $\alpha$ -amylase inhibitor present in pluchea  
446 tea may be proteinaceous in nature. Aleixandre et al. (2022) informed that phenolic acids  
447 have inhibition activity to  $\alpha$ -amylase enzyme depending their structures. Besides that,  
448 capability of phenolic acids to inhibit  $\alpha$ -amylase was determined by low half-maximum  
449 inhibitory concentration ( $IC_{50}$ ). There are C=C double bond conjugated with a carbonyl  
450 group of phenolic structures that stabilizes the binding forces to the active site of the  $\alpha$ -  
451 amylase. The hydroxyl groups of them are able to bind by non-covalent interaction, such  
452 as hydrogen binding, cation- $\pi$  interactions, salt bridge interactions, ionic interactions or  
453 electrostatic forces with amino acid residue at the active site in  $\alpha$ -amylase. The steeping  
454 temperature and storage time can remove hydroxyl groups of phenolic compounds that

455 can reduce the ability of enzyme inhibition. The phenolic acids with a greater number of  
456 hydroxyl groups are stronger capable to obstruct the  $\alpha$ -amylase enzyme.

457  $\alpha$ -glucosidase is an important enzyme in carbohydrates digestion, that catalysis  
458 the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and converts  
459 them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et al., 2014;  
460 Proenca et al., 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -glucosidase  
461 enzyme is used to determine antidiabetics activity. This is supported by Werdani and  
462 Widyawati (2018), that pluchea tea infusion has the potential as an antidiabetic agent.  
463 Widyawati et al. (2020) found that brewing fresh pluchea tea at 95°C for 5 minutes has  
464 an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857%.

465 The results showed, that the ability to inhibit the  $\alpha$ -glucosidase enzyme decreased  
466 with increasing brewing temperature and storage time. Brewing at 95°C for fresh pluchea  
467 tea (0 days of storage) obtained the lowest inhibitory ability, i.e.,  $48.32 \pm 1.27\%$ , and the  
468 highest inhibitory activity was found at 70°C brewing temperature for fresh pluchea tea,  
469 which was  $95.11 \pm 0.70\%$  (Figure 3). The test results showed that the ability to inhibit the  
470 enzyme  $\alpha$ -glucosidase tended to be higher than the ability to inhibit the enzyme  $\alpha$ -  
471 amylase. Li et al. (2018) informed that flavonoid compounds have the ability to inhibit the  
472 action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. This is due to the total flavonoids in  
473 brewing pluchea tea which tended to have the same pattern as the ability to inhibit the  
474 activity of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. Flavonoid compounds, such as  
475 rutin, myricetin, kaempferol, and quercetin which have antioxidant and antihyperglycemic  
476 activities. The ability to inhibit the action of enzymes from flavonoid compounds is  
477 determined by the position and number of hydroxyl groups and the number of double

478 bonds in rings A and B and the heterocyclic ring in ring C. The ability to inhibit the  $\alpha$ -  
479 glucosidase enzyme from pluchea tea was significantly affected by the brewing  
480 temperature and storage time. The capability of pluchea tea infusion to obstruct the  $\alpha$ -  
481 glucosidase enzyme was greater than the  $\alpha$ -amylase enzyme because the mechanism  
482 of two enzymes was different, according to the opinion of McCue et al. (2005). Widyawati  
483 et al. (2017) informed that phenolic and non-phenolic compounds determine the inhibitory  
484 activity of the  $\alpha$ -glucosidase enzyme. The ability of bound phenolic compounds to inhibit  
485  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. The presence of  
486 polymerization and degradation reactions, that may be occurred in pluchea tea during  
487 storage, affects the structure and profile of phenolic and non-phenolic compounds.  
488 Asriningtyas et al. (2014) claimed that pluchea leaves contain 3,5-di-*O*-caffeoylquinic  
489 acid, 4,5-di-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid methyl  
490 ester, 3,4,5-tri-*O*-caffeoylquinic acid, and 1,3,4,5-tetra-*O*-caffeoylquinic acid. Quinic acid  
491 is methyl esterified with the number of caffeic groups in the molecule that determines the  
492 activity of inhibiting the  $\alpha$ -glucosidase enzyme. Analysis of caffeoylquinic acids in pluchea  
493 tea infusion was obtained that the higher steeping temperature and longer storage time  
494 caused increased concentration of them, but the  $\alpha$ -glucosidase inhibition of them was  
495 reduced. Aleixandre et al. (2022) reported that the simple phenolic acids forming a dipole-  
496 dipole interaction of active site from  $\alpha$ -glucosidase enzyme are effectively inhibiting the  
497 enzyme.

498 This study was obtained information that the increasing of steeping temperature  
499 and storage time caused a degradation reaction of polyphenol compounds to produce  
500 simple phenolic compounds, such as gallic acid, (+)-catechin, myricetin, quercetin,

501 kaempferol, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-  
502 caffeoylquinic acid, supported the results of total phenolic content and total tannin content  
503 assays. Increased concentration of simple phenolic compounds determined the ability of  
504 these compounds as antioxidant agents, but reduced their capability as antidiabetic  
505 agents.

506

## 507 CONCLUSION

508 The steeping temperature and storage time of pluchea tea determined antioxidant  
509 and antidiabetic activities. Profile of phenolic compounds of pluchea tea infusion  
510 influenced antioxidant and antidiabetic activities. Storage time and brewing temperature  
511 caused degradation reaction of polyphenols that resulted simple phenolic compounds.  
512 Gallic acid, (+)-catechin, quercetin, myricetin, kaempferol, 3,4-di-O-caffeoylquinic acid,  
513 3,5-di-O-caffeoylquinic acid and 4,5-di-O-caffeoylquinic acid were simple phenolic  
514 compounds detected from steeping pluchea tea. Increasing of them determined  
515 antioxidant activity that correlated to total phenolic content and total tannin content. Total  
516 flavonoid content had a decreasing graph pattern with increasing storage time that was  
517 similar to the antidiabetic activity graph pattern, which means that the antidiabetic activity  
518 of pluchea tea depended on the total flavonoid content and the structural complexity of  
519 the phenolic compounds.

520

## 521 DATA AVAILABILITY

522 Table and figure used to support of this study were included in the article.

523

524 CONFLICT OF INTEREST

525 The authors declare no conflict of interest.

526

527 ACKNOWLEDGEMENTS

528 The authors would like to thank the he Ministry of Education and Culture of the Republic  
529 of Indonesia for fundamental research grant to higher education institutions in 2022

530

531 REFERENCES

532 ACAR A, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food  
533 wastes:evaluation of temperature and time effects on quality parameters. Applied Food  
534 Res. 2(100087):1-8.

535 ALEIXANDRE A et al. 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -  
536 glucosidase and influence of reaction conditions. Food Chem. 372(131231):1-9.

537 ALI et al. 2005. Tea and herbal infusuions: their antioxidant activity and phenolic profile.  
538 Food Chem. 89(2005):27-36.

539 ALI A. et al. 2018. Impact of storage conditions on the stability of predominant phenolic  
540 constituents and antioxidant activity of dried *piper bettle* extracts. Molecules.23(24):1-13.

541 AL-TEMIMI A, CHOUDHARY R. 2013. Determination of antioxidant activity in different  
542 kinds of plants *in vivo* and *in vitro* by using diverse technical methods. J Nut. Food Sci.  
543 3(1):1-6.

544 AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and  
545 methanol extract and fractions of *Gongronema latifolium* (*Asclepidaceae*) leaves in  
546 alloxan diabetic rats. J Applied Pharm. Sci. 01(09): 99-102.

547 ARSININGTYAS IS. et al. 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves  
548 of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product  
549 use. *Natural Product Res.* 28(17):1350-1353.

550 ARYAL S et al. 2019. Total phenolic content, flavonoid content and antioxidant of wild  
551 vegetables from Western Nepal. *Plants.* 8(96): 1-12.

552 AYELE DT, AKELE ML, MELESE A.T. 2022. Analysis of total phenolic contents,  
553 flavonoids, antioxidant and antibacterial activities of *Croton macrostachyus* root extracts.  
554 *BMC Chem.* 16(30): 1-9.

555 BALAKY HH et al. 2021. Estimation of total tannin and total phenolic content in plant  
556 (*Crataegus azarolus* L) by orbital shaker technique. *Intern. J. Agric. Environ. Food Sci.*  
557 5(1):1-6.

558 BALIYAN S et al. 2022. Determination of antioxidants by DPPH radical scavenging  
559 activity and quantitative phytochemical analysis of *Ficus religiosa*. *Molecules.* 27(1326):  
560 1-19.

561 CASTIGLIONI S et al. 2015. Influence of steeping conditions (time, temperature, and  
562 particle size) on antioxidant properties and sensory attributes of some white and green  
563 teas. *Intern. J. Food Sci. Nut. Online* 1-7.

564 Chan EWC et al. 2022. *Pluchea indica*: An updated review of its botany, uses, bioactive  
565 compounds and pharmacological properties. *Pharmaceutical Sciences Asia.* 49(1): 77-  
566 85 (2022)

567 CHANDRA S et al. 2014. Assessment of total phenolic and flavonoid content, antioxidant  
568 properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit



569 crops: A Comparative study. Hindawi Publishing Corporation Evidence-Based  
570 Complementary Alter. Med. 2014:1-9 (2014)

571 CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic, flavonoids,  
572 tannin and chlorophyll content of leaves of *Strobilanthes Kunthiana (Neelakurinji)*. J.Med.  
573 Plants Studies. 4(4):282-286.

574 FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional  
575 properties and visual appearance of Dampit Robusta coffee leaves tea. Advances in Food  
576 Sci. Sustain. Agric. Agro-industrial Engineering. 4(1):25-32.

577 FU L et al. 2017. Total phenolic contents and antioxidant capacities of herbal and tea  
578 infusions. Intern. J. Molecular Sci. 12:2112-2124.

579 GAO MR et al. 2019. A theoretical and experimental study: the influence of different  
580 standards on the determination of total phenol content in the Folin–Ciocalteu assay. J.  
581 Food Measurement and Characteristic. 13:1349–1356.

582 HUANG D, OU B, PRIOR RL. 2005. The Chemistry behind antioxidant capacity assays.  
583 J. Agric. Food Chem. 53:1841-1856.

584 ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties  
585 of rosehip tea beverage: study by response surface methodology. J. Food Sci. Tech.  
586 54(11):3737–3743.

587 JAYANI NIE et al. 2022. Evaluation of stability and quality characteristics of moringa  
588 (*Moringa oleifera*) herbal tea during storage. Food Res. 6(4):399-406.

589 KIM JM et al. 2020. Effect of storage temperature on the antioxidant activity and catechins  
590 stability of Matcha (*Camellia sinensis*). Food Science and Biotechnology. 29(9):1261–  
591 1271.

592 KILIC et al. 2017. Antioxidant properties of some herbal teas (green tea, senna, corn silk,  
593 rosmery) brewed at different temperatures. Intern. J. Secondary Metabolite. 4(3):142-248  
594 Special Issue 1.

595 KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of  
596 extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves  
597 from different provenances in Thailand. Revista Brasileira de Farmacognosia. 28(2018):  
598 145–150.

599 KUMAR N, GOEL N. 2019. Phenolic acids: natural versatile molecules with promising  
600 therapeutic applications. Biotechnology Reports. 24(e00370):1-10.

601 LI K et al. 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the flavonoids  
602 rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity  
603 relationship of its eight flavonoids by a refined assign score method. Chem. Central J.  
604 12(82):1-11.

605 LIN YS. et al. 2020. Effects of storage time and temperature on antioxidants in juice from  
606 *Momordica charantia* L. and *Momordica charantia* L. var. abbreviata Ser. Molecules.  
607 25(3614): 1-12.

608 MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content  
609 and antioxidant activity test of avocado seed extract (*Persea Americana* Mill). Jurnal MIPA  
610 Unsrat Online. 1(1): 5-10. [https://media.neliti.com/media/publications/116214-ID-  
611 penentuan-kandungan-tanin-dan-uji-aktivi.pdf](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf) [In Bahasa Indonesia]

612 MARTINEZ-SOLIS J et al. 2022. Antidiabetic and toxicological effect of the tea infusion  
613 of summer collection from *Annona cherimola* Miller leaves. Plants. 11(3224):1-17.

614 MATHUR R, VIJAYVERGIA R. 2017. Determination of total flavonoid and phenol content  
615 in *Mimusops Elengi* Linn. Intern. J. Pharm. Sci. Res. 8(12): 5282-5285.

616 MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of  
617 sprouted and solid-state bioprocessed soybean. Asia Pacific J. Clinical Nut. 14(2): 145-  
618 152.

619 MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant  
620 activity: A Review. Intern. J. Molecular Sci. 22(3380):1-30.

621 NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus*  
622 *sabdariffa* L.): Effect of dying temperature and brewing conditions on total soluble solid,  
623 phenolic content, antioxidant capacity and sensory quality. Beverages.6(2):1-11 (2020).

624 NOREEN H. et al. 2017. Measurement of total phenolic content and antioxidant activity  
625 of aerial parts of medicinal plant *Coronopus didymus*. Asian Pacific J. Tropical Med. 10(8):  
626 792–801.

627 NURCHOLIS GW et al. 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity in-  
628 vitro and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum pictum*  
629 (L.). Current Biochem. 1(2): 58-65.

630 OH J et al. 2013. Antioxidant and antimicrobial activities of various leafy herbal teas. Food  
631 Control. 31(2013):403-409.

632 PROENCA C et al. 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an in vitro and in silico  
633 structure–activity relationship study. J. Enzyme Inhibition Med. Chem. 32(1): 1216–1228.

634 PUREWAL SS et al. 2022. Unraveling the effect of storage duration on antioxidant  
635 properties, physicochemical and sensorial parameters of ready to serve Kinnow-aml  
636 beverages. Applied Food Res. 2(1): 1-8.

637 RAMPHINWA ML et al. 2023. Eco-physiological response of secondary metabolites of  
638 teas: review of quality attributes of herbal tea. *Frontiers in Sustain. Food Systems*.  
639 7(990334):1-14.

640 REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids.  
641 *Czech J. Food Sci.* 30(2):171-177 (2012)

642 RUAN J et al. 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea*  
643 *indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel  
644 column chromatography fractions. *Molecules*. 24(2784):1-20.

645 RUSITA YD et al. 2019. Comparison of tannin concentration on fresh and dried tea leaf  
646 (*Camelia sinensis* L.) extract using UV-visible spectrophotometric method. 1st  
647 International Conference of Health, Science & Technology (ICOHETECH) 2019 E-ISBN:  
648 978-623-92207-0-9. November 16<sup>th</sup>, 2019.LPPM Universitas Duta Bangsa Surakarta,  
649 Surakarta.

650 SANGEETHA R, VEDASREE N. 2012. In Vitro  $\alpha$ -Amylase inhibitory activity of the leaves  
651 of *Thespesia populnea*. *Intern. Scholarly Res.Network*. 2012: 1-4 (2012)

652 SHRAIM AM et al. 2021. Determination of total flavonoid content by aluminum chloride  
653 assay: A critical evaluation. *LWT Food Sci. Tech.* 150(111932):1-11.

654 SILVA-RAMIREZ KA et al. 2020. Effect of drying and steeping temperature on the  
655 phenolic content, antioxidant activity, aromatic compounds and sensory properties of  
656 *Culila polyantha* Benth. *Infusions. Processes*. 8(1378):2-20.

657 SRISOOK K et al. 2012. Antioxidant and anti-inflammatory activities of hot water extract  
658 from *Pluchea indica* Less. herbal tea. *J. Med. Plants Res.* 6(23):4077-4081.

659 SURIYAPHAN O. 2014. Nutrition, health benefits and applications of *Pluchea indica* (L)  
660 Less leaves. Pharm. Sci. Asia. 41:1–10.

661 SU D et al. 2019. Impact of thermal processing and storage temperature on phenolic  
662 profile and antioxidant activity of different varieties of lychee juice. LWT-Food Tech.  
663 116(108578):1-9.

664 THANAJIRUSCHAYA P et al. 2010. Effect of storage time and temperature on antioxidant  
665 components and properties of milled rice. Khon Kaen Univ. Res. J.15(9):843-851.

666 VONGSAK B et al. 2018. Comparison of active constituents, antioxidant capacity, and  
667 αglucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. Food  
668 Biosci. 25: 68–73.

669 WANG SC. et al. 2022. Effect of brewing conditions on polyphenols in the dark tea  
670 (*Camellia sinensis* L.) infusions: content, composition and antioxidant activities. Food  
671 Sci.Tech. 42(e36322):1-9.

672 WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica*  
673 Less as functional beverage in diabetic patients. ASSEHR. 98:164-167.

674 WIDYAWATI PS et al. 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract  
675 as antioxidant and antiwarmed over flavor (WOF) of duck meat. International Congress  
676 on Challenges of Biotechnological Research in Food and Health. November 15<sup>th</sup>  
677 2014.Agricultural Technology Faculty, Slamet Riyadi University, Surakarta.

678 WIDYAWATI PS et al. 2016. The physicochemical and antioxidant properties of *Pluchea*  
679 *indica* Less drink in tea bag packaging. IJFNS. 5: 13–120.

680 WIDYAWATI PS et al. 2017. In vitro antioxidant capacities and antidiabetic properties of  
681 pluchea leaves. IJPPS. 9(8): 203-208.

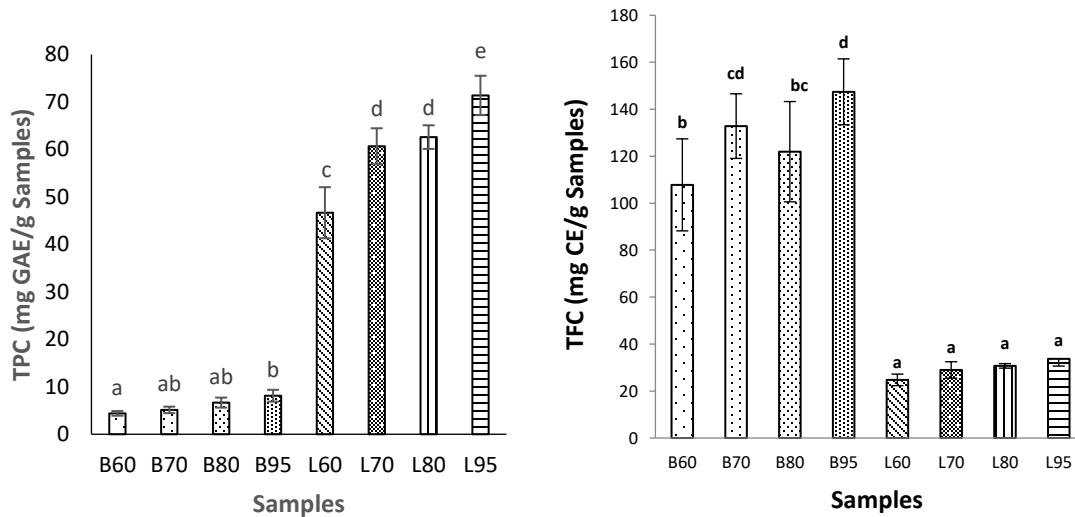
682 WIDYAWATI PS et al. 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica*  
683 Less beverage. CJFST.12(4): 125-139.

684 WIDYAWATI PS ET AL. 2022. The effect of  $\kappa$ -carrageenan proportion and hot water  
685 extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of stink  
686 lily (*Amorphophallus muelleri*) wet noodles. Molecules. 27(5062):1-16.

687 XU P, CHEN L, WANG Y. 2019. Effect of storage time on antioxidant activity and inhibition  
688 on  $\alpha$ -amylase and  $\alpha$ -glucosidase of white tea. Food Sci. Nut. 7(2): 636-644.

689 ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction time on caffeine in  
690 coffee. Lantanida J. 6(2):103-202. [https://jurnal.ar-](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811)  
691 [raniry.ac.id/index.php/lantanida/article/view/3811](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811). [In Bahasa Indonesia]

692 ZHANG Y et al. 2022. Effect of storage conditions and time on the polyphenol content of  
693 wheat flours. Processes. 9(248):1-11.



694

695

(a)

(b)

696

697

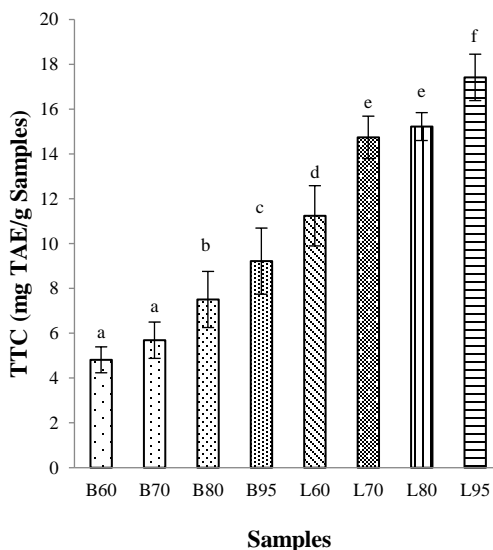
698

699

700

701

702



703

704

(c)

705

706

707

708

709

Figure 1. Bioactive compound content of pluchea tea at different steeping temperature and storage time (a) Total phenolic content (b) Total flavonoid content (c) Total tannin content (Values were means  $\pm$  standard deviations (n=6).

Different supercripts in graph showed a significant difference based on the DMRT test ( $P \leq 5\%$ )

710 Table 1. Phenolic Compound Profile of Pluchea Tea Infusion at Different Steeping Temperature and Storage Time

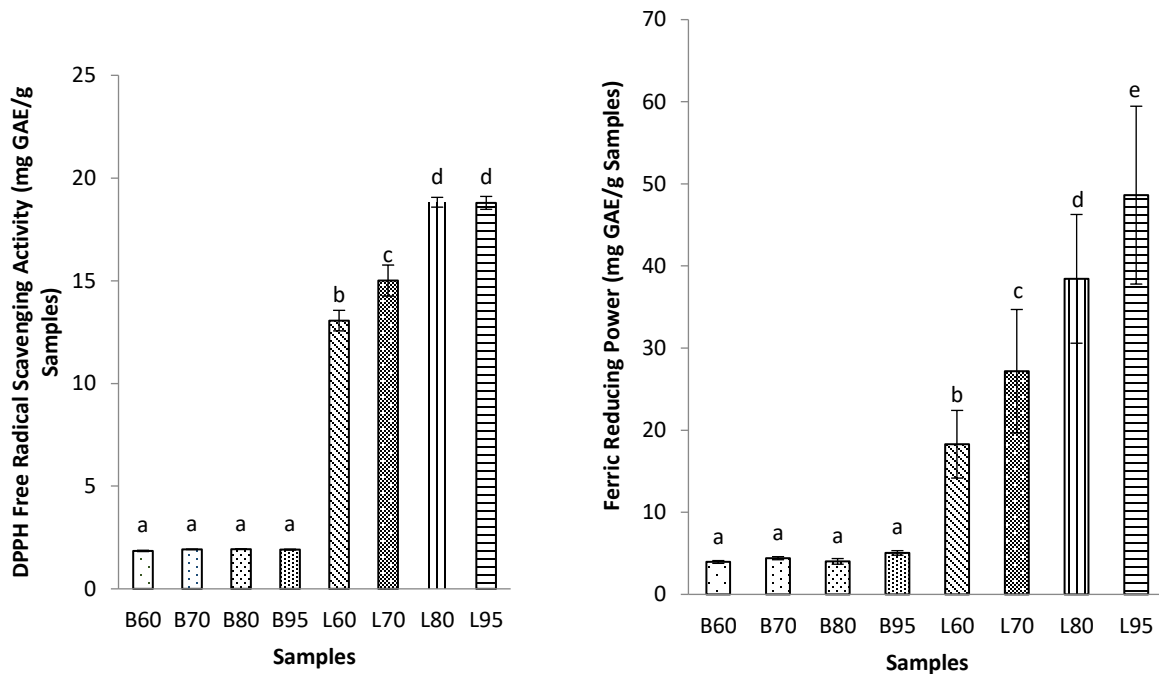
Samples	Gallic Acid ( $\mu\text{g/g}$ samples)	(+)-Catechin ( $\mu\text{g/g}$ samples)	Myricetin ( $\mu\text{g/g}$ samples)	Quercetin ( $\mu\text{g/g}$ samples)	Kaempferol ( $\mu\text{g/g}$ samples)	3,4-di- <i>O</i> -	3,5-di- <i>O</i> -	4,5-di- <i>O</i> -
						Caffeoylquinic acid ( $\mu\text{g/g}$ samples)	Caffeoylquinic acid ( $\mu\text{g/g}$ samples)	Caffeoylquinic acid ( $\mu\text{g/g}$ samples)
B60	0.2132 $\pm$ 0.0027	0.3425 $\pm$ 0.0110	0.1756 $\pm$ 0.1234	0.0220 $\pm$ 0.0268	0.1394 $\pm$ 0.0202	0.6103 $\pm$ 0.0628	0.6635 $\pm$ 0.0628	0.4906 $\pm$ 0.0060
B70	0.2157 $\pm$ 0.0013	0.3260 $\pm$ 0.0265	0.2587 $\pm$ 0.0160	0.1530 $\pm$ 0.0511	0.0514 $\pm$ 0.0037	0.6271 $\pm$ 0.0099	0.6162 $\pm$ 0.0099	0.4807 $\pm$ 0.0034
B80	0.2234 $\pm$ 0.0122	0.3240 $\pm$ 0.0222	0.4175 $\pm$ 0.0104	0.3666 $\pm$ 0.0103	0.3699 $\pm$ 0.0924	0.7967 $\pm$ 0.03060	0.6601 $\pm$ 0.0306	0.5299 $\pm$ 0.0053
B95	0.2316 $\pm$ 0.0104	0.4039 $\pm$ 0.0320	0.8786 $\pm$ 0.0434	0.6559 $\pm$ 0.0570	0.5913 $\pm$ 0.0239	1.5386 $\pm$ 0.0668	0.6642 $\pm$ 0.0668	1.0018 $\pm$ 0.0526
L60	0.2364 $\pm$ 0.0015	0.5085 $\pm$ 0.0111	1.4762 $\pm$ 0.0271	0.6220 $\pm$ 0.0706	0.3675 $\pm$ 0.0183	2.4863 $\pm$ 0.0270	0.9449 $\pm$ 0.0501	1.1842 $\pm$ 0.0120
L70	0.2324 $\pm$ 0.0214	0.5448 $\pm$ 0.0006	1.4245 $\pm$ 0.2526	1.0708 $\pm$ 0.0289	0.3726 $\pm$ 0.0944	2.3403 $\pm$ 0.0325	0.9485 $\pm$ 0.0794	1.0089 $\pm$ 0.0736
L80	0.2347 $\pm$ 0.0078	0.5023 $\pm$ 0.0773	1.457 $\pm$ 0.0925	0.8629 $\pm$ 0.0815	0.7966 $\pm$ 0.0366	2.6278 $\pm$ 0.0211	0.9099 $\pm$ 0.0387	1.2382 $\pm$ 0.1435
L95	0.2402 $\pm$ 0.0169	0.5995 $\pm$ 0.0372	2.6138 $\pm$ 0.0695	2.0230 $\pm$ 0.0573	0.9478 $\pm$ 0.0287	4.0211 $\pm$ 0.0851	1.3156 $\pm$ 0.0166	1.3797 $\pm$ 0.2170

711 Note : data of phenolic compound profile was obtained from two replicates that displayed as mean $\pm$ SD

712

713





714

(a)

(b)

715

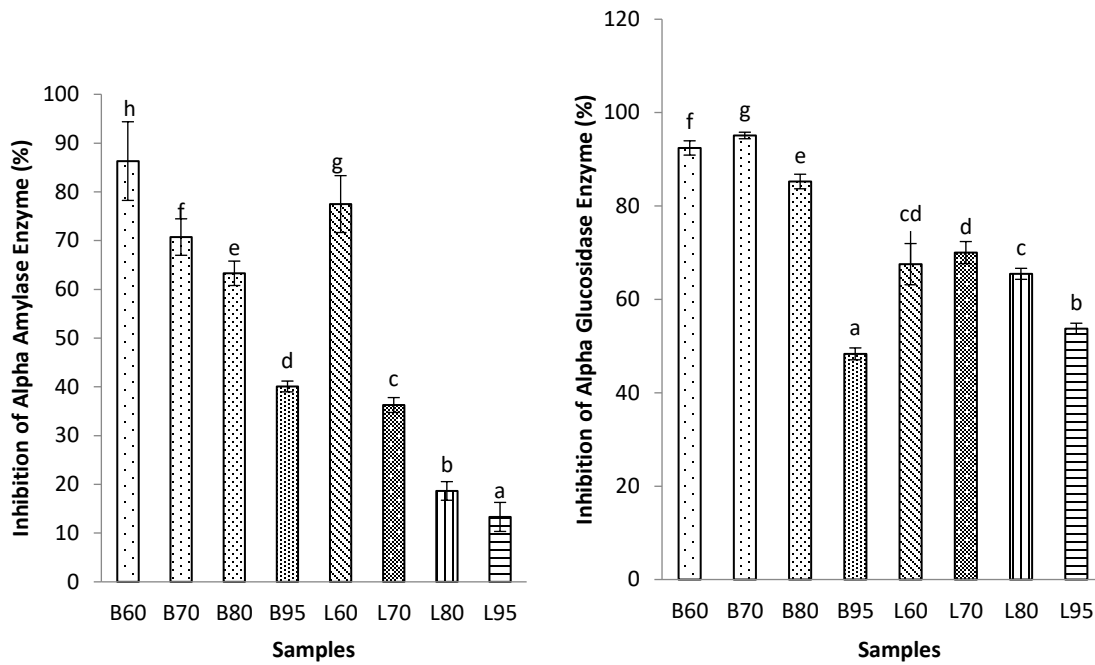
716 Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and

717 storage time (a) DPPH (b) FRAP (Values were means  $\pm$  standard deviations

718 (n=6). Different supercripts in graph showed a significant difference based on the

719 DMRT test ( $P \leq 5\%$ )

720



(a)

(b)

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage time (a)  $\alpha$ -amylase (b)  $\alpha$ -glucosidase (Values were means  $\pm$  standard deviations (n=6). Different superscripts in graph showed a significant difference based on the DMRT test ( $P \leq 5\%$ ))

721

722

723

724

725

726

727

2. First Revision: Format Revision Based on Philippine Journal of Science (28-4-2023)

- Correspondence
- Document
- Cover Letter
- List Reviewer
- Authorship Statement



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**(no subject)**

---

**Philippine Journal of Science** <philjournsci@gmail.com>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Fri, Apr 28, 2023 at 12:23 PM

Dear Dr. Widyawati,

Thank you very much for considering the Philippine Journal of Science (PJS) as the venue for publishing your research efforts! We wish to inform you that a complete submission must include the following:

[2] Manuscript

- It must strictly adhere to the PJS format in writing articles, particularly in the references section (*i.e.* the journal name must not be punctuated by a period, the volume and issue numbers must be separated from the page numbers by a space, the page range must be indicated using an en dash – instead of a hyphen -, *etc.*).

You can find the additional descriptions of the requirements in the PJS Author's Guide (<https://philjournalsci.dost.gov.ph/author-s-guide>). A reference number will be provided once you have complied with all requirements. Incomplete submissions will not be reviewed.

Again, thank you for your interest in PJS! We look forward to your complete submission.

Sincerely,  
Mr. ALLYSTER A. ENDOZO  
Managing Editor

For Dr. CAESAR A. SALOMA  
Editor-in-Chief

[Quoted text hidden]

--



**Philippine Journal of Science**  
**Science and Technology Information Institute**  
**Department of Science and Technology**  
**DOST Complex, Gen. Santos Ave., Bicutan 1631**  
**Taguig City, Metro Manila, Philippines**  
Telephone no. : 837 - 2191  
Email: [pjs@stii.dost.gov.ph](mailto:pjs@stii.dost.gov.ph); [philjournsci@gmail.com](mailto:philjournsci@gmail.com)  
Website: <https://philjournalsci.dost.gov.ph>  
Scopus: <https://www.scopus.com/sourceid/19700175735>

1 **Effect of Steeping Temperature and Storage Time on the Bioactive Compounds,**  
2 **Antioxidant and Antidiabetic Activities of *Pluchea Indica* Less Tea**

3 Painsi Sri Widyawati<sup>1\*)</sup>, Yufita Ratnasari Wilianto<sup>2)</sup>

4 <sup>1)</sup>Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala  
5 Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

6 <sup>2)</sup>Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic  
7 University, Kalisari Street Number 1, Surabaya 60272, Indonesia

8 Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea*  
9 *indica* Less, storage time

10

11

12

13

14

15

16

17

18

19

## 20 ABSTRACT

21 This study was done to determine effect of steeping temperature and storage time on the  
22 bioactive compounds, antioxidant and antidiabetic activities of *Pluchea indica* Less tea  
23 infusion. The research used a randomized block design with two factors, i.e., steeping  
24 temperature (60, 70, 80, and 95 °C) and storage time (0 and 5 years). The steeping  
25 temperature and storage time influenced the bioactive compounds, antioxidant and  
26 antidiabetic activities of samples. Total phenolic content and total tannin content went up  
27 along with increased antioxidant activity. Treatment resulted simple phenolic compounds,  
28 such as gallic acids, (+)-catechins, kaempferols, myricetins, quercetins, 3,4-di-O-  
29 caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, 4,5-di-O-caffeoylquinic acids. Total  
30 flavonoid content was decreased for storage time and significant different at higher  
31 steeping temperatures. The total flavonoid content had graph pattern similar with  $\alpha$ -  
32 amylase and  $\alpha$ -glucosidase inhibition activities. This means, the antidiabetic activity was  
33 largely determined by the total flavonoid content and structure of phenolic compounds. In  
34 order, to get high antioxidant activity, it was chosen pluchea tea stored at high steeping  
35 temperature, but high antidiabetic activity was fresh pluchea tea steeped at a low  
36 temperature.

37

## 38 INTRODUCTION

39 *Pluchea* tea is a product of *pluchea* leaf processing introduced by world people (Srisook  
40 *et al.* 2012; Widyawati *et al.* 2016) because of the efficacy of the active components in  
41 *pluchea* leaves, as an herbal plant that has been widely used for traditional medicine and  
42 food (Chan *et al.* 2022). *Pluchea* tea is composed many nutrients and bioactive

43 compounds useful to body health. The nutrient compositions in the pluchea tea include  
44 protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates, calcium,  $\beta$ -carotene, and  
45 vitamin C, whereas bioactive compounds is comprised, i.e., chlorogenic acid, caffeic acid,  
46 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 3,4-di-O-  
47 caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, quercetin,  
48 myricetin, kaempferol, total anthocyanin,  $\beta$ -carotene, and total carotenoid (Suriyaphan  
49 2014; Vongsak *et al.* 2018; Ruan *et al.* 2019; Widyawati *et al.* 2022, Chan *et al.* 2022).

50 Steeping process of pluchea tea leaves can be performed with fresh or dry leaves  
51 infusion by hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez *et al.*  
52 2020; Jayani *et al.* 2022). In Asian area, especially in Indonesian, people usually consume  
53 the pluchea tea with brewing of powdered pluchea leaves in tea bag by hot water or  
54 boiling water. Each tea bag contained 2 g of pluchea leaf powder is steeped with 100 mL  
55 hot water or boiling water. Widyawati *et al.* (2016) claimed that steeping of 2 g pluchea  
56 tea at 95 °C for 5 minutes results total phenolic content, total flavonoid content, the ability  
57 to scavenge DPPH free radicals, and the capability of reduce ferric ions 9.3 mg gallic acid  
58 equivalent (GAE)/g samples, 22.0 mg gallic acid equivalent (GAE)/g samples, 27.2 mg  
59 gallic acid equivalent (GAE)/g samples, and 10.2 mg gallic acid equivalent (GAE)/g  
60 samples, respectively. Werdani and Widyawati (2018) reported that drinking of pluchea  
61 tea in the morning and evening regularly (2 g/100 mL) can decline blood sugar levels.

62 Steeping pluchea tea with hot water at 95 °C for 5 min certainly determines the  
63 stability and amount of extracted bioactive compounds, that influences the biological  
64 activity, especially antioxidant and antidiabetic activities. Silva-Ramirez *et al.* (2020)  
65 reported that the infusion process can influence their content and composition of the

66 bioactive compounds and antioxidant activity of tea. Acar *et al.* (2022) informed that  
67 infusion quality of herb tea extract depends on several factors, i.e., time and temperature.  
68 Polyphenol profile and antioxidant properties of herb tea infusion decline with an increase  
69 in steeping/brewing and storage temperatures and longer exposure times.

70         Several studies have mentioned the effect of steeping temperature to bioactive  
71 compounds and antioxidant activity, such as some white and green teas are effective with  
72 hot water at 90 °C for 7 min (Castiglioni *et al.* 2015), roseship tea is effectively at infusion  
73 time around 6-8 min at temperatures of 84-86 °C (Ilyasoglu and Arpa 2017), the coffee  
74 brewing temperature influences the caffeine content extracted (Zarwinda and Sartika  
75 2018), the steeping of dark tea at 92 °C for 27 min results the highest total phenol content  
76 and antioxidant activity (Wang *et al.* 2022). The study of the effect of steeping temperature  
77 to pluchea tea infusion was carried out to afford information about preparation of pluchea  
78 tea most efficiently to get higher the bioactive compounds, antioxidant and antidiabetic  
79 activities.

80         On the other hand, storage time of pluchea tea also affects the levels of the  
81 bioactive compounds and biological activity because this tea usually is stored for a  
82 several months until years (Jayani *et al.* 2022). Tea or herbal tea is generally stored in  
83 ambient temperature and packed in tea bag or Alu foil standing proud or a combination  
84 of both. Many researchers informed that storage time decreases the bioactive  
85 compounds, antioxidant and antidiabetic activities, i.e., juice from *Momordica charantia* L.  
86 (Lin *et al.* 2020), dried *Piper bettle* extracts (Ali *et al.* 2018), white tea (Xu *et al.* 2019),  
87 kinnow-amlam beverages (Purewal *et al.* 2022), whole wheat flour (Zhang *et al.* 2021).  
88 Therefore, this research studied effect of steeping temperature and storage time on the



89 bioactive compounds, antioxidant and antidiabetic properties of pluchea tea. The study  
90 was emphasized to determine total phenolic content, total flavonoid content, total tannin  
91 content, scavenging activity of DPPH free radical, ferric reducing power,  $\alpha$ -amylase and  
92  $\alpha$ -glucosidase inhibition activities, and phenolic compound profile.

93

## 94 MATERIALS AND METHODS

### 95 MATERIALS

96 The pluchea leaves were collected from gardens in Mangrove areas, Wonorejo,  
97 Surabaya, Indonesia. The pluchea plants were included in Asteraceae family with  
98 specification according to the GBIF taxon ID number database:3132728. Then, the  
99 material was treated based on Widyawati *et al.* (2022) method and Widyawati *et al.*  
100 (2023). The pluchea tea packed in tea bag (2 g/tea bag) was steeped with hot water  
101 temperatures of 60, 70, 80, and 95 °C for 5 min and storage times of 0 (control) and 5  
102 years (stored) with infusion method. Then, the samples preparation was done based on  
103 Widyawati *et al.* (2016) and Widyawati *et al.* (2022) methods.

104

### 105 REAGENTS

106 The compounds used to analyze including 2,2-diphenyl-1-picrylhydrazyl (DPPH),  
107 sodium carbonate, gallic acid,  $\alpha$ -amylase,  $\alpha$ -glucosidase, pNPG (p-nitrophenyl- $\alpha$ -  
108 glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic  
109 acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and (+)-catechin were  
110 purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, Folin–Ciocalteu’s Phenol,  
111 sodium nitric, aluminum chloride, ferric chloride, sodium dihydrogen phosphate, sodium

112 phosphate, potassium ferricyanide, starch, acetic acid, and sodium hydroxide were  
113 purchased from Merck (Kenilworth, NJ, USA). Aquadest and aquabidest were purchased  
114 by PT Aqua Surabaya.

115

## 116 METHODOLOGY

### 117 TOTAL PHENOLIC CONTENT ANALYSIS

118 Total phenolic content of steeping pluchea tea was conducted by Gao *et al.* (2019)  
119 method based on spectrophotometric analysis. Total phenolic content assay using redox  
120 analysis between phenolic compounds and phosphomolybdic /phosphotungstic acid  
121 complexes is founded on the electron transfer in an alkaline medium from the phenolic  
122 compounds to result a blue colored solution because of phosphotungstic/  
123 phosphomolybdenum complex formation. Total phenolic content was measured by  
124 Spectrophotometer (spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  760 nm and  
125 a reference standard was a gallic acid. The results were expressed as mg gallic acid  
126 equivalentents (GAE)/g samples.

127

### 128 TOTAL FLAVONOID CONTENT ASSAY

129 Total flavonoid content of the samples was determined by the spectrophotometric  
130 method based on the reaction between  $\text{AlCl}_3$  and  $\text{NaNO}_2$  with an aromatic ring of  
131 flavonoid compounds, especially flavonol and flavon (Shraim *et al.* 2021). The reaction  
132 between  $\text{AlCl}_3$  and flavonoid compounds resulted a yellow solution. Then, the red solution  
133 was produced after NaOH solution addition that was measured by a spectrophotometer  
134 (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  510 nm. A (+) catechin was

135 used as a reference standard compound, and the results were expressed as mg catechin  
136 equivalents (CE)/g samples.

137

#### 138 TOTAL TANNIN CONTENT ANALYSIS

139 Total tannin content of the samples was analyzed by Folin-Ciocalteu method  
140 based on Chandran and Indira (2016). The reaction between the samples and reagents  
141 obtained blue dark color solution that measured by a spectrophotometer  
142 (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  760 nm. This analysis used a  
143 tannic acid as a reference standard and was expressed as mg tannic acid equivalents  
144 (TAE) /g samples.

145

#### 146 DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

147 The DPPH free radical scavenging activity was measured by the  
148 spectrophotometric method (Widyawati *et al.* 2017) to determine AA of the brewing of  
149 pluchea tea to donor hydrogen atom to nitrogen atom in DPPH resulting DPPH-H  
150 compound with a yellow-colored solution. The reaction between the DPPH in methanol  
151 solution with the samples was measured by a spectrophotometer (Spectrophotometer  
152 UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  517 nm. The reference standard compound was  
153 gallic acid and the results of analysis were expressed as mg gallic acid equivalents  
154 (GAE)/g samples.

155

#### 156 FERRIC REDUCING POWER ANALYSIS

157 Ferric reducing power was determined by Widyawati *et al.* (2014) method. Potency  
158 of the steeping pluchea tea reducing iron (III) to iron (II) ion was analyzed by  
159 spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  700 nm.  
160 The reducing capacity of antioxidant compounds of the steeping pluchea tea increased  
161 related to intensity of blue color solution. The bigger of reducing power, the higher of blue  
162 color intensity. The reference standard used as gallic acid, and the results were  
163 expressed as mg gallic acid equivalent (GAE)/g samples.

164

#### 165 $\alpha$ -AMYLASE ENZYME INHIBITION CAPACITY ASSAY

166 In vitro inhibition of  $\alpha$ -amylase enzyme was determined by Widyawati *et al.* (2020)  
167 method. Samples of steeping pluchea tea at various steeping temperatures and storage  
168 times were analyzed by spectrophotometer UV-Vis (Spectrophotometer UV-Vis-1800,  
169 Shimadzu, Japan) based on reaction between bioactive compounds and  $\alpha$ -amylase  
170 enzyme. Then, the residue enzyme was reacted with starch and the capacity of the  $\alpha$ -  
171 amylase enzyme hydrolyzed the starch to release glucose that could be analyzed based  
172 on absorbance at  $\lambda$  540 nm. The inhibition percentage of  $\alpha$ -amylase was assessed by  
173 the following formula:  $(ACb - ACa) - (As - Ab) / (ACb - ACa) \times 100 \%$ . Where, ACb was  
174 absorbance of 100% enzyme activity (only solvent with enzyme), ACa was absorbance  
175 of 0 % enzyme activity (only solvent without enzyme), As was absorbance of tested  
176 sample with enzyme, Ab was absorbance of tested sample without enzyme.

177

#### 178 $\alpha$ -GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

179 The analysis of the  $\alpha$ -glycosidase inhibitor activity was done by Widyawati *et al.*  
180 (2020) method with slight modification. The samples were reacted with the  $\alpha$ -glycosidase  
181 enzyme, and then the residue of this enzyme hydrolyzed p-nitrophenyl- $\alpha$ -D-  
182 glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The inhibitor activity of  
183 steeping pluchea tea to enzyme was measured by spectrophotometer UV-Vis  
184 (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at  $\lambda$  405 nm. The inhibition  
185 percentage of  $\alpha$ -glycosidase was assessed by the following formula:  $(ACb - ACa) - (As$   
186  $- Ab) / (ACb - ACa) \times 100 \%$  Where, ACb was absorbance of 100 % enzyme activity (only  
187 solvent with enzyme), ACa was absorbance of 0 % enzyme activity (only solvent without  
188 enzyme), As was absorbance of tested sample with enzyme, Ab was absorbance of  
189 tested sample without enzyme.

190

#### 191 HPLC ANALYSIS OF PHENOLICS

192 The phenolic compounds of samples were analyzed by HPLC based on  
193 Kongkiatpaiboon *et al.* (2018) method with modification. HPLC separation was achieved  
194 on LC-20AD series (Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence  
195 UFLC LC-20AD pump, SIL-20 ACHT autosampler, CTO-10AS VP column oven, CBM-20A  
196 system controller, and SPD-40 detector. The separation was done in a Shim-pack VP-  
197 ODS C18 column (5  $\mu$ m  $\times$  50 mm  $\times$  4.6 mm I.D.) with a GVP-ODS Cartridges (2 pcs)  
198 guard column (10 mm  $\times$  4.6 mm I.D.). The mobile phases were (A) 0.5 % acetic acid in  
199 water and (B) methanol using gradient elution: 10 % B in A to 50 % B in A for 40 min; 100  
200 % B for 20 min. This column was re-equilibrated with 10 % B in A for 10 min prior to each  
201 analysis and the flow rate was set at 1.0 mL/min with the controlled temperature at 40  $^{\circ}$ C.

202 SPD-40 detector was set at  $\lambda$  280 nm and injection volume was 20  $\mu$ L for every sample  
203 and reference standard.

204

## 205 EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

206 The research design used a randomized block design with two factors, i.e., the  
207 brewing temperature (B) and the storage time (L). The steeping temperature of pluchea  
208 tea consisted of four treatment levels, including 60 °C (B1), 70 °C (B2), 80 °C (B3), and  
209 95 °C (B4), and the storage time of pluchea tea was composed two levels, i.e., 0 year  
210 /fresh (L0), and 5 year/stored (L2). Each treatment was repeated six times in order to  
211 obtain 48 experiment units. The HPLC analysis of phenolic was repeated two times. The  
212 data of samples were analyzed by ANOVA at  $p \leq 5\%$ , and continued by DMRT (Duncan  
213 Multiple Range Test) at  $p \leq 5\%$ . Data were expressed as the mean  $\pm$  SD. The analysis  
214 used SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

215

## 216 RESULTS AND DISCUSSIONS

217 Pluchea leaf tea is produced by young pluchea leaf from 1-6 level on each branch  
218 the shoot (Widyawati *et al.* 2022), that is steeped at 95 °C for 5 min, has many biological  
219 activities, such as antioxidant activity (Widyawati *et al.* 2016), antidiabetic activity  
220 (Werdani and Widyawati, 2018), anti-inflammatory (Srisook *et al.* 2015). The chemical  
221 constituents in pluchea tea involve alkaloids, flavonoids, phenolics, sterols, cardiac  
222 glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2 g/100 mL  
223 steeping pluchea tea has total phenolic content 9.3 mg gallic acid equivalents (GAE)/g  
224 samples, total flavonoid content 22.0 mg catechin equivalents (CE)/g samples, DPPH

225 free radical scavenging activity 27.2 mg gallic acid equivalents (GAE)/g samples, and  
226 ferric reducing power 10.2 mg gallic acid equivalents (GAE)/g samples (Widyawati *et al.*  
227 2016). Previous research has informed related to the composition of phytochemical  
228 compounds in pluchea leaves, such as phenolic acids such as chlorogenic acids, caffeic  
229 acids, 3-O-caffeoylquinic acids, 4-O-caffeoylquinic acids, 5-O-caffeoylquinic acids, 3,4-  
230 di-O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic  
231 acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin;  $\beta$ -  
232 carotene; and total carotenoids (Suriyaphan 2014; Vongsak *et al.* 2018; Ruan *et al.* 2019;  
233 Chan *et al.* 2022; Widyawati *et al.* 2022). Presence of phytochemical compounds in herbal  
234 product were influenced by environmental factors, i.e., temperature, light exposure,  
235 oxygen level, pH and moisture. The structure of phytochemical compounds in herbal tea  
236 is very sensitive of the surrounding changes. The effect arising from these changes  
237 causes the structure of the phytochemical molecule to be degraded to produce smaller  
238 size molecules or to combine to produce larger size molecules (Ali *et al.* 2018; Jayani *et*  
239 *al.* 2022, Ramphinwa *et al.* 2023). Therefore, this study emphasized the effect of steeping  
240 temperature and storage time of pluchea tea on levels of the bioactive compounds,  
241 antioxidant and antidiabetic properties and phenolic compound profile.

242

## 243 BIOACTIVE COMPOUNDS

244 The bioactive compounds are active compounds in plants that are essential to  
245 protect a body health (Nguyen and Chuyen 2020). These compounds usually have many  
246 biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer,  
247 antimicrobial, antibacterial, anti-cholesterol and so on (Suriyaphan 2014; Acar *et al.*

248 2022). Phenolic compounds are potential bioactive compounds in plants, that have  
249 responsible redox properties to scavenge free radicals as cause with a number of chronic  
250 diseases (Noreen *et al.* 2017; Arya *et al.* 2019; Acar *et al.* 2022).

251 The steeping temperature (60, 70, 80 and 95 °C) and storage time (fresh and  
252 stored) determined total phenolic content, with values ranging from  $4.39 \pm 0.49$  to  $71.38$   
253  $\pm 4.14$  mg GAE/g samples. The total phenolic content of pluchea tea infused at different  
254 temperature and stored at different time that statistical analyzed by ANOVA at  $\alpha \leq 5 \%$   
255 shown at Figure 1. The total phenolic content of samples was significantly influenced by  
256 the steeping temperature and storage time. The highest total phenolic content was  
257 detected in the L 95 sample infused at 95 °C and stored for 5 years ( $71.38 \pm 4.14$  mg  
258 GAE/g samples) with was followed by L 80 sample infused at 80 °C and stored for 5 years  
259 ( $62.60 \pm 2.49$  mg GAE/g samples) and L70 sample infused at 70 °C and stored for 5 years  
260 ( $60.68 \pm 3.79$  mg GAE/g samples) and L60 sample infused at 60 °C and stored for 5 years  
261 ( $46.67 \pm 5.38$  mg GAE/g samples). The total phenolic contents of steeping fresh pluchea  
262 tea (B60) had a lower total phenolic content ( $4.39 \pm 0.48$  mg GAE/g samples) than the  
263 steeping stored pluchea tea for 5 years ( $48.67 \pm 5.38$  until  $71.38 \pm 4.14$  mg GAE/g  
264 samples). Fresh pluchea tea had a lower total phenolic content than stored pluchea tea  
265 for 5 years, besides that the higher the steeping temperature also caused the greater the  
266 extracted total phenolic content. The temperature of infusion influenced total phenolic  
267 content, it could relate to migration process of phenolic compounds to the water because  
268 of increasing contact between this compounds and water. The same phenomena also  
269 occurred in Castiglioni *et al.* (2015); Kilic *et al.* (2017), and Acar *et al.* (2022).



270 This occurrence showed that steeping temperature and storage time caused the  
271 process of degradation and oxidation of phenolic compounds. Su *et al.* (2019) reported  
272 that temperature treatment can stimulate the release of phenolic compounds and  
273 increase antioxidant activity of lychee juice stored at different temperatures of 4 and 45  
274 °C and different storage times (fresh and 72 hours). Hydrogen bonding is affected by  
275 temperature treatment because the hydrogen bond between phenolic compounds and  
276 proteins can be degraded that the measured levels of phenolic compounds are higher.  
277 The phenomena were supported by Ali *et al.* (2018); Jayani *et al.* (2022) and Ramphinwa  
278 *et al.* (2023). Zhang *et al.* (2021) reported that phenolic compounds present in plants are  
279 not completely stable, but are easily degraded during storage after harvest. Reblova  
280 (2012) claimed that antioxidant compounds can be slowly degraded with increasing  
281 temperature. Besides that, Fibrianto *et al.* (2021) also stated that the brewing  
282 temperature has an effect on the extracted antioxidant compounds, such as alkaloids,  
283 catechins and tannins. Thus, there is an assumption that the phenolic compounds in  
284 pluchea tea are degraded due to oxidation and hydrolysis because of temperature and  
285 storage time and can be easily extracted during brewing, thus increasing the phenolic  
286 content as the steeping temperature and storage time increase.

287 Based on using of a reference standard could be informed that phenolic  
288 compounds in steeping pluchea tea, including gallic acids, (+)-catechins, myricetins,  
289 quercetins, kaempferols, 3,4-di-O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and  
290 4,5-di-O-caffeoylquinic acids was showed in Table 1. Gallic acids and (+)-catechins were  
291 relative stable phenolic acid because of very small changes at different steeping  
292 temperature and storage time with concentration about  $0.21 \pm 0.00 - 0.24 \pm 0.02$  µg/g

293 samples and  $0.32 \pm 0.02 - 0.60 \pm 0.04 \mu\text{g/g}$  samples, respectively. However, myricetin,  
294 quercetin and 3,4-di-O-caffeoylquinic acid showed drastic increasing at higher steeping  
295 temperature and longer storage time. It's meant that these compounds tended relatively  
296 labile. Kaempferol, 3,5-di-O-caffeoylquinic acid and 4,5-di-O-caffeoylquinic acid  
297 underwent moderate changes compared to the other two groups of phenolic acids.  
298 Therefore myricetin, quercetin and 3,4-di-O-caffeoylquinic acid were easier to dissolve at  
299 higher steeping temperature and storage time can cause macromolecules of three  
300 phenolic acids in herbal tea convenient degradable to form simple phenolic compounds  
301 for storage, as explained by Su *et al.* (2019), Ali *et al.* (2018); Jayani *et al.* (2022);  
302 Ramphinwa *et al.* (2023), and Zhang *et al.* (2021). Degradable polyphenol compounds  
303 have a simple structure and free hydroxyl groups that can react with Folin-Ciocalteu's  
304 Phenol reagent, resulting complex blue solution that can detected as total phenolic  
305 content.

306 Flavonoids are the major phenolic compounds having potential as chemical and  
307 biological activities, especially as radical scavenging and antimicrobial activities (Ayele *et*  
308 *al.* 2022; Chandra *et al.* 2014). These compounds are the bioactive compounds that can  
309 protect the human body from the oxidative stress caused many degenerative diseases,  
310 especially cancer, cardiovascular problems and ageing (Mathur and Vijayvergia 2017).  
311 Total flavonoid content analysis for pluchea tea at various steeping temperatures and  
312 storage times were showed in Figure 1. The total flavonoids of steeping pluchea tea  
313 decreased with increasing storage time, but increased with increasing brewing  
314 temperature. The highest total flavonoid content was owned by fresh pluchea tea which  
315 was brewed at 95 °C ( $147.42 \pm 14.03 \text{ mg CE/g}$  samples) and the lowest was owned by

316 pluchea tea which had been stored for 5 years at various brewing temperatures (between  
317  $24.75 \pm 2.47$ - $33.71 \pm 3.06$  mg CE/g samples). Statistical analysis by ANOVA analysis at  
318  $\alpha \leq 5\%$  proven that brewing temperature and storage time of fresh pluchea tea had a  
319 significant effect on the total flavonoid content, but the stored pluchea tea (L) had no  
320 significant effect. Storage time had a significant effect on the total flavonoid content of  
321 brewing pluchea tea. Ali *et al.* (2018) reported that the degradation of bioactive  
322 compounds can take place through several stages, such as pre-treatment, processing,  
323 and storage, as is the case with medicinal plants which are dried, extracted and stored in  
324 the long term. Brewing temperature and storage time have an influence on the oxidation  
325 and polymerization processes that are stimulated by light. According to Noree *et al.*  
326 (2017), that the total flavonoid content test with  $\text{AlCl}_3$  and  $\text{NaNO}_2$  reagents measures  
327 flavone compounds, these compounds have activity due to the presence of a free  
328 hydroxyl functional group at position 4' in the compound. Degradation of flavone  
329 compounds due to temperature and storage causes the breaking of methylation bonds.  
330 Kim *et al.* (2020) also confirmed, that the total phenolic content and total flavonoid content  
331 of matcha are decreased with increasing brewing temperature and storage time. Xu *et al.*  
332 (2019) informed, that storage time can give a big impact on chemical composition  
333 changes with trending not the same.

334         The tannins have a various type of compounds are water-soluble polyphenols that  
335 are current in many plant foods and have a number of effects on health (Balaky *et al.*  
336 2021). Tannins are bioactive compounds that provide properties, such as astringent, anti-  
337 diarrheal, antibacterial and antioxidant (Malangngi *et al.* 2012). Data analysis showed,  
338 that the total tannin content of brewing pluchea tea increased with increasing brewing

339 temperature and storage time, as seen in Figure 1. Steeping pluchea tea contained  
340 tannins ranging from  $4.81 \pm 0.58$  -  $17.42 \pm 1.04$  (mg TAE/g samples). The tannin content  
341 increased with increasing storage time and brewing temperature. The results of the  
342 ANOVA statistical analysis at  $\alpha \leq 5\%$ , showed a significant increase in tannin content  
343 levels with increasing brewing temperature and storage time. The fresh pluchea tea  
344 brewed at  $60\text{ }^{\circ}\text{C}$  had the lowest tannin content level, was  $4.81 \pm 0.58$  mg TAE/g samples.  
345 The stored pluchea tea brewed at  $95\text{ }^{\circ}\text{C}$  had the highest tannin content level, was  $17.42$   
346  $\pm 1.04$  mg TAE/g samples. The results showed, that the higher the brewing temperature  
347 and the longer the storage time caused the tannin compound polymerization process to  
348 occur. Ali *et al.* (2018) said that pH, storage temperature, chemical structure and  
349 concentration, light, oxygen, enzymes and metal ions affect the presence of bioactive  
350 compounds in the material. Rusita *et al.* (2019) emphasized that tannins are a polar  
351 compound, that is resistant to heating, as a result the tannin content in pluchea tea  
352 increases with increasing brewing temperature and storage time, this is caused tannins  
353 are thermostable complex compounds.

354

#### 355 ANTIOXIDANT ACTIVITY

356 Antioxidant activity is capability of compounds to inhibit the oxidation of  
357 macromolecules from biological target that involve in oxidative chain reactions (Ali *et al.*  
358 2005; Oh *et al.* 2013). In the research, the antioxidant activity assay used was DPPH Free  
359 Radical Scavenging Activity (DPPH) and ferric reducing power (FRAP), Ali *et al.* (2005)  
360 and Huang *et al.* (2005) informed that phenolic compounds have antioxidant activity

361 because of their redox properties, such as hydrogen atom donor, electron transfer,  
362 reducing agent, and singlet oxygen quenchers.

363 DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate  
364 antioxidant activity because this method is very simple that is suitable to measure the  
365 donating hydrogen atoms capability of herbal tea. This reaction can cause the purple color  
366 of DPPH reduced to be yellow color (Munteanu and Apetrei, 2021; Baliyan *et al.* 2022).  
367 The result of DPPH assay in pluchea tea was showed in Figure 2. The DPPH values  
368 accrued with higher steeping temperature and longer storage time. Statistical analysis by  
369 ANOVA at  $\alpha \leq 5\%$  proven that the higher the steeping temperature of fresh pluchea tea  
370 (B60-B95) was consistent the ability to DPPH free radicals scavenging activity, whereas  
371 the stored pluchea tea resulted the higher activity and the values went up as rising of the  
372 infusion temperature. Pluchea tea storage at room temperature for 5 years resulted the  
373 DPPH free radical scavenging activity by more than 100 %. The steeping at higher  
374 temperatures could significantly increase the DPPH free radical scavenging activity in  
375 stored pluchea tea around 15 - 25 %. Brewing at 80 - 95 °C in stored pluchea tea  
376 insignificantly affected this antioxidant activity. Scavenging activity of DPPH free radicals  
377 was correlated with total phenolic and tannin levels, but inversely to total flavonoid levels.  
378 The phenomenon of the DPPH values in pluchea tea is contrary with the results of the  
379 study by Lin *et al.* (2020). However, this study was in accordance with Thanajiruschaya  
380 *et al.* (2010), claimed that during the storage process it is possible to form complex  
381 phenolic compounds which provide a high ability to scavenge DPPH free radicals. This  
382 research also demonstrated that longer storage time and higher infusion temperature  
383 produced many simple phenolic compounds with free hydroxyl groups capable to donor

384 hydrogen atom to DPPH free radical. Many phenolic acids, such as gallic acids, (+)-  
385 catechins, myricetins, kaempferols, quercetins, 3,5-di-O-caffeoylquinic acids, 3,4-di-O-  
386 caffeoylquinic acids, 4,5-di-O-caffeoylquinic acids have established potential antioxidant  
387 activity (Kumar and Goel 2019).

388 FRAP is method that identifies the antioxidant capacity of the phytochemical  
389 component through measured absorbance, as a result of the reaction among antioxidant  
390 compounds, potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a  
391 color complex, that can be measured at  $\lambda$  700 nm (Fu *et al.* 2011; Al-Temimi and  
392 Choudhary 2013). The principle of testing the ability to reduce iron ions is that antioxidants  
393 can reduce potassium ferrocyanide ( $\text{Fe}^{3+}$ ) to potassium ferrocyanide ( $\text{Fe}^{2+}$ ). Potassium  
394 ferrocyanide reacts with ferric chloride to form a ferric-ferrous complex and results green  
395 color solution (Widyawati *et al.* 2017).

396 The data showed, that the FRAP of pluchea tea became significantly different with  
397 going up brewing temperature and storage time (Figure 1). The FRAP value increased  
398 with higher steeping temperature and longer storage time, the lowest FRAP value was  
399 owned by pluchea tea which was brewed at 60 °C at  $3.95 \pm 0.17$  mg gallic acid equivalents  
400 (GAE)/g samples, and the highest was owned by pluchea tea which was stored for 5  
401 years at  $48.63 \pm 10.83$  mg gallic acid equivalents (GAE)/g samples. FRAP of the pluchea  
402 was significant correlated with the DPPH free radical scavenging activity, total phenolic  
403 and tannin contents. This case was contrast to the antioxidant activity of DPPH and FRAP  
404 on matcha, because the longer storage time reduces the levels of catechin content (Kim  
405 *et al.* 2020), and also the case of the effect of temperature and storage time in betel (*Piper*  
406 *bettle* L.) extract (Ali *et al.* 2018). Thanajiruschaya *et al.* (2010) revealed that the

407 antioxidant activity of rice stored at high temperatures is greater than that stored at low  
408 temperatures. The ferric reducing capability of pluchea tea infusion corresponded to  
409 simple phenolic acid values, presence of them in samples could accrue antioxidant  
410 activity because of ability of the electron transfer from free hydroxyl groups of phenolic  
411 acids.

412

### 413 ANTIDIABETIC ACTIVITY

414 Antidiabetic activity is potency of phenolic compounds to revise glucose uptake or  
415 keep away blood glucose go up.  $\alpha$ -amylase and  $\alpha$ -glucosidase are digestive enzymes  
416 which involve to control dietary carbohydrate and increase in postprandial blood glucose  
417 in human body (Fu *et al.* 2017). The phenolic compounds proven having the capability to  
418 bind protein that they can inhibit  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Hardoko *et al.*  
419 2019; Martinez-Solis *et al.* 2022). Previous research of Werdani and Widyawati (2018),  
420 claimed that pluchea tea infusion is potential as antidiabetic agents. This observation test  
421 is based on the breakdown ability of the substrate to produce a colored product, which is  
422 measured at  $\lambda = 540$  nm. The results showed, that the steeping pluchea tea was able to  
423 inhibit the action of the  $\alpha$ -amylase enzymes (Figure 3). The pluchea tea infusion had very  
424 good activity, more than 50 % and even almost 100 % for fresh pluchea tea which was  
425 brewed at 60, 70 and 80 °C and stored pluchea tea which steeped at 60 °C. Whereas  
426 fresh pluchea tea brewed at 95 °C for 5 min had an activity of inhibiting the alpha amylase  
427 enzyme of less than 50 %, which was equal to  $40.08 \pm 1.12\%$ . Widyawati *et al.* (2017)  
428 detected the ability to inhibit the  $\alpha$ -amylase enzyme from fresh pluchea tea brewed at 95  
429 °C for 5 min by 28.79 %. Increasing the brewing temperature and storage time reduced

430 the ability to inhibit the  $\alpha$ -amylase enzyme. The results of the analysis based on the  
431 ANOVA statistical test at  $\alpha \leq 5\%$  showed, that the brewing temperature and storage time  
432 had a significant effect on the ability to inhibit the  $\alpha$ -amylase enzyme. This ability was  
433 inversely proportional to the levels of total phenolic content, total tannin content, DPPH,  
434 and FRAP. This inhibitory activity was thought to be contributed by other bioactive  
435 compounds, besides phenolics which are sensitive to brewing temperature and storage  
436 time. Li et al. (2018) stated that there are flavonoid compounds that contribute to the  
437 ability to inhibit the  $\alpha$ -amylase enzyme. Flavonoid compounds with a hydroxyl structure  
438 at C-4' in ring B are more effective than C-6 in ring A. Akah et al. (2011) informed that the  
439 phytochemical compounds, such as terpenoids, saponins, flavonoids, glycosides and  
440 carbohydrate, and alkaloids are good antidiabetic metabolites. Sangeetha and Vedaşree  
441 (2012) explained, that the ability to inhibit the  $\alpha$ -amylase enzyme was determined by the  
442 content of the phenolic compound and protein. The  $\alpha$ -amylase inhibitor present in pluchea  
443 tea may be proteinaceous in nature. Aleixandre et al. (2022) informed that phenolic acids  
444 have inhibition activity to  $\alpha$ -amylase enzyme depending their structures. Besides that,  
445 capability of phenolic acids to inhibit  $\alpha$ -amylase was determined by low half-maximum  
446 inhibitory concentration ( $IC_{50}$ ). There are C=C double bond conjugated with a carbonyl  
447 group of phenolic structures that stabilizes the binding forces to the active site of the  $\alpha$ -  
448 amylase. The hydroxyl groups of them are able to bind by non-covalent interaction, such  
449 as hydrogen binding, cation- $\pi$  interactions, salt bridge interactions, ionic interactions or  
450 electrostatic forces with amino acid residue at the active site in  $\alpha$ -amylase. The steeping  
451 temperature and storage time can remove hydroxyl groups of phenolic compounds that



452 can reduce the ability of enzyme inhibition. The phenolic acids with a greater number of  
453 hydroxyl groups are stronger capable to obstruct the  $\alpha$ -amylase enzyme.

454  $\alpha$ -glucosidase is an important enzyme in carbohydrates digestion, that catalysis  
455 the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and converts  
456 them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et al., 2014;  
457 Proenca et al., 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -glucosidase  
458 enzyme is used to determine antidiabetics activity. This is supported by Werdani and  
459 Widyawati (2018), that pluchea tea infusion has the potential as an antidiabetic agent.  
460 Widyawati et al. (2020) found that brewing fresh pluchea tea at 95 °C for 5 minutes has  
461 an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857%.

462 The results showed, that the ability to inhibit the  $\alpha$ -glucosidase enzyme decreased  
463 with increasing brewing temperature and storage time. Brewing at 95°C for fresh pluchea  
464 tea (0 days of storage) obtained the lowest inhibitory ability, i.e.,  $48.32 \pm 1.27\%$ , and the  
465 highest inhibitory activity was found at 70 °C brewing temperature for fresh pluchea tea,  
466 which was  $95.11 \pm 0.70\%$  (Figure 3). The test results showed that the ability to inhibit the  
467 enzyme  $\alpha$ -glucosidase tended to be higher than the ability to inhibit the enzyme  $\alpha$ -  
468 amylase. Li *et al.* (2018) informed that flavonoid compounds have the ability to inhibit the  
469 action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. This is due to the total flavonoids in  
470 brewing pluchea tea which tended to have the same pattern as the ability to inhibit the  
471 activity of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. Flavonoid compounds, such as  
472 rutin, myricetin, kaempferol, and quercetin which have antioxidant and antihyperglycemic  
473 activities. The ability to inhibit the action of enzymes from flavonoid compounds is  
474 determined by the position and number of hydroxyl groups and the number of double

475 bonds in rings A and B and the heterocyclic ring in ring C. The ability to inhibit the  $\alpha$ -  
476 glucosidase enzyme from pluchea tea was significantly affected by the brewing  
477 temperature and storage time. The capability of pluchea tea infusion to obstruct the  $\alpha$ -  
478 glucosidase enzyme was greater than the  $\alpha$ -amylase enzyme because the mechanism  
479 of two enzymes was different, according to the opinion of McCue *et al.* (2005). Widyawati  
480 *et al.* (2017) informed that phenolic and non-phenolic compounds determine the inhibitory  
481 activity of the  $\alpha$ -glucosidase enzyme. The ability of bound phenolic compounds to inhibit  
482  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. The presence of  
483 polymerization and degradation reactions, that may be occurred in pluchea tea during  
484 storage, affects the structure and profile of phenolic and non-phenolic compounds.  
485 Asriningtyas *et al.* (2014) claimed that pluchea leaves contain 3,5-di-*O*-caffeoylquinic  
486 acid, 4,5-di-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid methyl  
487 ester, 3,4,5-tri-*O*-caffeoylquinic acid, and 1,3,4,5-tetra-*O*-caffeoylquinic acid. Quinic acid  
488 is methyl esterified with the number of caffeic groups in the molecule that determines the  
489 activity of inhibiting the  $\alpha$ -glucosidase enzyme. Analysis of caffeoylquinic acids in pluchea  
490 tea infusion was obtained that the higher steeping temperature and longer storage time  
491 caused increased concentration of them, but the  $\alpha$ -glucosidase inhibition of them was  
492 reduced. Aleixandre *et al.* (2022) reported that the simple phenolic acids forming a dipole-  
493 dipole interaction of active site from  $\alpha$ -glucosidase enzyme are effectively inhibiting the  
494 enzyme.

495 This study was obtained information that the increasing of steeping temperature  
496 and storage time caused a degradation reaction of polyphenol compounds to produce  
497 simple phenolic compounds, such as gallic acid, (+)-catechin, myricetin, quercetin,

498 kaempferol, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-  
499 caffeoylquinic acid, supported the results of total phenolic content and total tannin content  
500 assays. Increased concentration of simple phenolic compounds determined the ability of  
501 these compounds as antioxidant agents, but reduced their capability as antidiabetic  
502 agents.

503

## 504 CONCLUSION

505 The steeping temperature and storage time of pluchea tea determined antioxidant  
506 and antidiabetic activities. Profile of phenolic compounds of pluchea tea infusion  
507 influenced antioxidant and antidiabetic activities. Storage time and brewing temperature  
508 caused degradation reaction of polyphenols that resulted simple phenolic compounds.  
509 Gallic acid, (+)-catechin, quercetin, myricetin, kaempferol, 3,4-di-O-caffeoylquinic acid,  
510 3,5-di-O-caffeoylquinic acid and 4,5-di-O-caffeoylquinic acid were simple phenolic  
511 compounds detected from steeping pluchea tea. Increasing of them determined  
512 antioxidant activity that correlated to total phenolic content and total tannin content. Total  
513 flavonoid content had a decreasing graph pattern with increasing storage time that was  
514 similar to the antidiabetic activity graph pattern, which means that the antidiabetic activity  
515 of pluchea tea depended on the total flavonoid content and the structural complexity of  
516 the phenolic compounds.

517

## 518 DATA AVAILABILITY

519 Table and figure used to support of this study were included in the article.

520

521 CONFLICT OF INTEREST

522 The authors declare no conflict of interest.

523

524 ACKNOWLEDGEMENTS

525 The authors would like to thank the he Ministry of Education and Culture of the Republic  
526 of Indonesia for fundamental research grant to higher education institutions in 2022

527

528 REFERENCES

529 ACAR A, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food  
530 wastes:evaluation of temperature and time effects on quality parameters. Applied Food  
531 Res 2(100087): 1-8.

532 ALEIXANDRE A *et al.* 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -  
533 glucosidase and influence of reaction conditions. Food Chem 372(131231): 1-9.

534 ALI *et al.* 2005. Tea and herbal infusuions: their antioxidant activity and phenolic profile.  
535 Food Chem 89(2005): 27-36.

536 ALI A. *et al.* 2018. Impact of storage conditions on the stability of predominant phenolic  
537 constituents and antioxidant activity of dried *piper betle* extracts. Molecules 23(24): 1-13.

538 AL-TEMIMI A, CHOUDHARY R. 2013. Determination of antioxidant activity in different  
539 kinds of plants *in vivo* and *in vitro* by using diverse technical methods. J Nut Food Sci  
540 3(1): 1-6.

541 AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and  
542 methanol extract and fractions of *Gongronema latifolium* (*Asclepidaceae*) leaves in  
543 alloxan diabetic rats. J Applied Pharm Sci 01(09): 99-102.

544 ARSININGTYAS IS. *et al.* 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves  
545 of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product  
546 use. *Natural Product Res* 28(17):1350-1353.

547 ARYAL S *et al.* 2019. Total phenolic content, flavonoid content and antioxidant of wild  
548 vegetables from Western Nepal. *Plants* 8(96): 1-12.

549 AYELE DT, AKELE ML, MELESE A.T. 2022. Analysis of total phenolic contents,  
550 favonoids, antioxidant and antibacterial activities of *Croton macrostachyus* root extracts.  
551 *BMC Chem* 16(30): 1-9.

552 BALAKY HH *et al.* 2021. Estimation of total tannin and total phenolic content in plant  
553 (*Crataegus azarolus* L) by orbital shaker technique. *Intern J Agric Environ Food Sci* 5(1):  
554 1-6.

555 BALIYAN S *et al.* 2022. Determination of antioxidants by DPPH radical scavenging  
556 activity and quantitative phytochemical analysis of *Ficus religiosa*. *Molecules* 27(1326):  
557 1-19.

558 CASTIGLIONI S *et al.* 2015. Influence of steeping conditions (time, temperature, and  
559 particle size) on antioxidant properties and sensory attributes of some white and green  
560 teas. *Intern J Food Sci Nut Online* 1-7.

561 Chan EWC *et al.* 2022. *Pluchea indica*: An updated review of its botany, uses, bioactive  
562 compounds and pharmacological properties. *Pharmaceutical Sciences Asia* 49(1): 77-85.

563 CHANDRA S *et al.* 2014. Assessment of total phenolic and flavonoid content, antioxidant  
564 properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit  
565 crops: A Comparative study. Hindawi Publishing Corporation Evidence-Based  
566 Complementary Alter Med 2014: 1-9.

567 CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic, flavonoids,  
568 tannin and chlorophyll content of leaves of *Strobilanthes Kunthiana* (*Neelakurinji*). *J Med*  
569 *Plants Studies* 4(4): 282-286.

570 FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional  
571 properties and visual appearance of Dampit Robusta coffee leaves tea. *Advances in Food*  
572 *Sci Sustain Agric Agro-industrial Engineering* 4(1): 25-32.

573 FU L *et al.* 2017. Total phenolic contents and antioxidant capacities of herbal and tea  
574 infusions. *Intern J Molecular Sci* 12: 2112-2124.

575 GAO MR *et al.* 2019. A theoretical and experimental study: the influence of different  
576 standards on the determination of total phenol content in the Folin–Ciocalteu assay. *J*  
577 *Food Measurement and Characteristic* 13: 1349–1356.

578 HUANG D, OU B, PRIOR RL. 2005. The Chemistry behind antioxidant capacity assays.  
579 *J Agric Food Chem* 53: 1841-1856.

580 ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties  
581 of rosehip tea beverage: study by response surface methodology. *J Food Sci Tech*  
582 54(11): 3737–3743.

583 JAYANI NIE *et al.* 2022. Evaluation of stability and quality characteristics of moringa  
584 (*Moringa oleifera*) herbal tea during storage. *Food Res* 6(4): 399-406.

585 KIM JM *et al.* 2020. Effect of storage temperature on the antioxidant activity and catechins  
586 stability of Matcha (*Camellia sinensis*). *Food Science and Biotechnology* 29(9): 1261–  
587 1271.

588 KILIC *et al.* 2017. Antioxidant properties of some herbal teas (green tea, senna, corn silk,  
589 rosmery) brewed at different temperatures. Intern J Secondary Metabolite. 4(3): 142-248  
590 Special Issue 1.

591 KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of  
592 extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves  
593 from different provenances in Thailand. Revista Brasileira de Farmacognosia 28(2018):  
594 145–150.

595 KUMAR N, GOEL N. 2019. Phenolic acids: natural versatile molecules with promising  
596 therapeutic applications. Biotechnology Reports 24(e00370): 1-10.

597 LI K *et al.* 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the flavonoids  
598 rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity  
599 relationship of its eight flavonoids by a refined assign score method. Chem Central J  
600 12(82): 1-11.

601 LIN YS. *et al.* 2020. Effects of storage time and temperature on antioxidants in juice from  
602 *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser. Molecules  
603 25(3614): 1-12.

604 MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content  
605 and antioxidant activity test of avocado seed extract (*Persea Americana* Mill). Jurnal MIPA  
606 Unsrat Online. 1(1): 5-10. [https://media.neliti.com/media/publications/116214-ID-  
607 penentuan-kandungan-tanin-dan-uji-aktivi.pdf](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf) [In Bahasa Indonesia]

608 MARTINEZ-SOLIS J *et al.* 2022. Antidiabetic and toxicological effect of the tea infusion  
609 of summer collection from *Annona cherimola* Miller leaves. Plants 11(3224): 1-17.

610 MATHUR R, VIJAYVERGIA R. 2017. Determination of total flavonoid and phenol content  
611 in *Mimusops Elengi* Linn. Intern. J. Pharm. Sci. Res. 8(12): 5282-5285.

612 MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of  
613 sprouted and solid-state bioprocessed soybean. Asia Pacific J Clinical Nut 14(2): 145-  
614 152.

615 MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant  
616 activity: A Review. Intern J Molecular Sci 22(3380): 1-30.

617 NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus*  
618 *sabdariffa* L.): Effect of dying temperature and brewing conditions on total soluble solid,  
619 phenolic content, antioxidant capacity and sensory quality. Beverages 6(2):1-11.

620 NOREEN H. *et al.* 2017. Measurement of total phenolic content and antioxidant activity  
621 of aerial parts of medicinal plant *Coronopus didymus*. Asian Pacific J Tropical Med 10(8):  
622 792–801.

623 NURCHOLIS GW *et al.* 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity in-  
624 vitro and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum pictum*  
625 (L.). Current Biochem 1(2): 58-65.

626 OH J *et al.* 2013. Antioxidant and antimicrobial activities of various leafy herbal teas. Food  
627 Control 31(2013): 403-409.

628 PROENCA C *et al.* 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an in vitro and in silico  
629 structure–activity relationship study. J Enzyme Inhibition Med Chem 32(1): 1216–1228.

630 PUREWAL SS *et al.* 2022. Unraveling the effect of storage duration on antioxidant  
631 properties, physicochemical and sensorial parameters of ready to serve Kinnow-aml  
632 beverages. Applied Food Res 2(1): 1-8.



633 RAMPHINWA ML *et al.* 2023. Eco-physiological response of secondary metabolites of  
634 teas: review of quality attributes of herbal tea. *Frontiers in Sustain Food Systems*  
635 7(990334): 1-14.

636 REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids.  
637 *Czech J Food Sci* 30(2): 171-177.

638 RUAN J *et al.* 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea*  
639 *indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel  
640 column chromatography fractions. *Molecules* 24(2784): 1-20.

641 RUSITA YD *et al.* 2019. Comparison of tannin concentration on fresh and dried tea leaf  
642 (*Camelia sinensis* L.) extract using UV-visible spectrophotometric method. 1st  
643 International Conference of Health, Science & Technology (ICOHETECH) 2019 E-ISBN:  
644 978-623-92207-0-9. November 16<sup>th</sup>, 2019.LPPM Universitas Duta Bangsa Surakarta,  
645 Surakarta.

646 SANGEETHA R, VEDASREE N. 2012. In Vitro  $\alpha$ -Amylase inhibitory activity of the leaves  
647 of *Thespesia populnea*. *Intern. Scholarly Res.Network.* 2012: 1-4.

648 SHRAIM AM *et al.* 2021. Determination of total flavonoid content by aluminum chloride  
649 assay: A critical evaluation. *LWT Food Sci Tech* 150(111932): 1-11.

650 SILVA-RAMIREZ KA *et al.* 2020. Effect of drying and steeping temperature on the  
651 phenolic content, antioxidant activity, aromatic compounds and sensory properties of  
652 *Culila polyantha* Benth. *Infusions. Processes* 8(1378): 2-20.

653 SRISOOK K *et al.* 2012. Antioxidant and anti-inflammatory activities of hot water extract  
654 from *Pluchea indica* Less. herbal tea. *J Med Plants Res* 6(23): 4077-4081.

655 SURIYAPHAN O. 2014. Nutrition, health benefits and applications of *Pluchea indica* (L)  
656 Less leaves. Pharm Sci Asia 41: 1–10.

657 SU D *et al.* 2019. Impact of thermal processing and storage temperature on phenolic  
658 profile and antioxidant activity of different varieties of lychee juice. LWT-Food Tech  
659 116(108578): 1-9.

660 THANAJIRUSCHAYA P *et al.* 2010. Effect of storage time and temperature on antioxidant  
661 components and properties of milled rice. Khon Kaen Univ Res J15(9): 843-851.

662 VONGSAK B *et al.* 2018. Comparison of active constituents, antioxidant capacity, and  
663 αglucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. Food  
664 Bioscience 25: 68–73.

665 WANG SC. *et al.* 2022. Effect of brewing conditions on polyphenols in the dark tea  
666 (*Camellia sinensis* L.) infusions: content, composition and antioxidant activities. Food Sci  
667 Tech 42(e36322): 1-9.

668 WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica*  
669 Less as functional beverage in diabetic patients. ASSEHR 98: 164-167.

670 WIDYAWATI PS *et al.* 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract  
671 as antioxidant and anti-warmed-over flavor (WOF) of duck meat. International Congress  
672 on Challenges of Biotechnological Research in Food and Health. November 15<sup>th</sup>  
673 2014. Agricultural Technology Faculty, Slamet Riyadi University, Surakarta.

674 WIDYAWATI PS *et al.* 2016. The physicochemical and antioxidant properties of *Pluchea*  
675 *indica* Less drink in tea bag packaging. IJFNS 5: 13–120.

676 WIDYAWATI PS *et al.* 2017. In vitro antioxidant capacities and antidiabetic properties of  
677 pluchea leaves. IJPPS 9(8): 203-208.

678 WIDYAWATI PS *et al.* 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica*  
679 Less beverage. CJFST12(4): 125-139.

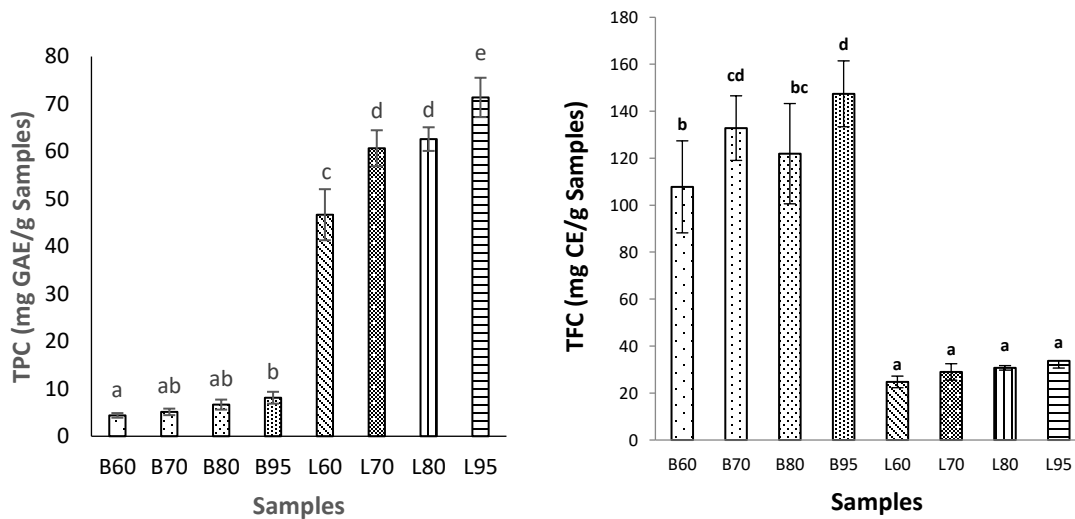
680 WIDYAWATI PS *et al.* 2022. The effect of  $\kappa$ -carrageenan proportion and hot water  
681 extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of stink  
682 lily (*Amorphophallus muelleri*) wet noodles. *Molecules* 27(5062): 1-16.

683 Widyawati PS *et al.* 2023. The effect of hot extract of *Pluchea indica* leaf powder on the  
684 physical, chemical and sensory properties of wet noodles. *CRNFS* 11(1): 276-293.

685 XU P, CHEN L, WANG Y. 2019. Effect of storage time on antioxidant activity and inhibition  
686 on  $\alpha$ -amylase and  $\alpha$ -glucosidase of white tea. *Food Sci Nut* 7(2): 636-644.

687 ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction time on caffeine in  
688 coffee. *Lantanida J* 6(2): 103-202. [https://jurnal.ar-](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811)  
689 [raniry.ac.id/index.php/lantanida/article/view/3811](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811). [In Bahasa Indonesia]

690 ZHANG Y *et al.* 2022. Effect of storage conditions and time on the polyphenol content of  
691 wheat flours. *Processes* 9(248): 1-11.



692

693

(a)

(b)

694

695

696

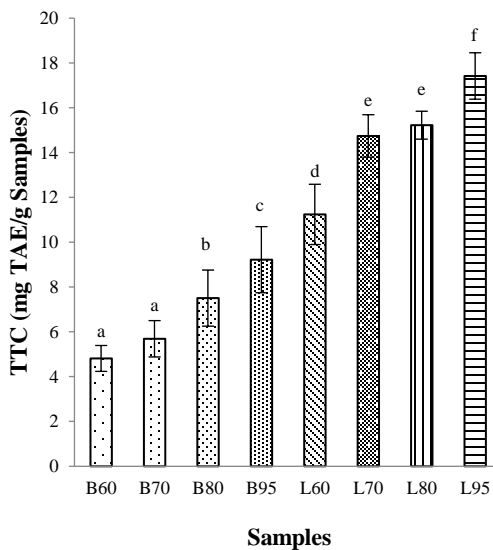
697

698

699

700

701



702

(c)

703

704

705

706

707

708

Figure 1. Bioactive compound content of pluchea tea at different steeping temperature and storage time (a) Total phenolic content (b) Total flavonoid content (c) Total tannin content (Values were means  $\pm$  standard deviations (n=6). Different superscripts in graph showed a significant difference based on the DMRT test ( $\alpha \leq 5\%$ ))

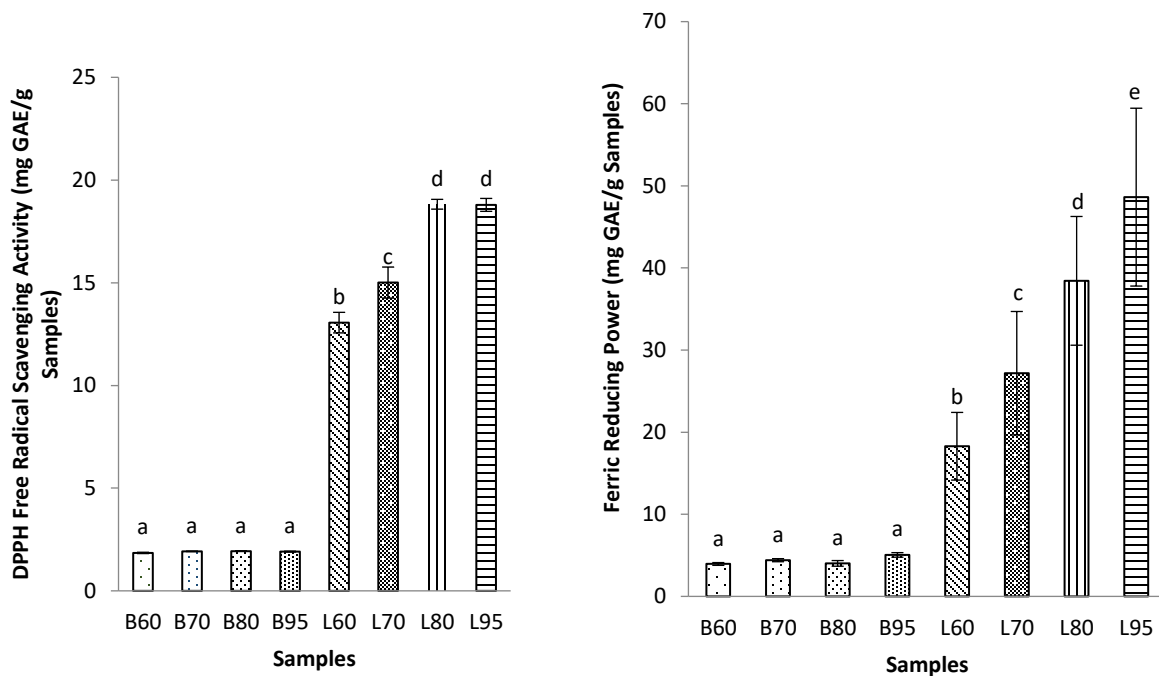
709 Table 1. Phenolic Compound Profile of Pluchea Tea Infusion at Different Steeping Temperature and Storage Time

Samples	Gallic Acid ( $\mu\text{g/g}$ samples)	(+)-Catechin ( $\mu\text{g/g}$ samples)	Myricetin ( $\mu\text{g/g}$ samples)	Quercetin ( $\mu\text{g/g}$ samples)	Kaempferol ( $\mu\text{g/g}$ samples)	3,4-di- <i>O</i> -	3,5-di- <i>O</i> -	4,5-di- <i>O</i> -
						Caffeoylquinic acid ( $\mu\text{g/g}$ samples)	Caffeoylquinic acid ( $\mu\text{g/g}$ samples)	Caffeoylquinic acid ( $\mu\text{g/g}$ samples)
B60	0.2132 $\pm$ 0.0027	0.3425 $\pm$ 0.0110	0.1756 $\pm$ 0.1234	0.0220 $\pm$ 0.0268	0.1394 $\pm$ 0.0202	0.6103 $\pm$ 0.0628	0.6635 $\pm$ 0.0628	0.4906 $\pm$ 0.0060
B70	0.2157 $\pm$ 0.0013	0.3260 $\pm$ 0.0265	0.2587 $\pm$ 0.0160	0.1530 $\pm$ 0.0511	0.0514 $\pm$ 0.0037	0.6271 $\pm$ 0.0099	0.6162 $\pm$ 0.0099	0.4807 $\pm$ 0.0034
B80	0.2234 $\pm$ 0.0122	0.3240 $\pm$ 0.0222	0.4175 $\pm$ 0.0104	0.3666 $\pm$ 0.0103	0.3699 $\pm$ 0.0924	0.7967 $\pm$ 0.03060	0.6601 $\pm$ 0.0306	0.5299 $\pm$ 0.0053
B95	0.2316 $\pm$ 0.0104	0.4039 $\pm$ 0.0320	0.8786 $\pm$ 0.0434	0.6559 $\pm$ 0.0570	0.5913 $\pm$ 0.0239	1.5386 $\pm$ 0.0668	0.6642 $\pm$ 0.0668	1.0018 $\pm$ 0.0526
L60	0.2364 $\pm$ 0.0015	0.5085 $\pm$ 0.0111	1.4762 $\pm$ 0.0271	0.6220 $\pm$ 0.0706	0.3675 $\pm$ 0.0183	2.4863 $\pm$ 0.0270	0.9449 $\pm$ 0.0501	1.1842 $\pm$ 0.0120
L70	0.2324 $\pm$ 0.0214	0.5448 $\pm$ 0.0006	1.4245 $\pm$ 0.2526	1.0708 $\pm$ 0.0289	0.3726 $\pm$ 0.0944	2.3403 $\pm$ 0.0325	0.9485 $\pm$ 0.0794	1.0089 $\pm$ 0.0736
L80	0.2347 $\pm$ 0.0078	0.5023 $\pm$ 0.0773	1.457 $\pm$ 0.0925	0.8629 $\pm$ 0.0815	0.7966 $\pm$ 0.0366	2.6278 $\pm$ 0.0211	0.9099 $\pm$ 0.0387	1.2382 $\pm$ 0.1435
L95	0.2402 $\pm$ 0.0169	0.5995 $\pm$ 0.0372	2.6138 $\pm$ 0.0695	2.0230 $\pm$ 0.0573	0.9478 $\pm$ 0.0287	4.0211 $\pm$ 0.0851	1.3156 $\pm$ 0.0166	1.3797 $\pm$ 0.2170

710 Note : data of phenolic compound profile was obtained from two replicates that displayed as mean  $\pm$  SD

711

712

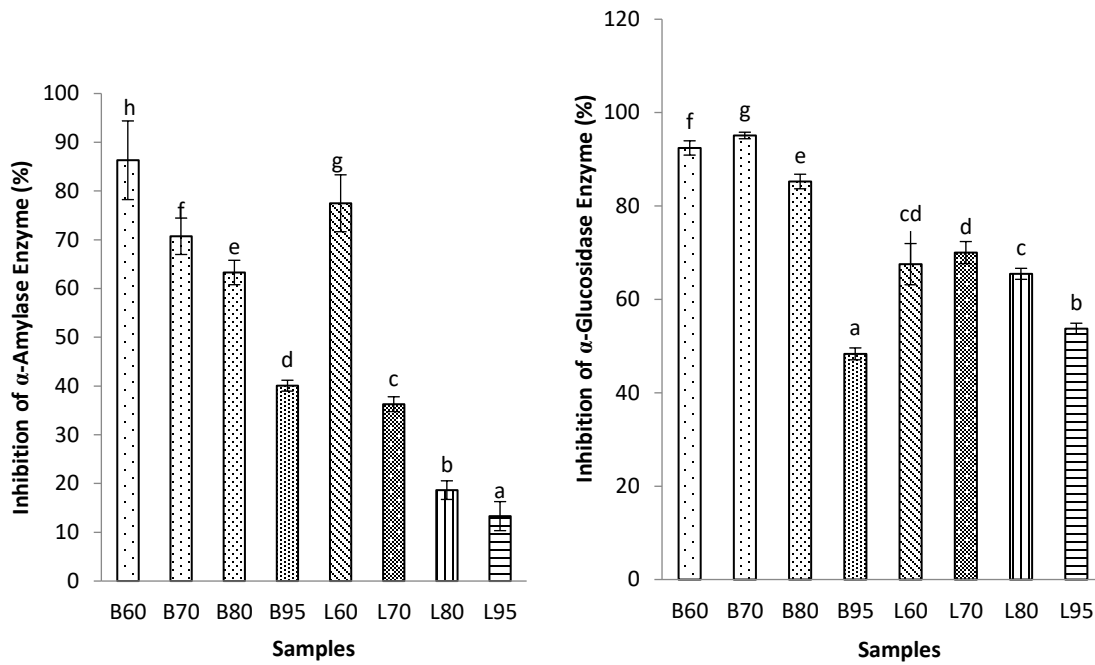


(a)

(b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage time (a) DPPH (b) FRAP (Values were means  $\pm$  standard deviations (n=6). Different supercripts in graph showed a significant difference based on the DMRT test ( $\alpha \leq 5\%$ ))

713  
714  
715  
716  
717  
718  
719



(a)

(b)

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage time (a)  $\alpha$ -amylase (b)  $\alpha$ -glucosidase (Values were means  $\pm$  standard deviations (n=6). Different supercripts in graph showed a significant difference based on the DMRT test ( $\alpha \leq 5\%$ ))



Paini Sri Widyawati <paini@ukwms.ac.id>

---

**(no subject)**

---

**Philippine Journal of Science** <philjournsci@gmail.com>

Fri, May 5, 2023 at 8:47 AM

To: Paini Sri Widyawati <paini@ukwms.ac.id>

Dear Dr. Widyawati,

This is to confirm the receipt of your latest manuscript submission. We will send another email to issue your reference number.

Thank you for considering the Philippine Journal of Science (PJS) as the venue for reporting your research findings!

Sincerely,

Ms. CARYL MARIA MINETTE I. ULAY

Editorial Assistant

For Dr. CAESARA A. SALOMA

Editor-in-Chief

[Quoted text hidden]





Paini Sri Widyawati <paini@ukwms.ac.id>

---

**(no subject)**

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <philjournsci@gmail.com>

Fri, May 5, 2023 at 8:52 AM

Dear Ms Caryl Maria Minette

Thanks for your attention

Regards

Paini Sri W

[Quoted text hidden]



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**Acknowledgment - PJS Paper Ms -158**


---

**Philippine Journal of Science** <philjournsci@gmail.com>  
 To: Paini Sri Widyawati <paini@ukwms.ac.id>  
 Cc: Caesar Saloma <caesar.saloma@gmail.com>

Fri, May

Dear Dr. Widyawati,

In relation to your manuscript titled "Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea Indica* Less Tea" submitted for publication in the Philippine Journal of Science (PJS), your reference number is Ms 23-158.

Your paper will be relayed to the reviewers and editors for evaluation. We will keep you informed regarding the status of your paper.

Thank you very much!

Sincerely,  
 Ms. CARYL MARIA MINETTE I. ULAY  
 Editorial Assistant

For Dr. CAESAR A. SALOMA  
 Editor-in-Chief

–  
**(no subject)**

6 messages

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
 To: Philippine Journal of Science <philjournsci@gmail.com>

Wed, Apr 26,

Dear Dr. CAESAR A. SALOMA  
 Editor-in-Chief in PJS

Sincerely,

I am interested in publishing my manuscript in PJS so that I send my manuscript with the title " Effect of Steeping Temperature and Storage Time on the Bioactive Compounds, Antioxidant and Antidiabetic Activities of *Pluchea Indica* Less Tea" . I also send a cover letter, list reviewer recommendation and form an authorship statement to be considered .

Thanks for attention


Regards

Paini Sri Widyawati

---

**4 attachments**

 COVER LETER.pdf  
 68K

 [PRO] Form - Authorship Statement3.pdf  
 86K

 LIST REVIEWER RECOMMENDATION.pdf  
 149K

 Effect of Brewing Temperature and Storage Time on Antioxidant and Antidiabetic Properties of Pluchea Tea Final.docx  
 92K

---

**Philippine Journal of Science** <philjournsci@gmail.com>  
 To: Paini Sri Widyawati <paini@ukwms.ac.id>

Fri, Apr 28,

Dear Dr. Widyawati,

Thank you very much for considering the Philippine Journal of Science (PJS) as the venue for publishing your research efforts! We wish to inform you that a complete submission must include the following:

[2] Manuscript

- It must strictly adhere to the PJS format in writing articles, particularly in the references section (*i.e.* the journal name must not be punctuated by a period, the volume and issue numbers separated from the page numbers by a space, the page range must be indicated using an en dash – instead of a hyphen -, *etc.*).

You can find the additional descriptions of the requirements in the PJS Author's Guide (<https://philjournsci.dost.gov.ph/author-s-guide>). A reference number will be provided once you have met all requirements. Incomplete submissions will not be reviewed.

Again, thank you for your interest in PJS! We look forward to your complete submission.

Sincerely,  
 Mr. ALLYSTER A. ENDOZO  
 Managing Editor

3. Status Manuscript: Information about the manuscript (8-7-2023)  
-Correspondence



Paini Sri Widyawati <paini@ukwms.ac.id>

---

## status my manuscript

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <philjournsci@gmail.com>

Sat, Jul 8, 2023 at 9:45 PM

Dear Ms. CARYL MARIA MINETTE I. ULAY  
Editorial Assistant

Please, inform me of the status of my manuscript titled "Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea Indica* Less Tea" (MS-158).

Thanks for attention

Regards

Paini Sri Widyawati



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**status my manuscript**

---

**Philippine Journal of Science** <philjournsci@gmail.com>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Sun, Jul 9, 2023 at 2:11 PM

Dear Dr. Widyawati,

Greetings! We appreciate you seeking the evaluation status of Ms 23-158. Fortunately, we have secured one (1) expert who commits to evaluating your manuscript. The reviewer is expected to turn over recommendations within 09 July 2023 (Sunday) unless requested for an extension of submission not longer than a month. As of the moment, we are actively inviting more experts to evaluate your manuscript. You will hear from us again once the first round of evaluation is completed. Thank you for understanding.

Sincerely,

Ms. CARYL MARIA MINETTE I. ULAY  
Editorial Assistant

[Quoted text hidden]

--



**Philippine Journal of Science**  
**Science and Technology Information Institute**  
**Department of Science and Technology**  
**DOST Complex, Gen. Santos Ave., Bicutan 1631**  
**Taguig City, Metro Manila, Philippines**  
Telephone no. : 837 - 2191  
Email: [pjs@stii.dost.gov.ph](mailto:pjs@stii.dost.gov.ph); [philjournsci@gmail.com](mailto:philjournsci@gmail.com)  
Website: <https://philjournalsci.dost.gov.ph>  
Scopus: <https://www.scopus.com/sourceid/19700175735>



Paini Sri Widyawati <paini@ukwms.ac.id>

---

## status my manuscript

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <philjournsci@gmail.com>

Sun, Jul 9, 2023 at 3:01 PM

Dear Ms Ms. CARYL MARIA MINETTE I. ULAY  
Editorial Assistant

Thanks for the information, I am glad to hear that.

Regards

Paini Sri Widyawati

[Quoted text hidden]

4. Second Revision: Major Revision (13-10-2023)
  - Correspondence
  - Review Note
  - Document



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**Fwd: Comments on PJS Paper Ms 23-158**

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Fri, Oct 13, 2023 at 4:26 PM

----- Forwarded message -----

From: **Philippine Journal of Science** <philjournsci@gmail.com>  
Date: Wed, Sep 20, 2023 at 12:28 PM  
Subject: Comments on PJS Paper Ms 23-158  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

DR. PAINI SRI WIDYAWATI  
Food Technology Study Program  
Agricultural Technology Faculty  
Widya Mandala Surabaya Catholic University  
Surabaya, Indonesia

Dear Dr. Widyawati,

This refers to your paper titled "Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea indica* Less Tea" [Ms 23-158] that was submitted for possible publication in the Philippine Journal of Science (PJS).

On behalf of Dr. Caesar A. Saloma, we are sending you the letter from the Editor-in-Chief and the comments of the reviewers regarding its need for revision. Also attached is a copy of your manuscript with the reviewer's comments or suggestions.

Please submit an itemized list of your answers to the said comments together with the revised version of your paper. You may also provide your rebuttal should you not agree with the comments. Let us also know if you have received this letter.

Thank you very much! We look forward to receiving your revised paper.

Sincerely,  
Ms. CARYL MARIA MINETTE I. ULAY  
Editorial Assistant

For Dr. CAESARA. SALOMA  
Editor-in-Chief



REPUBLIC OF THE PHILIPPINES  
**PHILIPPINE JOURNAL  
OF SCIENCE**

**Philippine Journal of Science**  
**Science and Technology Information Institute**  
**Department of Science and Technology**  
**DOST Complex, Gen. Santos Ave., Bicutan 1631**  
**Taguig City, Metro Manila, Philippines**



Telephone no. : 837 - 2191

Email: [pjs@stii.dost.gov.ph](mailto:pjs@stii.dost.gov.ph); [philjournsci@gmail.com](mailto:philjournsci@gmail.com)

Website: <https://philjournalsci.dost.gov.ph>

Scopus: <https://www.scopus.com/sourceid/19700175735>



REPUBLIC OF THE PHILIPPINES  
**PHILIPPINE JOURNAL  
OF SCIENCE**

**Philippine Journal of Science**  
**Science and Technology Information Institute**  
**Department of Science and Technology**  
**DOST Complex, Gen. Santos Ave., Bicutan 1631**  
**Taguig City, Metro Manila, Philippines**

Telephone no. : 837 - 2191

Email: [pjs@stii.dost.gov.ph](mailto:pjs@stii.dost.gov.ph); [philjournsci@gmail.com](mailto:philjournsci@gmail.com)

Website: <https://philjournalsci.dost.gov.ph>

Scopus: <https://www.scopus.com/sourceid/19700175735>

---

**2 attachments**



**Ms 23-158\_DRAFT [Review Notes].pdf**

164K



**R1 Ms 23-158 Reviewer 2 Comments on Manuscript.docx**

134K



Republic of the Philippines  
**DEPARTMENT OF SCIENCE AND TECHNOLOGY**  
**SCIENCE AND TECHNOLOGY INFORMATION INSTITUTE**



**ISO 9001:2015**  
Certificate No. 50500772 QM15

20 September 2023

**DR. PAINI SRI WIDYAWATI**

Food Technology Study Program  
Agricultural Technology Faculty  
Widya Mandala Surabaya Catholic University  
Surabaya, Indonesia

Dr. Widyawati,

Thank you for considering the **Philippine Journal of Science (PJS)** as a venue for publication of your research paper.

After a thorough evaluation of specialists in your field, it is recommended that your paper titled **"Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea indica* Less Tea" [Ms 23-158]** can be considered for publication only after the following revisions or comments are answered and complied with.

Attached is a copy of the reviewers' comments and recommendations on your paper. Please submit a copy of your revised paper and a checklist of your point-for-point answers to reviewers' comments **not later than 30 calendar days** upon receipt of this letter. Otherwise, we will consider the paper as new submission. You may send it *via* email at [philjournsci@gmail.com](mailto:philjournsci@gmail.com) or [pjs@stii.dost.gov.ph](mailto:pjs@stii.dost.gov.ph).

Thank you! We hope to receive your revised manuscript soon.

Sincerely yours,

**CAESAR A. SALOMA**

Editor-in-Chief, PJS  
Professor, National Institute of Physics  
University of the Philippines Diliman  
Quezon City, Philippines  
Encl: a/s



## COMMENTS ON THE PAPER

### Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea indica* Less Tea

#### GENERAL

The manuscript determines the effect of steeping temperature and storage time on the bioactive compounds, antioxidant, and antidiabetic activities of *Pluchea indica* Less tea infusion. The research used a randomized block design with two factors, *i.e.*, steeping temperature (60, 70, 80, and 95 oC) and storage time (0 and 5 years). The steeping temperature and storage time influenced the bioactive compounds, antioxidant and antidiabetic activities of samples.

The manuscript, having gained varying comments from the reviewers requires minor and major revisions. The specific comments of the two reviewers are discussed herein.

#### Reviewer 1 (General)

My overall evaluation is that the whole paper is unacceptable because it needs an extensive revision; from the title to the literature cited to show the clarity in the appropriateness of the research design followed and validity of statistical analysis employed, an accurate description of the materials and method to ensure repeatability/reproducibility, accuracy in interpretation of the explanation of the findings, as well as accuracy in reconciling the RRL citations with the literature cited section. The tables and figures must be appropriately labelled for better readability. Only after such have been accomplished, paper may be again re-evaluated for possible acceptance. My major and minor comments are found in the manuscript.

#### Reviewer 2 (General)

Statistical analysis used in comparing means of fresh and stored samples should be a T-test because only 2 treatments were considered. Separated the statistical analysis for comparing the means to establish the effect of steeping temperature from the effect of fresh versus stored tea. The correlation coefficient of parameters analyzed should be statistically analyzed (Pearson, Kendall, or Spearman) and not be based on the similarity of trends observed. Because of the inappropriate statistical analysis used some discussions and conclusions are inappropriate which will be detailed in the specific comments and recommendations below. Tables and Figures should be easily understood by the reader. A legend should be provided in each table and figure to establish the condition meant by the B60, B70, B80, B95, L60, L70, L80, and L95. B is not obvious to fresh and L is not recognizable to be the stored treatment.



**Specific Comments and Recommendations**

<b>Page</b>	<b>Line</b>	<b>Comments and Recommendations</b>
14	312-313	The statement is not conclusive since only 1 storage condition was compared with the fresh sample, thus decreasing TFC with increasing storage time is inappropriate. The study should have considered at least 3 storage conditions to establish this claim. The statement to be restated that there is lower TFC for stored tea compared with the fresh counterpart. Moreover, T—test should have been used to compare the fresh with the stored sample,
15-16	337-339	Same comment as above the study was not able to establish the effect of increasing storage time since only 1 storage condition was compared with the fresh.
15-16	337-344	Statements keep on repeating. The paragraph should be reviewed to make concise statements. The author should ask a language editor to review the whole paper.
18	397-398	Same comment as above the study was not able to establish the effect of increasing storage time since only 1 storage condition was compared with the fresh.
18	401-403	Correlation coefficient of parameters analyzed should be statistically analyzed (Pearson, Kendall, or Spearman) to establish the claims in this statement.
20	432-434	Correlation coefficient of parameters analyzed should be statistically analyzed (Pearson, Kendall, or Spearman) to establish the claims in this statement.

1 **Effect of Steeping Temperature and Storage Time on the Bioactive Compounds,**  
2 **Antioxidant and Antidiabetic Activities of *Pluchea* *Pluchea Indica Less Tea***

3 Painsi Sri Widyawati<sup>1)</sup>, Yufita Ratnasari Wilianto<sup>2)</sup>

4 <sup>1)</sup>Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala  
5 Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

6 <sup>2)</sup>Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic  
7 University, Kalisari Street Number 1, Surabaya 60272, Indonesia

8 Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature,  
9 *Pluchea* *Pluchea indica Less*, storage time

**Commented [A1]:** Check the experimental samples that you used; it is not explicitly stated in the methodology. Assuming that it is the infusion that you analyzed. Suggested working title is **Effect of Steeping Temperature and Storage Time on the Bioactive Compounds, Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea Indica Less Leaf***

**Commented [A2]:** The term tea is a beverage/drink from the tea plant, *Thea sinensis* only. Infusion is drink from other plant sources like dried leaves flowers or fruits, etc other than the tea plant. Tea may also be described as an infusion.

20 ABSTRACT

21 This study was done to determine the effects of steeping temperature and storage time  
22 on the bioactive compounds, antioxidant and antidiabetic activities of *PlucheaPluchea*  
23 *indica* Less tea infusion. The research used a randomized block design with two  
24 factors, i.e., steeping temperature (60, 70, 80, and 95 °C) and storage time (0 and 5  
25 years). The steeping temperature and storage time influenced the bioactive compounds,  
26 antioxidant and antidiabetic activities of samples. Total phenolic content and total tannin  
27 contents went up along with increased antioxidant activity. Treatment resulted simple  
28 phenolic compounds, such as gallic acids, (+)-catechins, kaempferols, myricetins,  
29 quercetins, 3,4-di-O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, 4,5-di-O-  
30 caffeoylquinic acids. Total flavonoid content was decreased for storage time and  
31 significant different at higher steeping temperatures. The total flavonoid content had  
32 graph pattern similar with  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition activities. This means,  
33 the antidiabetic activity was largely determined by the total flavonoid content and structure  
34 of phenolic compounds. In order, to get high antioxidant activity, it was chosen  
35 *plucheaPluchea* tea stored at high steeping temperature, but high antidiabetic  
36 activity was fresh *plucheaPluchea* tea steeped at a low temperature.

38 INTRODUCTION

39 *PlucheaPluchea* tea is a product of *plucheaPluchea* leaf processing introduced by world  
40 people (Srisook *et al.* 2012; Widyawati *et al.* 2016) because of the efficacy of the active  
41 components in *plucheaPluchea* leaves, as an herbal plant that has been widely used for  
42 traditional medicine and food (Chan *et al.* 2022). *PlucheaPluchea* tea is composed many

**Commented [A3]:** Generally, lacks clarity and must be revised accordingly.

**Commented [A4]:** In what samples samples? ...increased with increasing ...rather than went

**Commented [A5]:** You cannot state this because you have no control (no steeping, no storage). The control may also contain the same simple/specific bioactive compounds

**Commented [A6]:** Statement is still vague, no sentence parallelism

**Commented [A7]:** Analyze again your graphs, they do not show similar pattern.

**Commented [A8]:** You do not have to include this because you did not looked into the structure; you merely assumed

**Commented [A9]:** Fresh leaf? Did you use fresh leaf for storage and steeping?

**Commented [A10]:** The abstract must give an overview of what the study is all about. More importantly, it should indicate the findings that answer the research questions, highlighting the significance of the treatment means. Conclusion and recommendation should also be stated. A readable abstract should not be more than 300 words.

The abstract as presented lacks clarity and content. As an experimental research, you need to show explicitly, control, independent and dependent variable

**Commented [A11]:** -too many incomplete observations in a sentence

**Commented [A12]:** Author mentioned were not properly cited in the list of References/Literature cited section. RRL cited in this section as well as in the other sections of this paper must be properly cited and must be listed in the References/Literature cited section.

**Commented [A13]:** For all literature review, kindly checked the samples used i.e., infusion/or drink; fresh leaf, dried leaf, powdered leaf

43 nutrients and bioactive compounds useful to body health. The nutrient compositions in  
44 the *Pluchea Pluchea* tea include protein, fat, ash, insoluble fiber, soluble fiber,  
45 carbohydrates, calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive compounds is  
46 comprised, i.e., chlorogenic acid, caffeic acid, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic  
47 acid, 5-O-caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid,  
48 4,5-di-O-caffeoylquinic acid, quercetin, myricetin, kaempferol, total anthocyanin,  $\beta$ -  
49 carotene, and total carotenoid (Suriyaphan 2014; Vongsak *et al.* 2018; Ruan *et al.* 2019;  
50 Widyawati *et al.* 2022, Chan *et al.* 2022).

51 Steeping process of *Pluchea Pluchea* tea leaves can be performed with fresh or dry  
52 leaves infusion by hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez  
53 *et al.* 2020; Jayani *et al.* 2022). In Asian area, especially in Indonesian, people usually  
54 consume the *Pluchea Pluchea* tea with brewing of powdered *Pluchea Pluchea* leaves in  
55 tea bag by hot water or boiling water. Each tea bag contained 2 g of *Pluchea Pluchea* leaf  
56 powder is steeped with 100 mL hot water or boiling water. Widyawati *et al.* (2016) claimed  
57 that steeping of 2 g *Pluchea Pluchea* tea at 95 °C for 5 minutes results in the total phenolic  
58 content, total flavonoid content, the ability to scavenge DPPH free radicals, and the  
59 capability of reduce ferric ions at 9.3 mg gallic acid equivalent (GAE)/g samples, 22.0 mg  
60 gallic acid equivalent (GAE)/g samples, 27.2 mg gallic acid equivalent (GAE)/g samples,  
61 and 10.2 mg gallic acid equivalent (GAE)/g samples, respectively. Werdani and  
62 Widyawati (2018) reported that drinking of *Pluchea Pluchea* tea in the morning and  
63 evening regularly (2 g/100 mL) can decline blood sugar levels.

64 Steeping *Pluchea Pluchea* tea with hot water at 95 °C for 5 min certainly determines  
65 the stability and amount of extracted bioactive compounds, that influences the biological

66 activity, especially antioxidant and antidiabetic activities. Silva-Ramirez *et al.* (2020)  
67 reported that the infusion process can influence their content and composition of the  
68 bioactive compounds and antioxidant activity of tea. Acar *et al.* (2022) informed that  
69 infusion quality of herb tea extract depends on several factors, i.e., time and temperature.  
70 Polyphenol profile and antioxidant properties of herb tea infusion decline with an increase  
71 in steeping/brewing and storage temperatures and longer exposure times.

72 Several studies have mentioned the effect of steeping temperature to bioactive  
73 compounds and antioxidant activity, such as some white and green teas are effective with  
74 hot water at 90 °C for 7 min (Castiglioni *et al.* 2015), roship tea is effectively at infusion  
75 time around 6-8 min at temperatures of 84-86 °C (Ilyasoglu and Arpa 2017), the coffee  
76 brewing temperature influences the caffeine content extracted (Zarwinda and Sartika  
77 2018), the steeping of dark tea at 92 °C for 27 min results the highest total phenol content  
78 and antioxidant activity (Wang *et al.* 2022). The study of the effect of steeping temperature  
79 to *plucheaePlucheae* tea infusion was carried out to afford information about preparation of  
80 *plucheaePlucheae* tea most efficiently to get higher the bioactive compounds, antioxidant  
81 and antidiabetic activities.

82 On the other hand, storage time of *plucheaePlucheae* tea also affects the levels of  
83 the bioactive compounds and biological activity because this tea usually is stored for a  
84 several months until years (Jayani *et al.* 2022). Tea or herbal tea is generally stored in  
85 ambient temperature and packed in tea bag or Alu foil standing proud or a combination  
86 of both. Many researchers informed that storage time decreases the bioactive  
87 compounds, antioxidant and antidiabetic activities, i.e., juice from *Momordica charantia* L.  
88 (Lin *et al.* 2020), dried *Piper bettle* extracts (Ali *et al.* 2018), white tea (Xu *et al.* 2019),





112 glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-O-caffeoylquinic  
113 acid, 4,5-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and (+)-catechin were  
114 purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, Folin–Ciocalteu’s Phenol,  
115 sodium nitric, aluminum chloride, ferric chloride, sodium dihydrogen phosphate, sodium  
116 phosphate, potassium ferricyanide, starch, acetic acid, and sodium hydroxide were  
117 purchased from Merck (Kenilworth, NJ, USA). All reagents used were of analytical grade  
118 except for Aquadest and aquabidest which were was purchased from by PT Aqua  
119 Surabaya.

120  
121 METHODOLOGY Describe the preparation of the Plucheae leaf either dried leaf or powder.

122 Describe the preparation of the infusion samples.

123 Describe sampling and extraction procedure.

124 ANALYSIS OF THE BIOACTIVE COMPOUNDS

125 Total Phenolic Content Analysis

126 Total phenolic content of ~~steeping-treated plucheae~~ Plucheae infusion tea was was  
127 carried out using the technique by conducted by Gao *et al.* (2019). method based on  
128 spectrophotometric analysis. Total phenolic content assay using redox analysis between  
129 phenolic compounds and phosphomolybdic/ phosphotungstic acid complexes is founded  
130 on the electron transfer in an alkaline medium from the phenolic compounds to result a  
131 blue colored solution because of phosphotungstic/ phosphomolybdenum complex  
132 formation. Color inrtensity was measured in the. Total phenolic content was measured by  
133 Spectrophotometer (spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  760 nm with

**Commented [A22]:** If you followed the procedure of Widyawati, then this is.....*Plucheae* leaf powder

**Commented [A23]:**  
Describe drying method employed :Artificial drying at what temp or sundrying or at ambient temp for how long preferably what type of material used, dimension of the bag

**Commented [A24]:** Preparation of the infusion samples- lines 102-105  
Describe the preparation of the infusion  
eg. The 2 gram powder bag was steeped in 100 mL distilled water for 5 minutes at various temperatures namely, 60, 70, 80 and 95C and *Plucheae* powder was stored at 0 and 5 year -storage period.

**Commented [A25]:** Describe the sampling . procedure and extraction methods of the bioactive compounds

Sampling and Extraction of the bioactive compounds -lines 105-107

Describe the sampling and extraction of the bioactive compounds of the treated samples and that of the control.

Were the bioactive compounds extracted from the infusion or powder itself?

**Commented [A26]:** Analyses

In the description of the analyses done, you can do any of the following:

1.) simply mention the source cited but the source **must be fully cited in the references/literature section**

eg. **Phytochemical Contents.** The total phenolic content was determined by the Folin-Ciocalteu Assay while the total tannin analysis was conducted using the modified vanillin method. The total flavonoid concentration was measured using a calorimetric assay developed by Zhishen et al. (1999) while the method of Hosttetman and Marso (1995) for the analysis of saponins was used. For the alkaloids contents, the method developed by Hultin and Torsell (1965) was used in the analysis.

2 Describe briefly but completely the procedure. Source must also be well cited in the reference/literature cited section.

**Commented [A27R26]:**

**Commented [A28]:** There are many vague description or statements that are found. Clarity is needed. Revise according to the original source or literature. If there is modification, state briefly. Present the formula used and units of expression of the measured samples. Descriptions of all methods of analysis presented need revisions based ...

**Commented [A29]:** For clarity, for each of the methods, described clearly but briefly the principle that is involved.

134 ~~gallic acid as the and a~~ reference standard ~~was a gallic acid~~. The total phenolic content  
135 was calculated using the formula:

136 \_\_\_\_\_ (formula)

137 3The results were expressed as mg gallic acid equivalents (GAE)/g samples.

Formatted: Indent: First line: 0"

138

### 139 TOTAL FLAVONOID CONTENT ASSAY

Commented [A30]:

140 Total flavonoid content of the samples was ~~determined~~ measured based on the  
141 reaction by the spectrophotometric method based on the reaction between AlCl<sub>3</sub> and  
142 NaNO<sub>2</sub> with an aromatic ring of flavonoid compounds, especially flavonol and flavon  
143 (Shraim *et al.* 2021). The reaction between AlCl<sub>3</sub> and flavonoid compounds resulted a  
144 yellow solution. Then, the red solution was produced after NaOH solution addition that  
145 was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu,  
146 Japan) at λ 510 nm ~~with catechin as~~ A (+) catechin was used as a reference standard  
147 compound, and the results were expressed as mg catechin equivalents (CE)/g samples  
148 using the formula-

149 (show the formula)

150

151

### 152 TOTAL TANNIN CONTENT ANALYSIS

153 Total tannin content of the samples was analyzed by Folin-Ciocalteu method  
154 ~~based on~~ (Chandran and Indira, (2016). The reaction between the samples and reagents  
155 (specify the reagents) obtained ~~resulted in~~ blue dark color solution that was measured ~~by~~  
156 ~~a spectrophotometer UV-Vis~~ (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ

157 760 nm with tannic acid as the reference standard. ~~This analysis used a tannic acid as a~~  
158 ~~reference standard and was expressed as mg tannic acid equivalents (TAE)/g samples.~~  
159 Calculation of the total tannin content expressed as tannic acid equivalents (TAE)/g  
160 sample used the formula: (show the formula)

161

## 162 ANALYSIS OF THE ANTI-OXIDANT POTENTIAL

### 163 DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

164 The DPPH free radical scavenging activity was measured by the  
165 spectrophotometric method (Widyawati *et al.* 2017) to determine AA of the ~~brewing of~~  
166 ~~pluchea~~ Pluchea tea leaf infusion to donor hydrogen atom to nitrogen atom in DPPH  
167 resulting DPPH-H compound with a yellow-colored solution. The reaction between the  
168 DPPH in methanol solution with the samples was measured by a spectrophotometer  
169 (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  517 nm. The reference  
170 standard compound was gallic acid and the results of analysis were expressed as mg  
171 gallic acid equivalents (GAE)/g samples.

172

### 173 FERRIC REDUCING POWER ANALYSIS

174 Ferric reducing power was determined by following the method used by Widyawati  
175 *et al.* (2014) method. Potency of the steeping pluchea tea the samples reducing iron (III)  
176 to iron (II) ion was ~~..... Intensity of the blue color formed was analyzed measured by using~~  
177 ~~spectrophotometer UV-Vis~~ (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  700  
178 nm. ~~The reducing capacity of antioxidant compounds of the steeping pluchea tea~~  
179 ~~increased related to intensity of blue color solution~~ Intensity of the blue color indicated

Formatted: Indent: First line: 0"

Commented [A31]: Lines 163 -168 Description of the assay is not clear. Revised based on the procedure described by Widyawati *et al.* 2017. Show the formula used.

Commented [A32]: Lines 173-177 needs revision for clarity.

Commented [A33]: Described the reaction

Formatted: Font: (Default) Arial, 12 pt

Formatted: Font: 12 pt

180 ~~higher reducing capacity. The bigger of reducing power, the higher of blue color~~  
181 ~~intensity. The reference standard used as gallic acid, and~~ With gallic acid as the standard  
182 ~~compound,~~ the ~~results-reducing power were~~ expressed as mg gallic acid equivalent  
183 (GAE)/g samples. ~~was calculated using the formula: (show formula).~~

#### 185 $\alpha$ -AMYLASE ENZYME INHIBITION CAPACITY ASSAY

186 In vitro inhibition of  $\alpha$ -amylase enzyme ~~followed the procedure as described by~~ was  
187 ~~determined by~~ Widyawati *et al.* (2020). ~~method. Samples of steeping pluchea tea at~~  
188 ~~various steeping temperatures and storage times were analyzed by spectrophotometer~~  
189 ~~UV-Vis (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) based on reaction between~~  
190 ~~bioactive compounds and  $\alpha$ -amylase enzyme.~~ Then, the residue enzyme was reacted  
191 with starch and the capacity of the  $\alpha$ -amylase enzyme hydrolyzed the starch to release  
192 glucose that could be analyzed based on absorbance at  $\lambda$  540 nm. The inhibition  
193 percentage of  $\alpha$ -amylase was assessed ~~by using the following~~ formula:  $(ACb - ACa) -$   
194  $(As - Ab) (ACb - ACa) \times 100 \%$ . Where, ACb ~~was is the~~ absorbance of 100% enzyme  
195 activity (~~only solvent?~~ with ~~the~~ enzyme), ACa ~~was is the~~ absorbance of 0 % enzyme  
196 activity (~~only solvent?~~ without ~~the~~ enzyme), As ~~is the was~~ absorbance of tested sample  
197 with enzyme, Ab ~~was is~~ absorbance of tested sample without enzyme.

**Commented [A34]:** Describe the reactions leading to the release of glucose.

**Commented [A35]:** Check the formula

#### 199 $\alpha$ -GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

200 The analysis of the  $\alpha$ -glycosidase inhibitor activity was done by Widyawati *et al.*  
201 (2020) method ~~with slight modification.~~ The samples were reacted with the  $\alpha$ -glycosidase  
202 enzyme, and then the residue of this enzyme hydrolyzed p-nitrophenyl- $\alpha$ -D-

**Commented [A36]:** Description of the procedure is not clear. Revise based on the source.

**Commented [A37]:** Describe the modification

203 glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The inhibitor activity of  
204 steeping ~~pluche~~*Pluche* tea to enzyme was measured by spectrophotometer UV-Vis  
205 (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at  $\lambda$  405 nm. The inhibition  
206 percentage of  $\alpha$ -glycosidase was assessed by the following formula:  $(ACb - ACa) - (As$   
207  $- Ab) (ACb - ACa) \times 100 \%$  Where, ACb was absorbance of 100 % enzyme activity (only  
208 solvent with enzyme), ACa was absorbance of 0 % enzyme activity (only solvent without  
209 enzyme), As was absorbance of tested sample with enzyme, Ab was absorbance of  
210 tested sample without enzyme.

**Commented [A38]:** Must be revised, sentences must be restructured for clarity. Describe the principle clearly in the method.

## 212 HPLC ANALYSIS OF PHENOLICS

**Commented [A39]:** Description of the method, lines 218-223, is not clear.

213 The phenolic compounds of samples were analyzed by HPLC based on  
214 Kongkiatpaiboon *et al.* (2018) method with modification. HPLC separation was achieved  
215 on LC-20AD series (Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence  
216 UFLC LC-20AD pump, SIL-20 ACHT autosampler, CTO-10AS VP column oven, CBM-20A  
217 system controller, and SPD-40 detector. The separation was done in a Shim-pack VP-  
218 ODS C18 column (5  $\mu$ m x 50 mm x 4.6 mm I.D.) with a GVP-ODS Cartridges (2 pcs)  
219 guard column (10 mm x 4.6 mm I.D.). The mobile phases were (A) 0.5 % acetic acid in  
220 water and (B) methanol using gradient elution: 10 % B in A to 50 % B in A for 40 min; 100  
221 % B for 20 min. This column was re-equilibrated with 10 % B in A for 10 min prior to each  
222 analysis and the flow rate was set at 1.0 mL/min with the controlled temperature at 40 °C.  
223 SPD-40 detector was set at  $\lambda$  280 nm and injection volume was 20  $\mu$ L for every sample  
224 and reference standard.

**Commented [A40]:** Describe the modification

**Commented [A41]:** Confusing statements. Re-check the procedure.

How was the specific phenolic compounds identified?

226 EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

227 The research design used a randomized block design with two factors, i.e., the  
228 brewing temperature (B) and the storage time (L). ~~The Dried Plucheae leaf blades were~~  
229 ~~subjected to 4 steeping temperatures namely, of plucheae tea consisted of four treatment~~  
230 ~~levels, including~~ 60 °C (B1), 70 °C (B2), 80 °C (B3), and 95 °C (B4), and the storage time  
231 ~~of plucheae tea was composed two levels, i.e.,~~ 0 year /fresh (L0), and 5 year/stored (L2).  
232 Each treatment was repeated six times in order to obtain 48 experiment units. The HPLC  
233 analysis of phenolic was repeated two times. The data ~~of samples~~ were analyzed by  
234 ANOVA at  $p \leq 5 \%$ , and ~~if treatment means were significant, this was continued followed~~  
235 by ~~DMRT (Duncan Multiple Range Test) DMRT~~ at  $p \leq 5 \%$ , ~~to determine the significant~~  
236 ~~differences between the treatment means. Treatment means of the specific phenolic~~  
237 ~~compounds that were identified Data~~ were expressed as the mean  $\pm$  SD. The analysis  
238 used SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

240 RESULTS AND DISCUSSIONS

241 ~~PlucheaePlucheae~~ leaf infusion tea is produced by young ~~plucheaePlucheae~~ leaf from  
242 1-6 level on each branch the shoot (Widyawati *et al.* 2022), that is steeped at 95 °C for 5  
243 min, has many biological activities, such as antioxidant activity (Widyawati *et al.* 2016),  
244 antidiabetic activity (Werdani and Widyawati, 2018), anti-inflammatory (Srisook *et al.*  
245 2015). The chemical constituents in ~~plucheaePlucheae~~ tea involve alkaloids, flavonoids,  
246 phenolics, sterols, cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and  
247 saponins, where 2 g/100 mL steeping ~~plucheaePlucheae~~ tea has total phenolic content 9.3  
248 mg gallic acid equivalents (GAE)/g samples, total flavonoid content 22.0 mg catechin

**Commented [A42]:** Experimental research must have a control group, treated group, dependent variables and independent variables.

**Commented [A43]:** confusing

**Commented [A44]:** Were all 48 experimental units considered replicates and statistically analyzed?

**Commented [A45]:** How many replicates?

**Commented [A46]:**

**Commented [A47]:** Source (author cited) are not found in the list of references/literature cited section.

Lacks focus and organization thus, discussion relative to the results are not clear ie hard to comprehend

Data shown in the table and graphs must not be repeated in the text not unless there is a need to highlight a particular data observed. Observation must always be accompanied by statistical significance. Observation must be followed by an explanation.

eg. There was a significant increase in the total phenolic content at in samples steeped at 95C than at 60C (Table 1a). This implies that steeping at high temperature resulted in greater amount of phenolic compounds in the infusion which could be due to the fact .....(explanation, and cite literature that showed the same or related findings).

Suggestion (format of this section)

RESULTS AND DISCUSSION

RESULTS (Data and observations only)

Effect of steeping temperature

Effect of storage

Effect of combined steeping temp and storage

Show results of stat analysis in the table and figure (ANOVA, DMRT, ASSOCIATION/CORRELATION)

DISCUSSION (Explain the observations as indicated in table and graphs, state reasons behind such observations)

Or

RESULTS AND DISCUSSION

Effect of steeping temperature

Effect of storage

Effect of combined steeping temp and storage

Under each subtopic, results are presented then followed by explanation .

Show results of stat analysis in the table and figure (ANOVA, DMRT, ASSOCIATION/CORRELATION)

249 equivalents (CE)/g samples, DPPH free radical scavenging activity 27.2 mg gallic acid  
250 equivalents (GAE)/g samples, and ferric reducing power 10.2 mg gallic acid equivalents  
251 (GAE)/g samples (Widyawati *et al.* 2016). Previous research has informed related to the  
252 composition of phytochemical compounds in ~~pluchea~~*Pluchea* leaves, such as phenolic  
253 acids such as chlorogenic acids, caffeic acids, 3-O-caffeoylquinic acids, 4-O-  
254 caffeoylquinic acids, 5-O-caffeoylquinic acids, 3,4-di-O-caffeoylquinic acids, 3,5-di-O-  
255 caffeoylquinic acids, and 4,5-di-O-caffeoylquinic acids; total flavonoids which cover  
256 quercetin, kaempferol, myricetin, anthocyanin;  $\beta$ -carotene; and total carotenoids  
257 (Suriyaphan 2014; Vongsak *et al.* 2018; Ruan *et al.* 2019; Chan *et al.* 2022; Widyawati *et*  
258 *al.* 2022). Presence of phytochemical compounds in herbal product were influenced by  
259 environmental factors, i.e., temperature, light exposure, oxygen level, pH and moisture.  
260 The structure of phytochemical compounds in herbal tea is very sensitive of the  
261 surrounding changes. The effect arising from these changes causes the structure of the  
262 phytochemical molecule to be degraded to produce smaller size molecules or to combine  
263 to produce larger size molecules (Ali *et al.* 2018; Jayani *et al.* 2022, Ramphinwa *et al.*  
264 2023). Therefore, this study emphasized the effect of steeping temperature and storage  
265 time of ~~pluchea~~*Pluchea* tea on levels of the bioactive compounds, antioxidant and  
266 antidiabetic properties and phenolic compound profile.

Commented [A48]: Not measurable

Commented [A49]: Include in the Introduction. Select only those that will further support Table 1.

## 268 BIOACTIVE COMPOUNDS

### 269 Phenolic Compounds

270 The bioactive compounds are active compounds in plants that are essential to  
271 protect a body health (Nguyen and Chuyen 2020). These compounds usually have many



272 biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer,  
273 antimicrobial, antibacterial, anti-cholesterol and so on (Suriyaphan 2014; Acar *et al.*  
274 2022). Phenolic compounds ~~are have~~ potential ~~bioactive compounds in plants, that have~~  
275 ~~responsible~~ redox properties ~~to that can~~ scavenge free radicals ~~as that can~~ cause with a  
276 number of chronic diseases (Noreen *et al.* 2017; Arya *et al.* 2019; Acar *et al.* 2022).

277 ~~The steeping temperature (60, 70, 80 and 95 °C) and storage time (fresh and~~  
278 ~~stored) determined total phenolic content, with values ranging from 4.39 ± 0.49 to 71.38~~  
279 ~~± 4.14 mg GAE/g samples.—~~The total phenolic content of ~~pluche~~*Pluchea* ~~tea~~  
280 ~~infused~~infusion at different ~~steeping~~ temperature and storage period ~~ed at different time~~  
281 ~~that statistical analyzed by ANOVA at α ≤ 5 % shown at Figure 1, generally, significantly~~  
282 ~~increased with increasing steeping temperatures and storage periods. .Steeped and~~  
283 ~~stored -infusion had significantly higher amounts of phenolic compounds that the samples~~  
284 ~~were steeped and unstored. Further, The total phenolic content of samples was~~  
285 ~~significantly influenced by the steeping temperature and storage time.—~~The highest total  
286 phenolic content was ~~detected~~observed in ~~the~~the L-95 sample infused at 95 °C and  
287 stored for 5 years ~~{at 71.38 ± 4.14 mg GAE/g samples}~~ while the lowest was measured  
288 in in the unstored samples and infused at 60C. Phenolic content of the samples that  
289 ~~were infused at different temperatures then stored were significantly higher than the~~  
290 ~~steeped unstored samples while samples that were steeped only at 60 and 95C also~~  
291 ~~showed a significant increase in their phenolic. This implies that the steeping temperature~~  
292 ~~and the storage periods significantly resulted in the high amounts of the phenolic~~  
293 ~~compounds of the infusions. Results also indicated that phenolic compounds were~~  
294 ~~generally, greater in the infusion at high steeping temperatures and long storage (Figure~~

**Commented [A50]:** Include in the introduction as this shows the importance of bioactive compounds.

**Commented [A51]:** Fresh leaves? Do you mean 0 storage

295 1a). This could have been due to that fact that during during steeping with was followed  
296 by L 80 sample infused at 80 °C and stored for 5 years (62.60 ± 2.49 mg GAE/g samples)  
297 and L70 sample infused at 70 °C and stored for 5 years (60.68 ± 3.79 mg GAE/g samples)  
298 and L60 sample infused at 60 °C and stored for 5 years (46.67 ± 5.38 mg GAE/g samples).  
299 The total phenolic contents of steeping fresh pluchea tea (B60) had a lower total phenolic  
300 content (4.39 ± 0.48 mg GAE/g samples) than the steeping stored pluchea tea for 5 years  
301 (48.67 ± 5.38 until 71.38 ± 4.14 mg GAE/g samples). Fresh *pluchea Pluchea* tea had a  
302 lower total phenolic content than stored *pluchea Pluchea* tea for 5 years, besides that the  
303 higher the steeping temperature also caused the greater the extracted total phenolic  
304 content. The temperature of infusion influenced total phenolic content, it could relate to  
305 migration process of phenolic compounds to the water because of increasing contact  
306 between this compounds and water. The same phenomena also occurred in Castiglioni  
307 *et al.* (2015); Kilic *et al.* (2017), and Acar *et al.* (2022).

308 This occurrence showed that steeping temperature and storage time caused the  
309 process of degradation and oxidation of phenolic compounds. Su *et al.* (2019) reported  
310 that temperature treatment can stimulate the release of phenolic compounds and  
311 increase antioxidant activity of lychee juice stored at different temperatures of 4 and 45  
312 °C and different storage times (fresh and 72 hours). Hydrogen bonding is affected by  
313 temperature treatment because the hydrogen bond between phenolic compounds and  
314 proteins can be degraded that the measured levels of phenolic compounds are higher.  
315 The phenomena were supported by Ali *et al.* (2018); Jayani *et al.* (2022) and Ramphinwa  
316 *et al.* (2023). Zhang *et al.* (2021) reported that phenolic compounds present in plants are  
317 not completely stable, but are easily degraded during storage after harvest. Reblova

**Commented [A52]:** Do not repeat data found in the table or graphs; show only data that you want to highlight. Improve your graphs by properly labelling instead of sample codes. After doing this, match your observations/discussion with the available data shown in the graph or table. Improve also title and footnotes. See my comments.

Sentences have to be reconstructed for clarity

**Commented [A53]:** Reconstruct to support/explain your observations

318 (2012) claimed that antioxidant compounds can be slowly degraded with increasing  
319 temperature. Besides that, Fibrianto *et al.* (2021) also stated that the brewing  
320 temperature has an effect on the extracted antioxidant compounds, such as alkaloids,  
321 catechins and tannins. Thus, there is an assumption that the phenolic compounds in  
322 ~~pluche~~*Pluche*a tea are degraded due to oxidation and hydrolysis because of  
323 temperature and storage time and can be easily extracted during brewing, thus increasing  
324 the phenolic content as the steeping temperature and storage time increase.

325 Based on using of a reference standard could be informed that phenolic  
326 compounds in steeping ~~pluche~~*Pluche*a tea, including gallic acids, (+)-catechins,  
327 myricetins, quercetins, kaempferols, 3,4-di-O-caffeoylquinic acids, 3,5-di-O-  
328 caffeoylquinic acids, and 4,5-di-O-caffeoylquinic acids was showed in Table 1. Gallic  
329 acids and (+)-catechins were relative stable phenolic acid because of very small changes  
330 at different steeping temperature and storage time with concentration about  $0.21 \pm 0.00$   
331  $- 0.24 \pm 0.02$   $\mu\text{g/g}$  samples and  $0.32 \pm 0.02 - 0.60 \pm 0.04$   $\mu\text{g/g}$  samples, respectively.  
332 However, myricetin, quercetin and 3,4-di-O-caffeoylquinic acid showed drastic increasing  
333 at higher steeping temperature and longer storage time. It's meant that these compounds  
334 tended relatively labile. Kaempferol, 3,5-di-O-caffeoylquinic acid and 4,5-di-O-  
335 caffeoylquinic acid underwent moderate changes compared to the other two groups of  
336 phenolic acids. Therefore myricetin, quercetin and 3,4-di-O-caffeoylquinic acid were  
337 easier to dissolve at higher steeping temperature and storage time can cause  
338 macromolecules of three phenolic acids in herbal tea convenient degradable to form  
339 simple phenolic compounds for storage, as explained by Su *et al.* (2019), Ali *et al.* (2018);  
340 Jayani *et al.* (2022); Ramphinwa *et al.* (2023), and Zhang *et al.* (2021). Degradable

**Commented [A54]:** Sentences have to be restructured for clarity

**Commented [A55]:** Indicate Stat treatment means significance

**Commented [A56]:** Same as gallic and catechin

**Commented [A57]:** Vague; improve sentence construction.

341 polyphenol compounds have a simple structure and free hydroxyl groups that can react  
342 with Folin-Ciocalteu's Phenol reagent, resulting complex blue solution that can detected  
343 as total phenolic content.

344

### 345 Flavonoid Content

346 Flavonoids are the major phenolic compounds ~~having that have~~ potential as  
347 chemical and biological activities, ~~especially such~~ as radical scavenging and antimicrobial  
348 activities (Ayele *et al.* 2022; Chandra *et al.* 2014). ~~These compounds are the bioactive~~  
349 ~~compounds~~ that can protect the human body from the oxidative stress caused many  
350 degenerative diseases, especially cancer, cardiovascular problems and ageing (Mathur  
351 and Vijayvergia 2017). ~~Total flavonoid content analysis for pluchea tea at various~~

352 ~~steeping temperatures and storage times were showed in Figure 1.~~ The total flavonoids  
353 ~~content of steeped pluchea Pluchea tea infusion~~ decreased with ~~longer increasing~~  
354 ~~storage periodtime.~~ ~~Unstored samples exhibited higher flavonoid content than the stored~~  
355 ~~samples, but increased with increasing brewing temperature.~~ The highest total flavonoid  
356 content was ~~owned exhibited~~ by ~~fresh pluchea tea which was brewestored samples~~

357 ~~steeped~~ at 95 °C ~~(at 147.42 ± 14.03 mg CE/g samples)~~ Total flavonoid content was  
358 ~~significantly lower in the stored regardless of steeping temperature than those of the~~  
359 ~~unstored and steeped samples at 24.75 ± 2.47 to 33.71 ± 3.06 mg CE/g samples implying~~  
360 ~~that the increase in the flavonoid content of the infusion was affected primarily by the~~  
361 ~~steeping temperature. and the lowest was.~~ This implies that ~~owned by pluchea tea which~~  
362 ~~had been stored for 5 years at various brewing temperatures (24.75 ± 2.47 to 33.71 ±~~  
363 ~~3.06 mg CE/g samples between 24.75 ± 2.47-33.71 ± 3.06 mg CE/g samples).~~ Statistical

**Commented [A58]:** Not shown

**Commented [A59]:** Include in the analysis section

**Commented [A60]:** Start this part by describing observations in Table 1 highlighting significant differences. If possible do ANOVA, then DMRT to support your general observations and implications.

Follow with explanation and evidences from the RRL.

**Commented [A61]:** Replace tea to infusion.

**Formatted:** Indent: First line: 0"

**Commented [A62]:** ????

364 analysis by ANOVA analysis at  $\alpha \leq 5\%$  proven that brewing temperature and storage  
365 time of fresh pluchea tea had a significant effect on the total flavonoid content, but the  
366 stored pluchea tea (L) had no significant effect. Storage time had a significant effect on  
367 the total flavonoid content of brewing pluchea tea. Ali *et al.* (2018) reported that the  
368 degradation of bioactive compounds can take place through several stages, such as pre-  
369 treatment, processing, and storage, as is the case with medicinal plants which are dried,  
370 extracted and stored in the long term. Brewing temperature and storage time have an  
371 influence on the oxidation and polymerization processes that are stimulated by light.  
372 According to Noree *et al.* (2017), that the total flavonoid content test with  $\text{AlCl}_3$  and  $\text{NaNO}_2$   
373 reagents measures flavone compounds, these compounds have activity due to the  
374 presence of a free hydroxyl functional group at position 4' in the compound. Degradation  
375 of flavone compounds due to temperature and storage causes the breaking of methylation  
376 bonds. Kim *et al.* (2020) also confirmed, that the total phenolic content and total flavonoid  
377 content of matcha are decreased with increasing brewing temperature and storage time.  
378 Xu *et al.* (2019) informed, that storage time can give a big impact on chemical composition  
379 changes with trending not the same.

**Commented [A63]:** Is this fresh leaf

**Commented [A64]:** Overall suggestion, interpret the Figure 1B matched with the text. Then provide support and discussion.

**Commented [A65]:** Rather than stating ANOVA, use significant differences

**Commented [A66]:** This is related literature brew and infusion are different.

### 380 Tannin content

381 The tannins have a various type of compounds are water-soluble polyphenols that  
382 are current in many plant foods and have a number of effects on health (Balaky *et al.*  
383 2024). Tannins are bioactive compounds that provide properties, such as astringent, anti-  
384 diarrheal, antibacterial and antioxidant (Malangngi *et al.* 2012). Data analysis Generally,  
385 results indicated showed, that the total tannin content of brewing pluchea *Pluchea* tea  
386 infusion significantly increased with increasing steeping brewing temperature and storage

**Commented [A67]:** Needs revision, sentences reconstruction

**Commented [A68]:** Do these support your observations

**Commented [A69]:** Reconstruct to support the findings

**Formatted:** Indent: First line: 0"

387 periodtime (, as seen in Figure 1). Steeping pluchea tea contained tannins ranging from  
388  $4.81 \pm 0.58$  –  $17.42 \pm 1.04$  (mg TAE/g samples). The tannin content increased with  
389 increasing storage time and brewing temperature. The results of the ANOVA statistical  
390 analysis at  $\alpha \leq 5\%$ , showed a significant increase in tannin content levels with increasing  
391 brewing temperature and storage time. Among, the unstored steeped samples, theThe  
392 fresh pluchea tea brewed tannin content was significantly lowest in samples infused at 60  
393 °C had the lowest tannin content level, was at  $4.81 \pm 0.58$  mg TAE/g samples which is  
394 significantly different lower from the lowest tannin content of the stored samples. Among  
395 the stored and steeped samples, the highest tannin content was observed at samples  
396 steeped The stored pluchea tea brewed at 95 °C had the highest tannin content level,  
397 was  $17.42 \pm 1.04$  mg TAE/g samples and is significantly different from that of the highest  
398 tannin content of the unstored steeped samples at .- Indicating that the  
399 tannin content was affected by both high steeping temperature and long storage period  
400 and that the presence of high tannin content was primarily brought about by long storage  
401 period. The results showed, that the higher the brewing temperature and the longer the  
402 storage time caused the tannin compound polymerization process to occur. Ali *et al.*  
403 (2018) said that pH, storage temperature, chemical structure and concentration, light,  
404 oxygen, enzymes and metal ions affect the presence of bioactive compounds in the  
405 material. Rusita *et al.* (2019) emphasized that tannins are a polar compound, that is  
406 resistant to heating, as a result the tannin content in plucheaPluchea tea increases with  
407 increasing brewing temperature and storage time, this is caused tannins are thermostable  
408 complex compounds. |

409

**Commented [A70]:** This is a better write up but still Needs improvement.

**Commented [A71]:** Kindly

**Commented [A72]:** Add more support as regard the observations like phenolase activity aside from heat and storage; what is the structure of all the bioactive compounds that can possibly explain either the decrease or increase

410 ANTIOXIDANT ACTIVITY

411  
412 Antioxidant activity is capability of compounds to inhibit the oxidation of  
413 macromolecules from biological target that involve in oxidative chain reactions (Ali *et al.*  
414 2005; Oh *et al.* 2013). In the research, the antioxidant activity assay used was DPPH Free  
415 Radical Scavenging Activity (~~DPPH~~) and ferric reducing power (FRAP), Ali *et al.* (2005)  
416 and Huang *et al.* (2005) informed that phenolic compounds have antioxidant activity  
417 because of their redox properties, such as hydrogen atom donor, electron transfer,  
418 reducing agent, and singlet oxygen quenchers.

Commented [A73]: Improve the sentence

419

420 DPPH Free Radical Scavenging Activity

Formatted: Indent: First line: 0"

421 DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate  
422 antioxidant activity because this method is ~~very~~-simple that is suitable to measure the  
423 donating hydrogen atoms capability of herbal infusiontea. This reaction can cause the  
424 purple color of DPPH ~~reduced to be~~ change to yellow color (Munteanu and Apetrei,  
425 2021; Baliyan *et al.* 2022). The result of DPPH assay indicate that the in-pluche tea was  
426 showed in Figure 2. Tindicate the DPPH values accrued ~~with~~ at higher steeping  
427 temperature and longer storage time. Statistical analysis by ANOVA at  $\alpha \leq 5\%$  proven  
428 that the higher the steeping temperature of fresh ~~pluche~~Pluche tea-tinfusion (B60-B95)  
429 was consistent the ability to DPPH free radicals scavenging activity, whereas the stored  
430 ~~pluche~~Pluche tea resulted in the higher activity and the values went up as rising of the  
431 infusion temperature. ~~Pluche~~Pluche tea-infusion storedage at room temperature for 5  
432 years resulted in the DPPH free radical scavenging activity by more than 100 %. ~~The~~S

Commented [A74]: No codes, name the sample

Commented [A75]: Where is the data?

433 steeping at higher temperatures could significantly increased the DPPH free radical  
434 scavenging activity in stored *plucheaplucheatea* infusion around 15 to -25 %. Brewing  
435 Steeping at 80 - 95 °C in stored *plucheaplucheatea* infusion insignificantly affected the  
436 free radical scavenging property of the bioactive compounds this antioxidant activity.  
437 (Figure 2a).

438 Scavenging activity of DPPH free radicals was correlated with total phenolic and  
439 tannin levels, but inversely to total flavonoid levels. The phenomenon of the DPPH values  
440 in *plucheatea* is contrary with the results of the study by Lin *et al.* (2020). However, this  
441 study was in accordance with Thanajiruschaya *et al.* (2010), claimed that during the  
442 storage process it is possible to form complex phenolic compounds which provide a high  
443 ability to scavenge DPPH free radicals (Thanajiruschaya *et al.* (2010)). This research also  
444 demonstrated that longer storage time and higher infusion temperature produced many  
445 simple phenolic compounds with free hydroxyl groups capable to donor hydrogen atom  
446 to DPPH free radical. Many phenolic acids, such as gallic acids, (+)-catechins, myricetins,  
447 kaempferols, quercetins, 3,5-di-O-caffeoylquinic acids, 3,4-di-O-caffeoylquinic acids, 4,5-  
448 di-O-caffeoylquinic acids have established potential antioxidant activity (Kumar and Goel  
449 2019). The DPPH free radical scavenging property observed in the study was not  
450 consistent with the results of the study by Lin *et al.* (2020).

#### 451 452 Ferric Reducing Antioxidant Power (FRAP)

453 FRAP is method that identifies the antioxidant capacity of the phytochemical  
454 component through measured absorbance, as a result of the reaction among antioxidant  
455 compounds, potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a

**Commented [A76]:** Present the correlation or association stat analysis

**Commented [A77]:** Improve the paragraph. Again, clearly describe your interpretation based on the graph highlighting statistical significance between and among samples. This is the part that you explain the observations by discussing the structure of the phenolic compounds.

**Commented [A78]:** Add the role of the structures.

**Commented [A79]:** State the results of the study, explain as it relates to your study.

**Formatted:** Indent: First line: 0"



456 color complex, that can be measured at  $\lambda$  700 nm (Fu *et al.* 2011; Al-Temimi and  
457 Choudhary 2013). The principle of testing the ability to reduce iron ions is that antioxidants  
458 can reduce potassium ferrocyanide ( $\text{Fe}^{3+}$ ) to potassium ferrocyanide ( $\text{Fe}^{2+}$ ). Potassium  
459 ferrocyanide reacts with ferric chloride to form a ferric-ferrous complex and results green  
460 color solution (Widyawati *et al.* 2017).

Commented [A80]: REvise

461 ~~The data showed, that the FRAP of pluchea tea became significantly different with~~  
462 ~~going up brewing temperature and storage time (Figure 1). The The results showed that~~  
463 ~~the ferric reducing antioxidant power (FRAP) value~~ increased with higher steeping  
464 temperature and longer storage time. ~~The lowest FRAP value was owned by observed~~  
465 ~~pluchea Pluchea tea infusion~~ which was brewed at 60 °C at  $3.95 \pm 0.17$  mg gallic acid  
466 equivalents (GAE)/g samples, and the highest was owned by ~~pluchea Pluchea~~ tea which  
467 was stored for 5 years at  $48.63 \pm 10.83$  mg gallic acid equivalents (GAE)/g samples  
468 (Figure 2b). FRAP of the ~~pluchea Pluchea~~ was significant correlated with the DPPH free  
469 radical scavenging activity, total phenolic and tannin contents. This case was contrast to  
470 the antioxidant activity of DPPH and FRAP on matcha, because the longer storage time  
471 reduces the levels of catechin content (Kim *et al.* 2020), and also the case of the effect of  
472 temperature and storage time in betel (*Piper bettle* L.) extract (Ali *et al.* 2018).  
473 Thanajiruschaya *et al.* (2010) revealed that the antioxidant activity of rice stored at high  
474 temperatures is greater than that stored at low temperatures. The ferric reducing  
475 capability of ~~pluchea Pluchea~~ tea infusion corresponded to simple phenolic acid values,  
476 presence of them in samples could accrue antioxidant activity because of ability of the  
477 electron transfer from free hydroxyl groups of phenolic acids.

Commented [A81]: Check your graph

Commented [A82]: Lower FRAP was observed in the unstored samoles regardless of steeping temperature. FRAP increased significantly as steeping temperature was increased. FRAP of the samples stored for 5 years was also significantly higher than the stored samples. Highest FRAP was exhibited by stored samples steeped at 95C

Commented [A83]: Show correlation stat analysis

Commented [A84]: The observation can be based on the chemical reaction s based on the structure of the bioactive compounds ie reducing structure, oxidation, stability to heat, olubility etc

479 ANTIDIABETIC ACTIVITY

480 Enzyme Inhibition Activity

481 Antidiabetic activity is a measure of the potency of phenolic compounds to regulate  
482 glucose uptake regulate the uptake of glucose by the cells ke or keep from the away blood  
483 glucose go up through the mediation of 2 digestive enzymes i.e., alpha-amylase and  
484 α-glucosidase, which are involved are digestive enzymes which involve to control in the  
485 control of dietary carbohydrate digestion and increase release in the postprandial blood  
486 glucose in human body (Fu *et al.* 2017). The phenolic compounds proven having have the  
487 capability to bind with the protein that they can inhibit component of α-amylase and α-  
488 glucosidase enzymes (Hardoko *et al.* 2019; Martinez-Solis *et al.* 2022) resulting in the  
489 reduced activity of the enzymes. Previous research of Werdani and Widawati (2018),  
490 claimed that pluchea tea infusion is potential as antidiabetic agents. This observation test  
491 is based on the breakdown ability of the substrate to produce a colored product, which is  
492 measured at λ = 540 nm. The results showed, that the steeping pluchea Pluchea tea  
493 infusion was able to inhibit the action of the α-amylase enzymes (Figure 3). The  
494 pluchea Pluchea tea infusion had very good activity, more than 50 % and even almost 100  
495 % for fresh pluchea Pluchea tea which was brewed at 60, 70 and 80 °C and stored  
496 pluchea Pluchea tea which steeped at 60 °C. Whereas fresh pluchea Pluchea tea brewed  
497 at 95 °C for 5 min had an activity of inhibiting the alpha amylase enzyme of less than 50  
498 %, which was equal to 40.08 ± 1.12%. Widyawati *et al.* (2017) detected the ability to inhibit  
499 the α-amylase enzyme from fresh pluchea Pluchea tea brewed at 95 °C for 5 min by 28.79  
500 %. Increasing the brewing temperature and storage time reduced the ability to inhibit the  
501 α-amylase enzyme. The results of the analysis based on the ANOVA statistical test at α

Commented [A85]: Check if this description agrees with Fu et al.

Commented [A86]: Check if consistent with the source, author cited

502 ≤ 5 % showed, that the brewing temperature and storage time had a significant effect on  
503 the ability to inhibit the α-amylase enzyme. This ability was inversely proportional to the  
504 levels of total phenolic content, total tannin content, DPPH, and FRAP. This inhibitory  
505 activity was thought to be contributed by other bioactive compounds, besides phenolics  
506 which are sensitive to brewing temperature and storage time. Li et al. (2018) stated that  
507 there are flavonoid compounds that contribute to the ability to inhibit the α-amylase  
508 enzyme. Flavonoid compounds with a hydroxyl structure at C-4' in ring B are more  
509 effective than C-6 in ring A. Akah et al. (2011) informed that the phytochemical  
510 compounds, such as terpenoids, saponins, flavonoids, glycosides and carbohydrate, and  
511 alkaloids are good antidiabetic metabolites. Sangeetha and Vedasree (2012) explained,  
512 that the ability to inhibit the α-amylase enzyme was determined by the content of the  
513 phenolic compound and protein. The α-amylase inhibitor present in ~~pluchea~~ *Pluchea* tea  
514 may be proteinaceous in nature. Aleixandre et al. (2022) informed that phenolic acids  
515 have inhibition activity to α-amylase enzyme depending their structures. Besides that,  
516 capability of phenolic acids to inhibit α-amylase was determined by low half-maximum  
517 inhibitory concentration (IC<sub>50</sub>). There are C=C double bond conjugated with a carbonyl  
518 group of phenolic structures that stabilizes the binding forces to the active site of the α-  
519 amylase. The hydroxyl groups of them are able to bind by non-covalent interaction, such  
520 as hydrogen binding, cation-π interactions, salt bridge interactions, ionic interactions or  
521 electrostatic forces with amino acid residue at the active site in α-amylase. The steeping  
522 temperature and storage time can remove hydroxyl groups of phenolic compounds that  
523 can reduce the ability of enzyme inhibition. The phenolic acids with a greater number of  
524 hydroxyl groups are stronger capable to obstruct the α-amylase enzyme.

Corresponding Author: paini@ukwms.ac.id

**Commented [A87]:** Discussion is not clear. Needs improvement. Reconstruct your sentences for clearer interpretation and explanation. Explain the figures Interpret the data as shown in Figure 3a as in observation between stored and unstored samples; observation within sample treatment ie unstored, steeped and stored steeped

Explain the decreasing trend in the enzyme inhibition activity in both the unstored, steeped and stored and steeped samples ie effect of storage; effect of temperature based on the physical, chemical and biochemical nature of both the enzymes and phenolic compounds

525  $\alpha$ -glucosidase is an important enzyme in carbohydrates digestion, that catalysis  
526 the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and converts  
527 them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et al., 2014;  
528 Proenca et al., 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -glucosidase  
529 enzyme is used to determine antidiabetics activity. This is supported by Werdani and  
530 Widyawati (2018), that *plucheaePlucheae* tea infusion has the potential as an antidiabetic  
531 agent. Widyawati et al. (2020) found that brewing fresh *plucheaePlucheae* tea at 95 °C for  
532 5 minutes has an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857%.

533 The results showed, that the ability to inhibit the  $\alpha$ -glucosidase enzyme decreased  
534 with increasing brewing temperature and storage time. Brewing at 95°C for fresh  
535 *plucheaePlucheae* tea (0 days of storage) obtained the lowest inhibitory ability, i.e.,  $48.32 \pm$   
536  $1.27\%$ , and the highest inhibitory activity was found at 70 °C brewing temperature for  
537 fresh *plucheaePlucheae* tea, which was  $95.11 \pm 0.70\%$  (Figure 3). The test results showed  
538 that the ability to inhibit the enzyme  $\alpha$ -glucosidase tended to be higher than the ability to  
539 inhibit the enzyme  $\alpha$ -amylase. Li *et al.* (2018) informed that flavonoid compounds have  
540 the ability to inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. This is due  
541 to the total flavonoids in brewing *plucheaePlucheae* tea which tended to have the same  
542 pattern as the ability to inhibit the activity of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes.  
543 Flavonoid compounds, such as rutin, myricetin, kaempferol, and quercetin which have  
544 antioxidant and antihyperglycemic activities. The ability to inhibit the action of enzymes  
545 from flavonoid compounds is determined by the position and number of hydroxyl groups  
546 and the number of double bonds in rings A and B and the heterocyclic ring in ring C. The  
547 ability to inhibit the  $\alpha$ -glucosidase enzyme from *plucheaePlucheae* tea was significantly

548 affected by the brewing temperature and storage time. The capability of *Pluchea*  
549 tea infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater than the  $\alpha$ -amylase  
550 enzyme because the mechanism of two enzymes was different, according to the opinion  
551 of McCue *et al.* (2005). Widyawati *et al.* (2017) informed that phenolic and non-phenolic  
552 compounds determine the inhibitory activity of the  $\alpha$ -glucosidase enzyme. The ability of  
553 bound phenolic compounds to inhibit  $\alpha$ -glucosidase enzymes was higher than free  
554 phenolic compounds. The presence of polymerization and degradation reactions, that  
555 may be occurred in *Pluchea* tea during storage, affects the structure and profile  
556 of phenolic and non-phenolic compounds. Asriningtyas *et al.* (2014) claimed that  
557 *Pluchea* leaves contain 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid  
558 methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid,  
559 and 1,3,4,5-tetra-*O*-caffeoylquinic acid. Quinic acid is methyl esterified with the number  
560 of caffeic groups in the molecule that determines the activity of inhibiting the  $\alpha$ -  
561 glucosidase enzyme. Analysis of caffeoylquinic acids in *Pluchea* tea infusion was  
562 obtained that the higher steeping temperature and longer storage time caused increased  
563 concentration of them, but the  $\alpha$ -glucosidase inhibition of them was reduced. Aleixandre  
564 *et al.* (2022) reported that the simple phenolic acids forming a dipole-dipole interaction of  
565 active site from  $\alpha$ -glucosidase enzyme are effectively inhibiting the enzyme.

566 This study was obtained information that the increasing of steeping temperature  
567 and storage time caused a degradation reaction of polyphenol compounds to produce  
568 simple phenolic compounds, such as gallic acid, (+)-catechin, myricetin, quercetin,  
569 kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-  
570 caffeoylquinic acid, supported the results of total phenolic content and total tannin content

571 assays. Increased concentration of simple phenolic compounds determined the ability of  
572 these compounds as antioxidant agents, but reduced their capability as antidiabetic  
573 agents.

574

## 575 CONCLUSION

576 The steeping temperature and storage time of *Pluchea* tea determined  
577 antioxidant and antidiabetic activities. Profile of phenolic compounds of *Pluchea*  
578 tea infusion influenced antioxidant and antidiabetic activities. Storage time and brewing  
579 temperature caused degradation reaction of polyphenols that resulted simple phenolic  
580 compounds. Gallic acid, (+)-catechin, quercetin, myricetin, kaempferol, 3,4-di-O-  
581 caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid and 4,5-di-O-caffeoylquinic acid were  
582 simple phenolic compounds detected from steeping *Pluchea* tea. Increasing of  
583 them determined antioxidant activity that correlated to total phenolic content and total  
584 tannin content. Total flavonoid content had a decreasing graph pattern with increasing  
585 storage time that was similar to the antidiabetic activity graph pattern, which means that  
586 the antidiabetic activity of *Pluchea* tea depended on the total flavonoid content  
587 and the structural complexity of the phenolic compounds.

588

## 589 DATA AVAILABILITY

590 Table and figures used to support of this the study were included in the article.

591

## 592 CONFLICT OF INTEREST

593 The authors declare no conflict of interest.

Corresponding Author: paini@ukwms.ac.id

**Commented [A88]:** Same as in the above. Discussion is not clear. Needs improvement. Reconstruct your sentences for clearer interpretation and explanation. Explain the figures Interpret the data as shown in Figure 3a as in observation between stored and unstored samples; observation within sample treatment ie unstored, steeped and stored steeped

Explain the decreasing trend in the enzyme inhibition activity in both the unstored, steeped and stored and steeped samples ie effect of storage; effect of temperature based on the physical, chemical and biochemical nature of both the enzymes and phenolic compounds

**Commented [A89]:** The way this is written is not conclusive but may written to show explicitly the results of the study. To do this, construct your research questions. Conclusions are more explicit if they answer the research questions.

**Commented [A90]:** No

**Commented [A91]:** No.

**Commented [A92]:** No. Data/Observations did not show.

**Commented [A93]:** There was no correlation that was statistically analyzed

**Commented [A94]:** No, there was significant differences

**Commented [A95]:** This was not observed, no data to prove this

594

595 ACKNOWLEDGEMENTS

596 The authors would like to ~~thank-acknowledge~~ the ~~he~~ Ministry of Education and Culture of  
597 the Republic of Indonesia for funding this research under fundamental research grant ~~to~~  
598 awarded to higher education institutions in 2022.

599

600 REFERENCES

601 ACAR A, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food  
602 wastes:evaluation of temperature and time effects on quality parameters. Applied Food  
603 Res 2(100087): 1-8.

604 ALEIXANDRE A *et al.* 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -  
605 glucosidase and influence of reaction conditions. Food Chem 372(131231): 1-9.

606 ALI *et al.* 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile.  
607 Food Chem 89(2005): 27-36.

608 ALI A. *et al.* 2018. Impact of storage conditions on the stability of predominant phenolic  
609 constituents and antioxidant activity of dried *piper betle* extracts. Molecules 23(24): 1-13.

610 AL-TEMIMI A, CHOUDHARY R. 2013. Determination of antioxidant activity in different  
611 kinds of plants *in vivo* and *in vitro* by using diverse technical methods. J Nut Food Sci  
612 3(1): 1-6.

613 AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and  
614 methanol extract and fractions of *Gongronema latifolium* (*Asclepidaceae*) leaves in  
615 alloxan diabetic rats. J Applied Pharm Sci 01(09): 99-102.

**Commented [A96]:** You may want to replace this section with Literature cited. In scientific writing report, all those listed in this section MUST be found in the manuscript also as well as source of literature found in the manuscript must be fully cited in this section. Make sure that both are reconciled.

616 ARSININGTYAS IS. *et al.* 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves  
617 of *PlucheaPluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural  
618 product use. *Natural Product Res* 28(17):1350-1353.

619 ARYAL S *et al.* 2019. Total phenolic content, flavonoid content and antioxidant of wild  
620 vegetables from Western Nepal. *Plants* 8(96): 1-12.

621 AYELE DT, AKELE ML, MELESE A.T. 2022. Analysis of total phenolic contents,  
622 favonoids, antioxidant and antibacterial activities of *Croton macrostachyus* root extracts.  
623 *BMC Chem* 16(30): 1-9.

624 BALAKY HH *et al.* 2021. Estimation of total tannin and total phenolic content in plant  
625 (*Crataegus azarolus* L) by orbital shaker technique. *Intern J Agric Environ Food Sci* 5(1):  
626 1-6.

627 BALIYAN S *et al.* 2022. Determination of antioxidants by DPPH radical scavenging  
628 activity and quantitative phytochemical analysis of *Ficus religiosa*. *Molecules* 27(1326):  
629 1-19.

630 CASTIGLIONI S *et al.* 2015. Influence of steeping conditions (time, temperature, and  
631 particle size) on antioxidant properties and sensory attributes of some white and green  
632 teas. *Intern J Food Sci Nut Online* 1-7.

633 Chan EWC *et al.* 2022. *PlucheaPluchea indica*: An updated review of its botany, uses,  
634 bioactive compounds and pharmacological properties. *Pharmaceutical Sciences Asia*  
635 49(1): 77-85.

636 CHANDRA S *et al.* 2014. Assessment of total phenolic and flavonoid content, antioxidant  
637 properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit



638 crops: A Comparative study. Hindawi Publishing Corporation Evidence-Based  
639 Complementary Alter Med 2014: 1-9.

640 CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic, flavonoids,  
641 tannin and chlorophyll content of leaves of *Strobilanthes Kunthiana (Neelakurinji)*. J Med  
642 Plants Studies 4(4): 282-286.

643 FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional  
644 properties and visual appearance of Dampit Robusta coffee leaves tea. Advances in Food  
645 Sci Sustain Agric Agro-industrial Engineering 4(1): 25-32.

646 FU L *et al.* 2017. Total phenolic contents and antioxidant capacities of herbal and tea  
647 infusions. Intern J Molecular Sci 12: 2112-2124.

648 GAO MR *et al.* 2019. A theoretical and experimental study: the influence of different  
649 standards on the determination of total phenol content in the Folin–Ciocalteu assay. J  
650 Food Measurement and Characteristic 13: 1349–1356.

651 HUANG D, OU B, PRIOR RL. 2005. The Chemistry behind antioxidant capacity assays.  
652 J Agric Food Chem 53: 1841-1856.

653 ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties  
654 of rosehip tea beverage: study by response surface methodology. J Food Sci Tech  
655 54(11): 3737–3743.

656 JAYANI NIE *et al.* 2022. Evaluation of stability and quality characteristics of moringa  
657 (*Moringa oleifera*) herbal tea during storage. Food Res 6(4): 399-406.

658 KIM JM *et al.* 2020. Effect of storage temperature on the antioxidant activity and catechins  
659 stability of Matcha (*Camellia sinensis*). Food Science and Biotechnology 29(9): 1261–  
660 1271.

Corresponding Author: paini@ukwms.ac.id

661 KILIC *et al.* 2017. Antioxidant properties of some herbal teas (green tea, senna, corn silk,  
662 rosmery) brewed at different temperatures. Intern J Secondary Metabolite. 4(3): 142-248  
663 Special Issue 1.

664 KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of  
665 extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea Pluchea indica*  
666 leaves from different provenances in Thailand. Revista Brasileira de Farmacognosia  
667 28(2018): 145–150.

668 KUMAR N, GOEL N. 2019. Phenolic acids: natural versatile molecules with promising  
669 therapeutic applications. Biotechnology Reports 24(e00370): 1-10.

670 LI K *et al.* 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the flavonoids  
671 rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity  
672 relationship of its eight flavonoids by a refined assign score method. Chem Central J  
673 12(82): 1-11.

674 LIN YS. *et al.* 2020. Effects of storage time and temperature on antioxidants in juice from  
675 *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser. Molecules  
676 25(3614): 1-12.

677 MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content  
678 and antioxidant activity test of avocado seed extract (*Persea Americana* Mill). Jurnal MIPA  
679 Unsrat Online. 1(1): 5-10. [https://media.neliti.com/media/publications/116214-ID-  
680 penentuan-kandungan-tanin-dan-uji-aktivi.pdf](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf) [In Bahasa Indonesia]

681 MARTINEZ-SOLIS J *et al.* 2022. Antidiabetic and toxicological effect of the tea infusion  
682 of summer collection from *Annona cherimola* Miller leaves. Plants 11(3224): 1-17.

Corresponding Author: paini@ukwms.ac.id

683 MATHUR R, VIJAYVERGIA R. 2017. Determination of total flavonoid and phenol content  
684 in *Mimusops Elengi* Linn. Intern. J. Pharm. Sci. Res. 8(12): 5282-5285.

685 MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of  
686 sprouted and solid-state bioprocessed soybean. Asia Pacific J Clinical Nut 14(2): 145-  
687 152.

688 MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant  
689 activity: A Review. Intern J Molecular Sci 22(3380): 1-30.

690 NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus*  
691 *sabdariffa* L.): Effect of dying temperature and brewing conditions on total soluble solid,  
692 phenolic content, antioxidant capacity and sensory quality. Beverages 6(2):1-11.

693 NOREEN H. *et al.* 2017. Measurement of total phenolic content and antioxidant activity  
694 of aerial parts of medicinal plant *Coronopus didymus*. Asian Pacific J Tropical Med 10(8):  
695 792–801.

696 NURCHOLIS GW *et al.* 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity in-  
697 vitro and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum pictum*  
698 (L.). Current Biochem 1(2): 58-65.

699 OH J *et al.* 2013. Antioxidant and antimicrobial activities of various leafy herbal teas. Food  
700 Control 31(2013): 403-409.

701 PROENCA C *et al.* 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an in vitro and in silico  
702 structure–activity relationship study. J Enzyme Inhibition Med Chem 32(1): 1216–1228.

703 PUREWAL SS *et al.* 2022. Unraveling the effect of storage duration on antioxidant  
704 properties, physicochemical and sensorial parameters of ready to serve Kinnow-aml  
705 beverages. Applied Food Res 2(1): 1-8.

Corresponding Author: paini@ukwms.ac.id

706 RAMPHINWA ML *et al.* 2023. Eco-physiological response of secondary metabolites of  
707 teas: review of quality attributes of herbal tea. *Frontiers in Sustain Food Systems*  
708 7(990334): 1-14.

709 REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids.  
710 *Czech J Food Sci* 30(2): 171-177.

711 RUAN J *et al.* 2019. Comprehensive chemical profiling in the ethanol extract of  
712 *PlucheaPluchea indica* aerial parts by liquid chromatography/mass spectrometry analysis  
713 of its silica gel column chromatography fractions. *Molecules* 24(2784): 1-20.

714 RUSITA YD *et al.* 2019. Comparison of tannin concentration on fresh and dried tea leaf  
715 (*Camelia sinensis* L.) extract using UV-visible spectrophotometric method. 1st  
716 International Conference of Health, Science & Technology (ICOHETECH) 2019 E-ISBN:  
717 978-623-92207-0-9. November 16<sup>th</sup>, 2019.LPPM Universitas Duta Bangsa Surakarta,  
718 Surakarta.

719 SANGEETHA R, VEDASREE N. 2012. In Vitro  $\alpha$ -Amylase inhibitory activity of the leaves  
720 of *Thespesia populnea*. *Intern. Scholarly Res.Network.* 2012: 1-4.

721 SHRAIM AM *et al.* 2021. Determination of total flavonoid content by aluminum chloride  
722 assay: A critical evaluation. *LWT Food Sci Tech* 150(111932): 1-11.

723 SILVA-RAMIREZ KA *et al.* 2020. Effect of drying and steeping temperature on the  
724 phenolic content, antioxidant activity, aromatic compounds and sensory properties of  
725 *Culila polyantha* Benth. *Infusions. Processes* 8(1378): 2-20.

726 SRISOOK K *et al.* 2012. Antioxidant and anti-inflammatory activities of hot water extract  
727 from *PlucheaPluchea indica* Less. herbal tea. *J Med Plants Res* 6(23): 4077-4081.

Corresponding Author: paini@ukwms.ac.id

728 SURIYAPHAN O. 2014. Nutrition, health benefits and applications of *PlucheaPluchea*  
729 *indica* (L) Less leaves. Pharm Sci Asia 41: 1–10.

730 SU D *et al.* 2019. Impact of thermal processing and storage temperature on phenolic  
731 profile and antioxidant activity of different varieties of lychee juice. LWT-Food Tech  
732 116(108578): 1-9.

733 THANAJIRUSCHAYA P *et al.* 2010. Effect of storage time and temperature on antioxidant  
734 components and properties of milled rice. Khon Kaen Univ Res J15(9): 843-851.

735 VONGSAK B *et al.* 2018. Comparison of active constituents, antioxidant capacity, and  
736 αglucosidase inhibition in *PlucheaPluchea indica* leaf extracts at different maturity stages.  
737 Food Bioscience 25: 68–73.

738 WANG SC. *et al.* 2022. Effect of brewing conditions on polyphenols in the dark tea  
739 (*Camellia sinensis* L.) infusions: content, composition and antioxidant activities. Food Sci  
740 Tech 42(e36322): 1-9.

741 WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *PlucheaPluchea*  
742 *indica* Less as functional beverage in diabetic patients. ASSEHR 98: 164-167.

743 WIDYAWATI PS *et al.* 2014. Potency of beluntas (*PlucheaPluchea indica* Less) leaves  
744 extract as antioxidant and anti-warmed-over flavor (WOF) of duck meat. International  
745 Congress on Challenges of Biotechnological Research in Food and Health. November  
746 15<sup>th</sup> 2014. Agricultural Technology Faculty, Slamet Riyadi University, Surakarta.

747 WIDYAWATI PS *et al.* 2016. The physicochemical and antioxidant properties of  
748 *PlucheaPluchea indica* Less drink in tea bag packaging. IJFNS 5: 13–120.

749 WIDYAWATI PS *et al.* 2017. In vitro antioxidant capacities and antidiabetic properties of  
750 *plucheaPluchea* leaves. IJPPS 9(8): 203-208.

Corresponding Author: paini@ukwms.ac.id

751 WIDYAWATI PS *et al.* 2020. Effect of lemon (*Citrus limon* L.) addition to *PlucheaPluchea*  
752 *indica* Less beverage. CJFST12(4): 125-139.

753 WIDYAWATI PS *et al.* 2022. The effect of κ-carrageenan proportion and hot water  
754 extract of the *PlucheaPluchea indica* Less leaf tea on the quality and sensory properties  
755 of stink lily (*Amorphophallus muelleri*) wet noodles. *Molecules* 27(5062): 1-16.

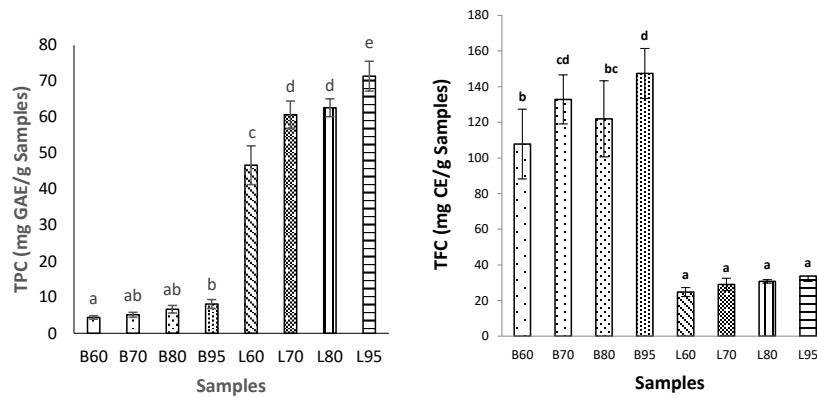
756 Widyawati PS *et al.* 2023. The effect of hot extract of *PlucheaPluchea indica* leaf powder  
757 on the physical, chemical and sensory properties of wet noodles. *CRNFS* 11(1): 276-293.

758 XU P, CHEN L, WANG Y. 2019. Effect of storage time on antioxidant activity and inhibition  
759 on α-amylase and α-glucosidase of white tea. *Food Sci Nut* 7(2): 636-644.

760 ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction time on caffeine in  
761 coffee. *Lantanida J* 6(2): 103-202. [https://jurnal.ar-](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811)  
762 [raniry.ac.id/index.php/lantanida/article/view/3811](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811). [In Bahasa Indonesia]

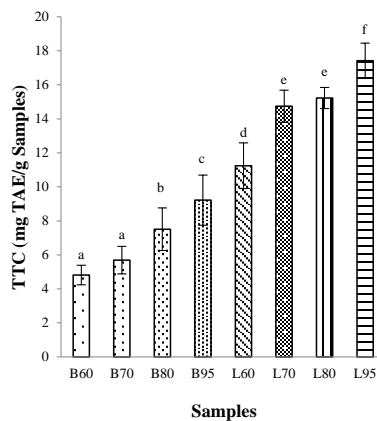
763 ZHANG Y *et al.* 2022. Effect of storage conditions and time on the polyphenol content of  
764 wheat flours. *Processes* 9(248): 1-11.

Commented [A97]: Reconcile list with text and vice versa.



(a)

(b)



(c)

Figure 1. Bioactive compound contents of *Pluchea tea-infusion* at different steeping temperature and storage time (a) Total phenolic content (b) Total flavonoid content (c) Total tannin content

(Values were Means are expressed as  $\pm$  standard deviations (n=6).

Means that share the same letter between columns are not significantly different at  $p < 0.05$

Samples: B60-Steeped at 60C, unstored; B70-Steeped at 70C, unstored; B80-Steeped at 80C, unstored; B95-Steeped at 95C, unstored; L60-Steeped at 60C, Stored for 5 years; L70-Steeped at 70C, Stored for 5 years; L80-Steeped at 80C, Stored for 5 years; L95-Steeped at 95C, Stored for 5 years

Formatted: Font: 10 pt

786

Different superscripts in graph showed a significant difference based on the

787

DMRT test ( $\alpha \leq 5\%$ )

Corresponding Author: [paini@ukwms.ac.id](mailto:paini@ukwms.ac.id)



788  
789

Table 1. Simple Phenolic Compound Profile-profile of Pluchea Pluchea Tea Infusion at dDifferent sSteeping tTemperature and sStorage tTime

Formatted: Line spacing: single

Samples	Gallic Acid (µg/g samples)	(+)-Catechin (µg/g samples)	Myricetin (µg/g samples)	Quercetin (µg/g samples)	Kaempferol (µg/g samples)	3,4-di-O-	3,5-di-O-	4,5-di-O-
						Caffeoylquinic acid (µg/g samples)	Caffeoylquinic acid (µg/g samples)	Caffeoylquinic acid (µg/g samples)
B60	0.2132±0.0027	0.3425±0.0110	0.1756±0.1234	0.0220±0.0268	0.1394±0.0202	0.6103±0.0628	0.6635±0.0628	0.4906±0.0060
B70	0.2157±0.0013	0.3260±0.0265	0.2587±0.0160	0.1530±0.0511	0.0514±0.0037	0.6271±0.0099	0.6162±0.0099	0.4807±0.0034
B80	0.2234±0.0122	0.3240±0.0222	0.4175±0.0104	0.3666±0.0103	0.3699±0.0924	0.7967±0.03060	0.6601±0.0306	0.5299±0.0053
B95	0.2316±0.0104	0.4039±0.0320	0.8786±0.0434	0.6559±0.0570	0.5913±0.0239	1.5386±0.0668	0.6642±0.0668	1.0018±0.0526
L60	0.2364±0.0015	0.5085±0.0111	1.4762±0.0271	0.6220±0.0706	0.3675±0.0183	2.4863±0.0270	0.9449±0.0501	1.1842±0.0120
L70	0.2324±0.0214	0.5448±0.0006	1.4245±0.2526	1.0708±0.0289	0.3726±0.0944	2.3403±0.0325	0.9485±0.0794	1.0089±0.0736
L80	0.2347±0.0078	0.5023±0.0773	1.457±0.0925	0.8629±0.0815	0.7966±0.0366	2.6278±0.0211	0.9099±0.0387	1.2382±0.1435
L95	0.2402±0.0169	0.5995±0.0372	2.6138±0.0695	2.0230±0.0573	0.9478±0.0287	4.0211±0.0851	1.3156±0.0166	1.3797±0.2170

790  
791  
792

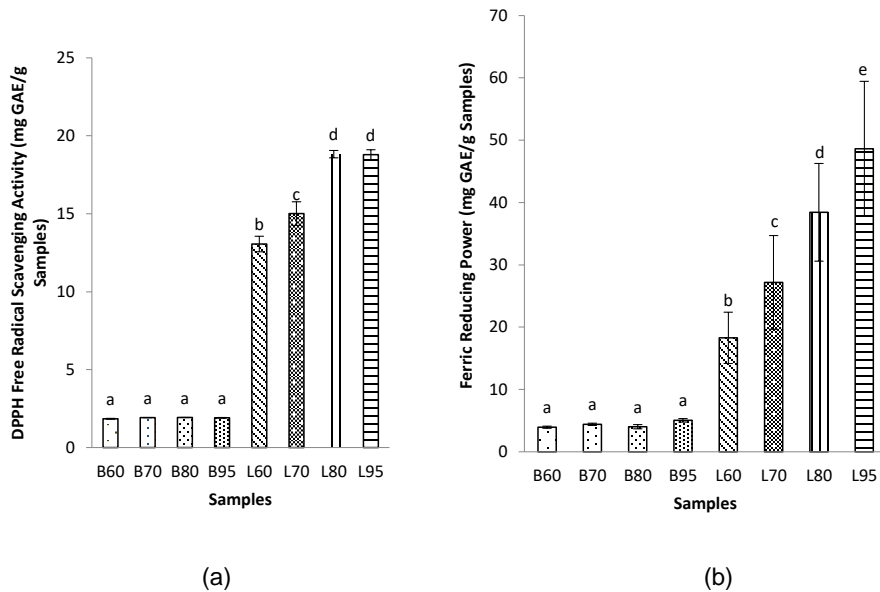
Note : data of phenolic compound profile Treatment means was obtained from two replicates that displayed expressed as mean ± SD. Steeping temperature, 60, 70, 80 and 95C; Storage Time- 0, 5 years

Formatted: Font: 11 pt

Formatted: Font: 11 pt

Formatted: Font: 9 pt

Formatted: Line spacing: single



793

794

795

796

797

798

799

800

801

802

803

804

805

806

807

808

809

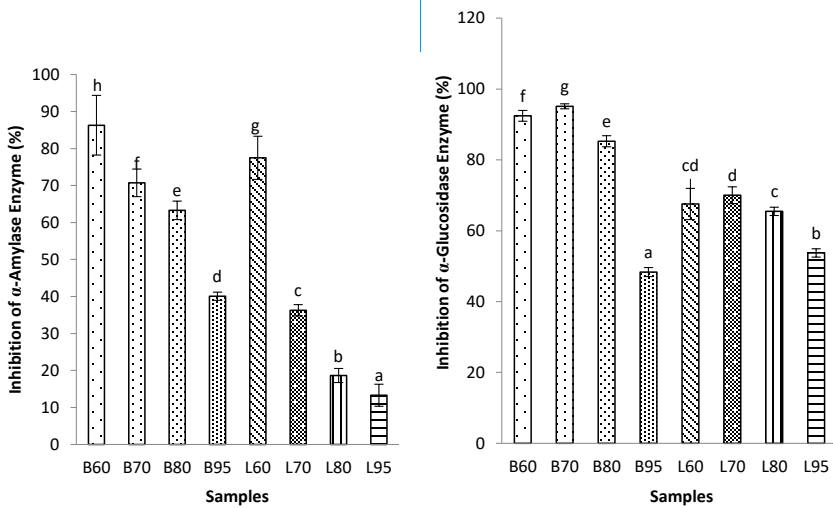
Figure 2. Antioxidant activity of *Pluchea tea* infusion at different steeping temperature and storage time (a) DPPH free radical scavenging activity (b) Ferric reducin antioxidant activity (FRAP) (Values Treatment means were means are expressed as  $\pm$  standard deviations (n=6). Means that share the same letter between columns are not significantly different at p (<0.5) Samples: B60-Steeped at 60C, unstored; B70-Steeped at 70C, unstored; B80-Steeped at 80C, unstored; B95-Steeped at 95C, unstored; L60-Steeped at 60C, Stored for 5 years; L70-Steeped at 70C, Stored for 5 years; L80-Steeped at 80C, Stored for 5 years; L95-Steeped at 95C, Stored for 5 years Different supercripts in graph showed a significant difference based on the DMRT test ( $\alpha \leq 5\%$ )

Formatted: Font: 11 pt

Formatted: Font: (Default) Arial, 11 pt

Formatted: Indent: Left: 0.5"

Formatted: Left, Line spacing: single



**Commented [A98]:** Delete line 810 red line above small letters (a) and (b).  
Delete lines 824 to 850

810

811

(a)

(b)

812

813

814

815

816

817

818

819

820

821

822

823

824

Figure 3. Antidiabetic activity Percent (%) inhibition of pluchea-Pluchea tea infusion at different steeping temperature and storage time (a)  $\alpha$ -amylase (b)  $\alpha$ -glucosidase  
Treatment means are expressed as  $\pm$  standard deviations (n=6).  
Means that share the same letter between columns are not significantly different at p (<0.5)  
Samples: B60-Steeped at 60C, unstored; B70-Steeped at 70C, unstored; B80-Steeped at 80C, unstored; B95-Steeped at 95C, unstored; L60-Steeped at 60C,Stored for 5 years,L70-Steeped at 70C,Stored for 5 years; L80-Steeped at 80C,Stored for 5 years; L95-Steeped at 95C,Stored for 5 years  
 (Values were means  $\pm$  standard deviations (n=6). Different supercripts in graph showed a significant difference based on the DMRT test ( $\alpha \leq 5\%$ )

825

826

827

828

829

830

831

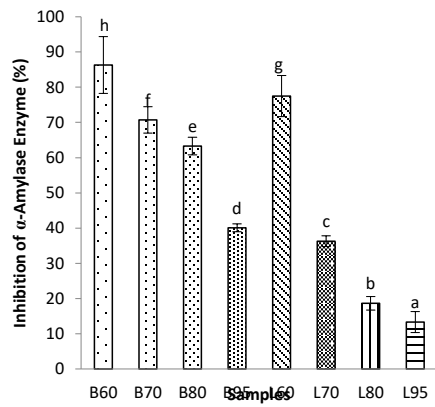
832

833

834

Corresponding Author: [paini@ukwms.ac.id](mailto:paini@ukwms.ac.id)

835



836

837

838 (a)

(b)

839

840

841

842

843

844

845

846 Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and

847 storage time (a)  $\alpha$ -amylase (b)  $\alpha$ -glucosidase (Values were means  $\pm$  standard

848 deviations (n=6). Different supercripts in graph showed a significant difference

849 based on the DMRT test ( $\alpha \leq 5\%$ )

850

Corresponding Author: paini@ukwms.ac.id



Paini Sri Widyawati <paini@ukwms.ac.id>

---

## Fwd: Comments on PJS Paper Ms 23-158

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Fri, Oct 13, 2023 at 10:45 PM

Dear Ms. CARYL MARIA MINETTE I. ULAY  
Editorial Assistant

Greeting,  
Thanks for information  
Regards

Paini Sri Widyawati  
[Quoted text hidden]



Paini Sri Widyawati <paini@ukwms.ac.id>

---

## Fwd: Comments on PJS Paper Ms 23-158

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Tue, Oct 24, 2023 at 10:49 AM

Dear Miss Caryl Maria

Greetings,

Related to revision of my manuscript, when does date line my manuscript revision?  
Please give me information.

Regards

Paini Sri Widyawati  
[Quoted text hidden]



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**Fwd: Comments on PJS Paper Ms 23-158**

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Tue, Oct 24, 2023 at 12:49 PM

Dear Dr. Widyawati,

Greetings! I wish to inform you that we have sent you the compiled review notes on September 20, 2023 (Wednesday). However, we received an email from your end asking us for an update regarding the evaluation. Hence, we sent the compiled review notes again on October 13, 2023 (Friday).

In this regard, we allow you to submit an itemized list of your answers to the said comments together with the revised version of your paper within 30 days after receiving the files. Please let us know if you need more time to prepare in revising your paper.

Sincerely,  
Editorial Assistant  
[Quoted text hidden]





Paini Sri Widyawati <paini@ukwms.ac.id>

---

## Fwd: Comments on PJS Paper Ms 23-158

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Tue, Oct 24, 2023 at 2:04 PM

Dear Ms Caryl Maria

Greetings,

Because I received an assignment from the Chancellor for a curriculum meeting until the end of the month, I ask permission to upload the revised manuscript until the end of November. However, I promise that once the assignment is complete, I will immediately revise it and send it back.

Thank you for your cooperation

Regards

Paini SW

[Quoted text hidden]



Paini Sri Widyawati <paini@ukwms.ac.id>

---

## Fwd: Comments on PJS Paper Ms 23-158

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Tue, Oct 24, 2023 at 3:07 PM

Dear Dr. Widyawati,

Greetings! This is to inform you that we at the PJS Editorial Office are amenable to your requested extension in the submission of your revision and itemized response to reviewers' comments on the Ms 23-158 paper.

We hope to receive your feedback on 30 November 2023 (Friday). Thank you!

Sincerely,

Ms. CARYL MARIA MINETTE I. ULAY  
Editorial Assistant

[Quoted text hidden]



Paini Sri Widyawati <paini@ukwms.ac.id>

---

## Fwd: Comments on PJS Paper Ms 23-158

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Tue, Oct 24, 2023 at 3:26 PM

Dear Ms Ceryl Maria

Greetings,

Thanks for your attention

Regards

Paini SW

[Quoted text hidden]



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**Fwd: Comments on PJS Paper Ms 23-158**

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Wed, Nov 22, 2023 at 6:37 PM

Dear Ms CARYL MARIA MINETTE I. ULAY

Greetings,

I sent my manuscript revision with the title "Effect of Steeping Temperature and Storage Time on the Bioactive Compounds, Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea Indica* Less. I have tried to fulfill the reviewer's suggestion .

Thanks for attention

The Best Regards

Paini Sri Widyawati  
[Quoted text hidden]



**Effect of Brewing Temperature and Storage Time on Antioxidant and Antidiabetic Properties of Pluchea Tea Final.docx**  
111K

1 **Effect of Steeping Temperature and Storage Time on the Bioactive Compounds,**  
2 **Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea Indica***  
3 **Less**

4 Painsi Sri Widyawati<sup>1\*)</sup>, Yufita Ratnasari Wilianto<sup>2)</sup>

5 <sup>1)</sup>Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala  
6 Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

7 <sup>2)</sup>Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic  
8 University, Kalisari Street Number 1, Surabaya 60272, Indonesia

9 Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea*  
10 *indica* Less, storage time

21 ABSTRACT

22 This study was done to determine the effects of steeping temperature and storage time  
23 on the bioactive contents, antioxidant and antidiabetic activities of *Pluchea* leaf infusion.  
24 The research used a randomized block design with two factors, i.e., steeping temperature  
25 (T) and storage time (B). The variety of the steeping temperatures included 60 (T1), 70  
26 (T2), 80 (T3), and 95 (T4) (°C) with the storage time of 0 (B1) and 5 (B2) (year). The  
27 research resulted 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2,  
28 T4B1, T4B2). Statistical analysis using a paired T test at  $\alpha \leq 0.05$  showed that treatments  
29 significantly influenced the bioactive contents (total phenol (TPC), total tannin (TTC), total  
30 flavonoid (TFC)), antioxidant (DPPH scavenging activity (DPPH) and ferric reducing  
31 antioxidant power (FRAP)) and antidiabetic ( $\alpha$ -amylase (AA) and  $\alpha$ -glucosidase (GA)  
32 inhibitors) activities of samples. The bioactive contents influenced antioxidant and  
33 antidiabetic activities. TFC was decreased for storage time and significant increased at  
34 higher steeping temperature. The AA and GA of *Pluchea* infusion increased until 70 °C  
35 of the steeping temperature, but decreased until 95 °C. The AA and GA were strongly and  
36 negatively correlated with TPC, TTC, DPPH and FRAP, but it was moderately and  
37 negatively correlated with TFC. Between the antioxidant activity of DPPH and FRAP and  
38 the antidiabetic activity of AA and GA of *Pluchea* infusion were strongly and positively  
39 correlated with correlation coefficient (r) values of 0.956 and 0.725, respectively. The  
40 treatments gave different effect of simple phenolic compounds, such as gallic acid,  
41 kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-caffeoylquinic acid, 3,5- di-O-  
42 caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid of *Pluchea* infusion at different  
43 steeping temperature and long storage. To obtain high antioxidant activity, *Pluchea*

44 infusion selected was stored and steeped at high temperature, however high antidiabetic  
45 activity obtained was fresh *Pluchea* infusion and steeped at low temperature.

46

## 47 INTRODUCTION

48 *Pluchea* herbal tea is a product of dried *Pluchea* leaf processing introduced by  
49 world people (Srisook et al., 2012; Widyawati et al., 2016) because of the efficacy of the  
50 active components in *Pluchea* leaves, as an herbal plant that has been widely used for  
51 traditional medicine and food (Chan et al., 2022). *Pluchea* leaves are composed many  
52 nutrients and bioactive compounds useful to body health. The nutrient compositions in  
53 the *Pluchea* leaves include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates,  
54 calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive compounds is comprised, i.e.,  
55 chlorogenic acid, caffeic acid, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-  
56 caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-  
57 caffeoylquinic acid, quercetin, myricetin, kaempferol, total anthocyanin,  $\beta$ -carotene, and  
58 total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et  
59 al., 2022, Chan et al., 2022).

60 Steeping process of *Pluchea* leaves can be performed with fresh or dry leaves  
61 infusion by hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et al.,  
62 2020; Jayani et al., 2022). In Asian area, especially in Indonesian, people usually  
63 consume the *Pluchea* infusion with brewing of powdered *Pluchea* leaves in tea bag by  
64 hot water or boiling water. Each tea bag contained 2 g of *Pluchea* leaf powder is steeped  
65 with 100 mL hot water or boiling water. Widyawati et al. (2016) claimed that steeping of 2  
66 g *Pluchea* leaf powder at 95 °C for 5 minutes results total phenolic content, total flavonoid

67 content, the ability to scavenge DPPH free radicals, and the capability of reduce ferric  
68 ions 9.3 mg gallic acid equivalent (GAE)/g samples, 22.0 mg gallic acid equivalent  
69 (GAE)/g samples, 27.2 mg gallic acid equivalent (GAE)/g samples, and 10.2 mg gallic  
70 acid equivalent (GAE)/g samples, respectively. Werdani and Widyawati (2018) reported  
71 that drinking of *Pluchea* leaf powder infusion in the morning and evening regularly (2  
72 g/100 mL) can decline blood sugar levels.

73 The steeping of *Pluchea* herbal tea with hot water at 95 °C for 5 minutes certainly  
74 determines the stability and amount of extracted bioactive compounds, that influences  
75 the biological activity, especially antioxidant and antidiabetic activities. Silva-Ramirez et  
76 al. (2020) reported that the infusion process can influence their content and composition  
77 of the bioactive compounds and antioxidant activity of tea. Acar et al. (2022) informed  
78 that infusion quality of herbal tea extract depends on several factors, i.e., time and  
79 temperature. Polyphenol profile and antioxidant properties of herbal tea infusion decline  
80 with an increase in steeping/brewing and storage temperatures and longer exposure  
81 times.

82 Several studies have mentioned the effect of steeping temperature to bioactive  
83 compound contents and antioxidant activity, such as some white and green teas are  
84 effective with hot water at 90 °C for 7 min (Castiglioni et al., 2015), roseship tea is  
85 effectively at infusion time around 6-8 min at temperatures of 84-86 °C (Ilyasoglu and  
86 Arpa, 2017), the coffee brewing temperature influences the caffeine content extracted  
87 (Zarwinda and Sartika, 2018), the steeping of dark tea at 92 °C for 27 min results the  
88 highest total phenol content and antioxidant activity (Wang et al., 2022). The study of the  
89 effect of steeping temperature to *Pluchea* infusion was carried out to afford information



90 about preparation of powdered *Pluchea* leaves most efficiently to get higher the bioactive  
91 compounds, antioxidant and antidiabetic activities.

92 On the other hand, storage time of *Pluchea* herbal tea also affects the levels of the  
93 bioactive compounds and biological activity because this herbal tea usually is stored for  
94 a several months until years (Jayani et al., 2022). Tea or herbal tea is generally stored in  
95 ambient temperature and packed in tea bag or Alu foil standing proud or a combination  
96 of both. Many researchers informed that storage time decreases the bioactive  
97 compounds, antioxidant and antidiabetic activities, i.e., juice from *Momordica charantia* L.  
98 (Lin et al., 2020), dried *Piper bettle* extracts (Ali et al., 2018), white tea (Xu et al., 2019),  
99 kinnow-amlam beverages (Purewal et al., 2022), whole wheat flour (Zhang et al., 2021).  
100 Therefore, this research studied the effect of steeping temperature and storage time on  
101 the bioactive compounds, antioxidant and antidiabetic activities of infusion from powdered  
102 *Pluchea* leaves. The study was done to determine total phenolic content (TPC), total  
103 flavonoid content (TFC), total tannin content (TTC), DPPH free radical scavenging activity  
104 (DPPH), ferric reducing antioxidant power (FRAP),  $\alpha$ -amylase (AA) and  $\alpha$ -glycosidase  
105 (GA) inhibition activities, and phenolic compound profile.

106

## 107 MATERIALS AND METHODS

### 108 RAW MATERIALS AND PREPARATION

109 The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya,  
110 East Java, Indonesia. The *Pluchea* plants were included in *Asteraceae* family with  
111 specification according to the GBIF taxon ID number database:3132728 (Ferraris, 2023).  
112 *Pluchea* leaves at 1-6 level of each branch of the shoot were collected, sorted, washed

113 and dried to get moisture content around  $11.16 \pm 0.09$  % dry base (Widyawati et al.,  
114 2022). The powdering of dried *Pluchea* leaves was done to get a 45-mesh size. And then,  
115 the heating of the *Pluchea* leaf powder was done using a drying oven (Binder, Merck  
116 KGaA, Darmstadt, Germany) at 120 °C for 10 min to reduce microbial organisms and  
117 packed using infusion bag that made from paper filter around 2 g/bag. And then all of  
118 samples called *Pluchea* herbal tea was stored for 0 and 5 years in standing pouch before  
119 analysis.

120 In the research, the one tea bag of *Pluchea* herbal tea that stored 0 (B1) and 5  
121 (B2) year, was steeped with 100 mL hot water at various temperatures, including 60 (T1),  
122 70 (T2), 80 (T3), and 95 (T4) °C for 5 min with infusion method that obtained 8 treatment  
123 combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2. After the  
124 temperature of *Pluchea* infusion similar to ambient temperature was analyzed further.

125

## 126 REAGENTS

127 The compounds used to analyze including 2,2-diphenyl-1-picrylhydrazyl (DPPH),  
128 sodium carbonate, gallic acid,  $\alpha$ -amylase,  $\alpha$ -glucosidase, pNPG (p-nitrophenyl- $\alpha$ -  
129 glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-O-caffeoylquinic  
130 acid, 4,5-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and (+)-catechin were  
131 purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, Folin–Ciocalteu’s Phenol,  
132 sodium nitric, aluminum chloride, ferric chloride, sodium dihydrogen phosphate, sodium  
133 phosphate, potassium ferricyanide, starch, acetic acid, and sodium hydroxide were  
134 purchased from Merck (Kenilworth, NJ, USA). All reagents used were of analytical grade  
135 except for distilled water which was purchased from PT Aqua Industry Surabaya.

136

## 137 METHODOLOGY

### 138 ANALYSIS OF THE BIOACTIVE COMPOUNDS

#### 139 TOTAL PHENOLIC CONTENT ANALYSIS

140 Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the  
141 technique by Gao et al. (2019). About 10  $\mu$ L *Pluchea* infusion and 1 mL Folin-Ciocalteu's  
142 phenol reagent 10 % were mixed in 10 mL volumetric flask and incubated for 5 min. And  
143 then 2 mL  $\text{Na}_2\text{CO}_3$  7.5 % was entered and distilled water was added until 10 mL volume.  
144 The color intensity of solution was measured in the spectrophotometer UV-Vis 1800  
145 (Shimadzu, Japan) at  $\lambda$  760 nm with gallic acid as the reference standard. The total  
146 phenolic content was calculated using the formula:  $y=0.00009x+0.008$  with  $R^2=0.9941$ .  
147 The results were expressed as mg gallic acid equivalent (GAE)/g samples.

148

#### 149 TOTAL FLAVONOID CONTENT ASSAY

150 Total flavonoid content (TFC) of the samples was measured based on the reaction  
151 between  $\text{AlCl}_3$  and  $\text{NaNO}_2$  with an aromatic ring of flavonoid compounds, especially  
152 flavonol and flavon (Shraim et al., 2021). The reaction between  $\text{AlCl}_3$  and flavonoid  
153 compounds resulted a yellow solution. About 30  $\mu$ L *Pluchea* infusion was mixed with 0.3  
154 mL  $\text{NaNO}_2$  5 % in 10 mL volumetric flask and incubated for 5 min. The mixture was added  
155 with 0.3 mL  $\text{AlCl}_3$  10 % for 5 min. And then, 2 mL  $\text{NaOH}$  1 M and distilled water were  
156 added until 10 mL volume. Then, the red solution was produced after  $\text{NaOH}$  solution  
157 addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800,  
158 Shimadzu, Japan) at  $\lambda$  510 nm with (+)-catechin as the reference standard compound,

159 and the results were expressed as mg catechin equivalents (CE)/g samples using the  
160 formula:  $y=0.00008x-0.0023$  with  $R^2= 0.9980$ .

161

## 162 TOTAL TANNIN CONTENT ANALYSIS

163 Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method  
164 (Chandran and Indira, 2016). Approximately 10  $\mu$ L *Pluchea* infusion was added 1 mL  
165 Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flask and incubated for 5 min.  
166 Then, the mixture was added 2 mL  $\text{Na}_2\text{CO}_3$  7.5 % and distilled water was added until  
167 10 mL volume. The blue dark color solution that measured UV-Vis spectrophotometer  
168 1800 (Shimadzu, Japan) at  $\lambda$  760 nm with tannic acid as the reference standard.  
169 Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/g samples used  
170 the formula:  $y=0.00009x+0.0021$  with  $R^2=0.9993$

171

## 172 ANALYSIS OF THE ANTIOXIDANT POTENTIAL

### 173 DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

174 The DPPH free radical scavenging activity (DPPH) was measured by the  
175 spectrophotometric method (Widyawati et al., 2017) to determine antioxidant activity of  
176 the *Pluchea* leaf infusion to donor hydrogen atom to nitrogen atom in DPPH resulting  
177 DPPH-H compound with a yellow-colored solution. About 25  $\mu$ L *Pluchea* leaf infusion was  
178 entered in reaction tube and added 3 mL DPPH solution (4 mg/100 mL). And then the  
179 solution was incubated for 15 min in a dark room and absorbance was measured by a  
180 spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$ . 517 nm.  
181 The reference standard compound was gallic acid and the results of analysis were

182 expressed as mg gallic acid equivalents (GAE)/g samples that calculated using formula:  
183  $y=0.146x+1.7896$  with  $R^2=0.9975$ .

184

#### 185 FERRIC REDUCING POWER ANALYSIS

186 Ferric reducing power (FRAP) was determined following the method used by  
187 Widyawati et al. (2014) method. Approximately 10  $\mu$ L of samples were added 2.5 mL  
188 phosphate buffer pH 6.6 and 2.5 mL potassium ferricyanide 1% in reaction tube. And then  
189 mixture was shaken and incubation for 20 min at 50 °C. Finally, 2.5 mL chloroacetic acid  
190 10% (w/v) was added. 2,5 mL supernatant was added 2.5 mL distilled water, 0.5 mL  
191 ferric chloride 0.1% w/v and incubated for 10 min. Potency of the samples reducing iron  
192 (III) to iron (II) ion was signed by intensity of blue color formed that measured using UV-  
193 Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  700 nm.  
194 Intensity of the blue color indicated higher reducing capacity. The reducing power  
195 expressed as mg gallic acid equivalent (GAE)/g samples was calculated using the  
196 formula:  $y=0.0002x+0,0256$  with  $R^2=0,9906$ .

197

#### 198 $\alpha$ -AMYLASE ENZYME INHIBITION CAPACITY ASSAY

199 In vitro inhibition of  $\alpha$ -amylase enzyme (AA) followed the procedure as described  
200 by Widyawati et al. (2020). Each 500  $\mu$ L of samples, starch 1 % (w/v) and sodium acetate  
201 buffer pH 5 were mixed. Then, each 250  $\mu$ L of mixture and  $\alpha$ -amylase solution (0.1 g of  
202 this enzyme 12.5 unit/mL was dissolved in 50 mL of 0.2 M sodium acetate pH 5) was  
203 shaken and added 2 mL sodium hydroxide 1M. Before analysis, this mixture was  
204 incubated at 37 °C for 10 min. Then, the capacity of the  $\alpha$ -amylase enzyme hydrolyzed

205 the starch to release glucose that could be analyzed based on absorbance at  $\lambda$  540 nm.  
206 The inhibition percentage of  $\alpha$ -amylase was assessed using the formula:  $(ACb - Aca) -$   
207  $(As - Ab) (ACb - Aca) \times 100 \%$ . Where, ACb is the absorbance of 100 % enzyme activity  
208 (solvent with the enzyme), Aca is the absorbance of 0 % enzyme activity (solvent without  
209 the enzyme), As is the absorbance of test sample with enzyme, Ab is absorbance of test  
210 sample without enzyme.

211

#### 212 $\alpha$ -GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

213 The analysis of the  $\alpha$ -glycosidase inhibitor activity (GA) was done by Widyawati et  
214 al. (2020) method with slight modification. About 150  $\mu$ L samples contained 100  $\mu$ L  
215 *Pluchea* infusion and 50  $\mu$ L pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH 7)  
216 were reacted with 50  $\mu$ L  $\alpha$ -glycosidase 2 mM (0.0833 unit/mL), and then the mixture was  
217 incubated at 37 °C for 15 min. Finally, the reaction was stopped with addition of 1000  $\mu$ L  
218 sodium carbonate 0.2 M. The residue of this enzyme hydrolyzed p-nitrophenyl- $\alpha$ -D-  
219 glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The inhibitor activity of  
220 steeping *Pluchea* tea to enzyme was measured by UV-vis spectrophotometer  
221 (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at  $\lambda$  405 nm. The inhibition  
222 percentage of  $\alpha$ -glycosidase was calculated using formula:  $(ACb - Aca) - (As - Ab) (ACb$   
223  $- Aca) \times 100 \%$ . Where, ACb is the absorbance of 100 % enzyme activity (solvent with  
224 enzyme), Aca is the absorbance of 0 % enzyme activity (solvent without enzyme), As is  
225 the absorbance of test sample with enzyme, Ab is the absorbance of test sample without  
226 enzyme.

227

## 228 HPLC ANALYSIS OF PHENOLICS

229 The phenolic compounds of the samples were analyzed by HPLC based on  
230 Kongkiatpaiboon et al. (2018) method with modifications. Each *Pluchea* infusion was  
231 sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter  
232 syringe (Whatmann, 0.2 µm, NYL). About 20 µL of sample was injected in an HPLC  
233 (LC20AD series, Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC  
234 LC-20AD pump, CTO-30A column oven, CBM-20A/20 Alite system controller, and SPD-  
235 20A/20 AV UV-Vis detector. Separation of phenolic compounds in samples was carried  
236 out using a Shim-pack VP-ODS C18 column (ID 5 µm × 50 mm × 4.6 mm) with a GVP-  
237 ODS Cartridge guard column (2 pieces) (ID 10 mm × 4.6 mm). Analytical conditions: the  
238 mobile phase used consisted of a solution of (A) 0.5 % acetic acid in water and (B)  
239 absolute methanol. Analysis was carried out using a gradient system in the following  
240 order: initial conditions of 10 % B in A to 50 % B in A were maintained for 40 minutes;  
241 then 100 % B was maintained for 20 minutes. Next the column was re-equilibrated with  
242 10 % B in A maintained for 10 minutes before analysis of the next sample. The sample  
243 flow rate was set at 1.0 ml/min with a controlled temperature at 40 °C. Detection was used  
244 at a wavelength of 280 nm. The reference standard used were gallic acid, (+)-catechin,  
245 myricetin, quercetin, kaempferol, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and  
246 4,5-dicaffeoylquinic acid. All of the reference standard was dissolved in distilled water  
247 and prepared similar to the samples before injected in HPLC.

248

## 249 EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

250 The research design used a randomized block design with two factors, i.e., the  
251 steeping temperature (T) and the storage time (B). *Pluchea* leaf blades were subjected  
252 to 4 steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95 °C (T4),  
253 and the storage time of 0 year /fresh (B1), and 5 year/stored (B2). The research resulted  
254 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The  
255 HPLC analysis of phenolic was repeated two times. The data of samples were analyzed  
256 by ANOVA at  $\alpha \leq 0.05$ , and continued analysis using a paired T test at  $\alpha \leq 0.05$ . treatment  
257 means of specific phenolic compounds that were identified were expressed as the mean  
258  $\pm$  SD. The analysis used SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

259

## 260 RESULTS AND DISCUSSIONS

261 *Pluchea* leaf infusion is produced by young *Pluchea* leaf from 1-6 level on each  
262 branch the shoot (Widyawati et al., 2022), that is steeped at 95 °C for 5 min, has many  
263 biological activities, such as antioxidant activity (Widyawati et al., 2016), antidiabetic  
264 activity (Werdani and Widyawati, 2018), anti-inflammatory (Srisook et al., 2015). The  
265 chemical constituents in *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols,  
266 cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2  
267 g/100 mL steeping *Pluchea* tea has total phenolic content 9.3 mg gallic acid equivalents  
268 (GAE)/g samples, total flavonoid content 22.0 mg catechin equivalents (CE)/g samples,  
269 DPPH free radical scavenging activity 27.2 mg gallic acid equivalents (GAE)/g samples,  
270 and ferric reducing power 10.2 mg gallic acid equivalents (GAE)/g samples (Widyawati et  
271 al., 2016). Previous research has informed related to the composition of phytochemical  
272 compounds in *Pluchea* leaves, such as phenolic acids such as chlorogenic acids, caffeic



273 acids, 3-O-caffeoylquinic acids, 4-O-caffeoylquinic acids, 5-O-caffeoylquinic acids, 3,4-  
274 di-O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic  
275 acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin;  $\beta$ -  
276 carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al.,  
277 2019; Chan et al., 2022; Widyawati et al., 2022). Presence of phytochemical compounds  
278 in herbal product were influenced by environmental factors, i.e., temperature, light  
279 exposure, oxygen level, pH and moisture. The structure of phytochemical compounds in  
280 herbal tea is very sensitive of the surrounding changes. The effect arising from these  
281 changes causes the structure of the phytochemical molecule to be degraded to produce  
282 smaller size molecules or to combine to produce larger size molecules (Ali et al., 2018;  
283 Jayani et al. 2022, Ramphinwa et al., 2023). Therefore, this study was conducted to  
284 determine the effect of steeping temperature and storage time of *Pluchea* tea on levels  
285 of the bioactive compounds, antioxidant and antidiabetic properties and phenolic  
286 compound profile.

287

## 288 BIOACTIVE COMPOUNDS

### 289 Phenolics Compounds

290 The bioactive compounds are active compounds in plants that are essential to  
291 protect a body health (Nguyen and Chuyen, 2020). These compounds usually have many  
292 biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer,  
293 antimicrobial, antibacterial, anti-cholesterol and so on (Suriyaphan, 2014; Acar et al.,  
294 2022). Phenolic compounds have potential redox properties that can scavenge free

295 radicals that can cause a number of chronic diseases (Noreen et al., 2017; Aryal et al.,  
296 2019; Acar et al., 2022).

297 The total phenolic content (TPC) of *Pluchea* infusion at different steeping  
298 temperature and storage period generally significantly increased with increasing steeping  
299 temperature and storage period based on paired T test at  $\alpha \leq 0.05$ . Steeped and stored  
300 infusion had significantly higher amounts of phenolic compounds than the samples were  
301 steeped and un-stored. Further, the highest total phenolic content was observed in  
302 samples infused at 95 °C and stored for 5 years (at  $71.38 \pm 4.14$  mg GAE/g samples) while  
303 the lowest was measured in the un-stored samples and infused at 60 °C. Phenolic content  
304 of samples that were infused at different temperatures then stored were steeped only at  
305 60 and 95 °C also showed a significant increase in their phenolic. This implies that the  
306 steeping temperature and the storage periods significantly resulted in the high amounts  
307 of the phenolic compounds of the infusions. Results also indicated that phenolic  
308 compounds were generally greater in the infusion at high steeping temperatures and long  
309 storage (Figure 1a). This could have been due to that fact that during steeping fresh  
310 *Pluchea* tea had a lower total phenolic content than stored *Pluchea* tea for 5 years,  
311 besides that the higher the steeping temperature also caused the greater the extracted  
312 total phenolic content. The temperature of infusion influenced total phenolic content, it  
313 could relate to migration process of phenolic compounds to the water because of  
314 increasing contact between these compounds and water. The same phenomena also  
315 occurred in Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al. (2022).

316 This occurrence showed that steeping temperature and storage period caused the  
317 process of degradation and oxidation of phenolic compounds. Su et al. (2019) reported

318 that temperature treatment can stimulate the release of phenolic compounds and  
319 increase antioxidant activity of lychee juice stored at different temperatures of 4 and 45  
320 °C and different long storage (fresh and 72 hours). Hydrogen bonding is affected by  
321 temperature treatment because the hydrogen bond between phenolic compounds and  
322 proteins can be degraded that the measured levels of phenolic compounds are higher.  
323 The phenomena were supported by Ali et al. (2018); Jayani et al. (2022) and Ramphinwa  
324 et al. (2023). Zhang et al. (2021) reported that phenolic compounds present in plants are  
325 not completely stable, but are easily degraded during storage after harvest. Reblova  
326 (2012) claimed that antioxidant compounds can be slowly degraded with increasing  
327 temperature. Besides that, Fibrianto et al. (2021) also stated that the brewing  
328 temperature has an effect on the extracted antioxidant compounds, such as alkaloids,  
329 catechins and tannins. Thus, there is an assumption that the phenolic compounds in  
330 *Pluchea* infusion are degraded due to oxidation and hydrolysis because of temperature  
331 and storage time and can be easily extracted during steeping, thus increasing the  
332 phenolic content as the steeping temperature and long storage increase.

333 Based on using of a reference standard could be informed that phenolic  
334 compounds in steeping *Pluchea* infusion, including gallic acids, (+)-catechins, myricetins,  
335 quercetins, kaempferols, 3,4-di-O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and  
336 4,5-di-O-caffeoylquinic acids was showed in Table 1. The results of statistical analysis  
337 using a paired T test at  $\alpha \leq 0.05$  showed that gallic acid and kaempferol of *Pluchea*  
338 infusion were insignificantly different at various steeping temperature and long storage.  
339 Nevertheless, the concentration of quercetin and 3,5-dicaffeoylquinic acid of *Pluchea*  
340 infusion was significantly different of two treatments except at 70 °C. The (+)-catechin

341 concentration of *Pluchea* infusion was significantly different at 95 °C, but the myricetin  
342 was different concentration at 80 and 95 °C. The 3,4-dicaffeoylquinic acid and 4,5-  
343 dicaffeoylquinic acid compounds from *Pluchea* infusion were significantly different at 60  
344 °C, however the concentration of 3,4-dicaffeoylquinic acid was also significantly different  
345 at 80 and 95 °C. Based on the analysis of concentration of simple phenolic compounds  
346 showed that gallic acids and kaempferol were relative stable phenolic acid because of **no**  
347 **changes** at different steeping temperature and storage time with concentration **about**  
348 **0.21± 0.00 to 0.24±0.02 µg/g** samples and **0.14±0.02 to 0.95±0.03 µg/g** samples,  
349 respectively. However, myricetin, (+)-catechin and 3,4-di-O-caffeoylquinic acid showed  
350 drastic increasing at higher steeping temperature and longer storage time. It's meant that  
351 these compounds tended relatively labile. Quercetin, 3,5-di-O-caffeoylquinic acid and  
352 4,5-di-O-caffeoylquinic acid underwent moderate changes compared to the other two  
353 groups of phenolic acids. Therefore, myricetin, (+)-catechin and 3,4-di-O-caffeoylquinic  
354 acid were easier to dissolve at higher steeping temperature and storage time can cause  
355 macromolecules of three phenolic acids in herbal tea convenient degradable to form  
356 simple phenolic compounds for storage, as explained by Su et al. (2019), Ali et al. (2018);  
357 Jayani et al. (2022); Ramphinwa et al. (2023), and Zhang et al. (2021). Degradable  
358 polyphenol compounds have a simple structure and free hydroxyl groups that can react  
359 with Folin-Ciocalteu's Phenol reagent, resulting complex blue solution that can detected  
360 as total phenolic content.

### 361 **Flavonoid Content** (TFC)

362 **Flavonoids are the major phenolic compounds that have potential chemical and**  
363 **biological activities, such as** radical scavenging and antimicrobial activities (Ayele et al.,

2022; Chandra et al., 2014) that can protect the human body from the oxidative stress caused many degenerative diseases, especially cancer, cardiovascular problems and ageing (Mathur and Vijayvergia, 2017). The total flavonoid content of steeped *Pluchea* infusion decreased with longer storage period. Un-stored samples exhibited higher flavonoid content than the stored samples. The statistical analysis using a paired T test at  $\alpha= 0.05$  showed that total flavonoid content of *Pluchea* infusion was significantly different between two treatments (Figure 1b). The highest total flavonoid content was exhibited by fresh samples steeped at 95 °C about 147.42±14.03 mg CE/g samples. Total flavonoid content was significantly lower in the stored regardless of steeping temperature than those of the un-stored around 24.75±2.47 to 33.71±3.06 mg CE/g samples implying that the increase in the flavonoid content of the infusion was affected primarily by the steeping temperature.

#### Tannin Content (TTC)

Tannins are bioactive compounds that provide properties, such as astringent, anti-diarrheal, antibacterial and antioxidant (Malangngi et al., 2012). Generally, results indicated that the total tannin content of *Pluchea* infusion significantly increased with increasing steeping temperature and storage period (Figure 1c). Among, the un-stored steeped samples, the tannin content was significantly lowest in samples infused at 60 °C about 4.81±0.58 to 17.42±1.04 mg TAE/g samples which is significantly different lower from the lowest tannin content of the stored samples. Among the stored and steeped samples, the highest tannin content was observed at samples steeped at 95 °C about 17.42 ± 1.04 mg TAE/g samples and was significantly different from that of the highest tannin content of the un-stored steeped samples at 95 °C about 9.22 ± 1.48 mg TAE/g

387 samples. Indicating that the tannin content was affected by both high steeping  
388 temperature and long storage period and that the presence of high tannin content was  
389 primarily brought about by long storage period. Kowalska et al. (2021) informed that the  
390 condensation of catechins to tannins of polyphenolic compounds is a dominant process  
391 occurred in tea leaves that is accelerated during maceration of raw material. However,  
392 the high temperature can degrade polyphenolic compounds to form simple phenolic  
393 compounds that is essential to body health. The results showed, that the higher the  
394 brewing temperature and the longer the storage time caused the tannin compound to  
395 degrade to result catechin compounds. This phenomenon is in line with the increase in  
396 total phenol levels and the concentration of (+)-catechin compounds. Ali et al. (2018) said  
397 that pH, storage temperature, chemical structure and concentration, light, oxygen,  
398 enzymes and metal ions affect the presence of bioactive compounds in the material.  
399 Nevertheless, Rusita et al. (2019) emphasized that tannins are a polar compound, that is  
400 resistant to heating, as a result the tannin content in *Pluchea* tea increases with increasing  
401 steeping temperature and storage time, this is caused tannins are thermostable complex  
402 compounds.

403

#### 404 ANTIOXIDANT ACTIVITY

405 Antioxidant activity is capability of compounds to inhibit the oxidation of  
406 macromolecules from biological target that involve in oxidative chain reactions (Ali et al.,  
407 2005; Oh et al., 2013). The antioxidant activity assay was done in this research using  
408 DPPH Free Radical Scavenging Activity (DPPH) and ferric reducing power (FRAP)  
409 methods. The phenolic compounds are an active antioxidant that have antioxidant

410 capability depend on their redox properties. The structure of phenolic compounds  
411 determine effectivity to donor hydrogen atom which is negatively correlated with the O-H  
412 phenolic bond strength. The higher antioxidant power of phenolic compounds is caused  
413 the weaker O-H phenolic bond (Kruk et al., 2022). The mechanism of phenolic  
414 compounds is involved as antioxidants through the ability to donate hydrogen atoms,  
415 transfer electrons, reducing agents and singlet oxygen quenchers (Ali et al., 2005; Huang  
416 et al. 2005).

#### 417 DPPH Free Radical Scavenging Activity

418 DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate  
419 antioxidant activity because this method is simple that is suitable to measure the donating  
420 hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of  
421 DPPH to change to yellow color (Munteanu and Apetrei, 2021; Baliyan et al., 2022). The  
422 result of DPPH assay indicates that the DPPH values accrued at higher steeping  
423 temperature and longer storage time. Statistical analysis by ANOVA using a paired T test  
424 at  $\alpha \leq 0.05$  proven that the higher the steeping temperature of fresh *Pluchea* infusion  
425 (T1B1, T2B1, T3B1, and T4B1) was consistent the ability to DPPH free radicals  
426 scavenging activity, whereas the stored *Pluchea* infusion resulted in the higher activity  
427 and the values went up as rising of the infusion temperature. *Pluchea* infusion stored  
428 room temperature for 5 years resulted in the DPPH free radical scavenging activity by  
429 more than 100 %. Steeping at higher temperatures significantly increased the DPPH free  
430 radical scavenging activity in stored *Pluchea* infusion around 15 to 25 %. Steeping at 80-  
431 95 °C in stored *Pluchea* infusion insignificantly affected the free radical scavenging  
432 property of the bioactive compounds (Figure 2a).

433 Scavenging activity of DPPH free radicals was strongly and positively correlated  
434 with total phenolic and tannin levels, but inversely to total flavonoid levels. Based on  
435 Pearson correlation at Table 2, the correlated coefficient values (r) between DPPH and  
436 TPC, TTC and TFC were 0.993, 0.942, and -0.940, respectively. During the storage  
437 process it is possible to form complex phenolic compounds which provide a high ability  
438 to scavenge DPPH free radicals (Thanajiruschaya et al., 2010). This research also  
439 demonstrated that longer storage time and higher infusion temperature produced many  
440 simple phenolic compounds with free hydroxyl groups capable to donor hydrogen atom  
441 to DPPH free radical. Many phenolic acids, such as gallic acids, (+)-catechins, myricetins,  
442 kaempferols, quercetins, 3,5-di-O-caffeoylquinic acids, 3,4-di-O-caffeoylquinic acids, 4,5-  
443 di-O-caffeoylquinic acids have established potential antioxidant activity (Kumar and Goel,  
444 2019). Kruk et al (2022) informed that the capability of phenolic compounds to donor  
445 hydrogen atom depends on chemical structure, number and position of hydroxyl groups  
446 attached to a benzene ring, a double bond between C2 and C3 rings and a carbonyl group  
447 (C=O) on the C ring at C4. The effectivity of antioxidant compounds donor hydrogen atom  
448 is determined by O-H bond dissociation energy.

449 The DPPH free radical scavenging property observed in the study was not  
450 consistent with the results of the study by Moraes-de-Souza et al. (2008). The research  
451 shows that total phenolic content of herbal infusion is low correlated with DPPH free  
452 radical scavenging activity. However, Dobrinas et al. (2021) informed that total phenolic  
453 content of tea infusion is positively and significantly correlated with Inhibitor activity of  
454 DPPH.

455



456 Ferric Reducing Antioxidant Power (FRAP)

457 FRAP is an analysis of antioxidant power of the phytochemical compounds based  
458 on the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic  
459 acid, and ferric chloride to produce a color complex, that can be measured at  $\lambda$  700 nm  
460 (Fu et al., 2011; Al-Temimi and Choudhary, 2013). The principle of the assay measures  
461 of the ability of antioxidant compounds to reduce iron ions of potassium ferrocyanide  
462 ( $\text{Fe}^{3+}$ ) to be potassium ferrocyanide ( $\text{Fe}^{2+}$ ). Potassium ferrocyanide reacts with ferric  
463 chloride to form a ferric-ferrous complex and results green color solution (Widyawati et  
464 al., 2017; Raharjo and Haryoto, 2019).

465 The results showed that the ferric reducing antioxidant power (FRAP) increased  
466 with higher steeping temperature and long storage time. The lowest FRAP was observed  
467 in the un-stored samples which was steeped at 60 °C at  $3.95 \pm 0.17$  mg gallic acid  
468 equivalents (GAE)/g samples, and the highest was owned by *Pluchea* infusion which was  
469 stored for 5 years at 95 °C at  $48.63 \pm 10.83$  mg gallic acid equivalents (GAE)/g samples  
470 (Figure 2b). FRAP increased significantly as steeping temperature was increased. FRAP  
471 of the samples stored for 5 years was also significantly higher than the stored samples at  
472  $\alpha \leq 0.05$ . Based on Pearson correlation, the FRAP of *Pluchea* infusion was strongly and  
473 positively significant correlated with the DPPH, TPC and TTC, but inversely to TFC. The  
474 correlated coefficient values (r) between FRAP and DPPH, TPC, TTC and TFC were  
475 0.956, 0.953, 0.948 and -0.826, respectively.

476 This case was contrast to the antioxidant activity of DPPH and FRAP on matcha,  
477 because the longer storage time reduces the levels of catechin content due to the  
478 catechins, such as epigallocatechin gallat (EGCG), epicatechin gallat (ECG),

479 epigallocatechin (EGC), and epicatechin (EC) are bioactive compounds that have high  
480 antioxidant activity (Kim et al. 2020), and also the case of the effect of temperature and  
481 storage time in betel (*Piper bettle* L.) extract. Light and temperature influence degradation  
482 of phenolic compounds of betel that determine antioxidant activity. Different structure of  
483 phenolic compounds determines their stability to degrade accelerating of light and  
484 temperature. Hydroxychavicol is the best stability of phenolic compounds of betel  
485 compared with eugenol, isoeugenol and allyl pyrocatechol (Ali et al., 2018).  
486 Thanajiruschaya et al. (2010) revealed that the antioxidant activity of rice stored at high  
487 temperatures is greater than that stored at low temperatures. The ferric reducing  
488 capability of *Pluchea* infusion corresponded to simple phenolic acid values, presence of  
489 them in samples could accrue antioxidant activity because of ability of the electron  
490 transfer from free hydroxyl groups of phenolic acids.

#### 491 ANTIDIABETIC ACTIVITY

##### 492 $\alpha$ -Amylase enzyme inhibition activity (AA)

493 Antidiabetic activity is a measure of the potency of phenolic compounds to regulate  
494 the uptake of glucose by the cells from the blood through the mediation of 2-degestive  
495 enzymes i.e.,  $\alpha$ -amylase and  $\alpha$ -glucosidase, which are involved the control of dietary  
496 carbohydrate digestion and release in the postprandial blood glucose in human body (Fu  
497 et al., 2017). The phenolic compounds have the capability to bind with the protein  
498 component of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Martinez-Solis et al., 2022)  
499 resulting in the reduced activity of the enzymes. The results showed, that the steeping  
500 *Pluchea* infusion was able to inhibit the action of the  $\alpha$ -amylase enzymes (Figure 3a). The  
501 *Pluchea* infusion had very good activity, more than 50 % and even almost 100 % for fresh

502 *Pluchea* infusion which was brewed at 60, 70 and 80 °C and stored *Pluchea* infusion  
503 which steeped at 60 °C. Whereas fresh *Pluchea* infusion steeped at 95 °C for 5 minutes  
504 had an activity of inhibiting the  $\alpha$ -amylase enzyme of less than 50 %, which was equal to  
505 40.08±1.12 %. Widyawati et al. (2017) detected the ability to inhibit the  $\alpha$ -amylase enzyme  
506 from fresh *Pluchea* infusion steeped at 95 °C for 5 minutes by 28.79 %. Increasing the  
507 steeping temperature and storage time reduced the ability to inhibit the  $\alpha$ -amylase  
508 enzyme. The results of the analysis based on a paired T test at  $\alpha \leq 0.05$  showed, that the  
509 steeping temperature and storage time had a significant effect on the ability to inhibit the  
510  $\alpha$ -amylase enzyme. Based on Pearson correlation, the AA of *Pluchea* infusion was  
511 strongly and negatively significant correlated with TPC, TTC, DPPH and FRAP, but it was  
512 moderately and negatively significant correlated with TFC. The correlated coefficient  
513 values (r) between AA and TPC, TTC, DPPH, FRAP and TFC were -0.708, -0.857, -0.696,  
514 -0.806 and 0.429, respectively.

515 This inhibitory activity was thought to be contributed by other bioactive compounds,  
516 besides phenolics which are sensitive to steeping temperature and storage time. Li et al.  
517 (2018) stated that there are flavonoid compounds that contribute to the ability to inhibit  
518 the  $\alpha$ -amylase enzyme. Flavonoid compounds with a hydroxyl structure at C-4' in ring B  
519 are more effective than C-6 in ring A. Akah et al. (2011) informed that the phytochemical  
520 compounds, such as terpenoids, saponins, flavonoids, glycosides and carbohydrate, and  
521 alkaloids are good antidiabetic metabolites. Sangeetha and Vedesree (2012) explained,  
522 that the ability to inhibit the  $\alpha$ -amylase enzyme was determined by the content of the  
523 phenolic compound and protein. The  $\alpha$ -amylase inhibitor enzyme present in *Pluchea*  
524 infusion may be proteinaceous in nature. Aleixandre et al. (2022) informed that phenolic

525 acids have inhibition activity to  $\alpha$ -amylase enzyme depending their structures. Besides  
526 that, capability of phenolic acids to inhibit  $\alpha$ -amylase enzyme was determined by low half-  
527 maximum inhibitory concentration ( $IC_{50}$ ). There are C=C double bond conjugated with a  
528 carbonyl group of phenolic structures that stabilizes the binding forces to the active site  
529 of the  $\alpha$ -amylase. The hydroxyl groups of them are able to bind by non-covalent  
530 interaction, such as hydrogen binding, cation- $\pi$  interactions, salt bridge interactions, ionic  
531 interactions or electrostatic forces with amino acid residue at the active site in  $\alpha$ -amylase  
532 enzyme. The steeping temperature and storage time can remove hydroxyl groups of  
533 phenolic compounds that can reduce the ability of enzyme inhibition. The phenolic acids  
534 with a greater number of hydroxyl groups are stronger capable to obstruct the  $\alpha$ -amylase  
535 enzyme.

#### 536 $\alpha$ -Glucosidase enzyme inhibition activity (GA)

537  $\alpha$ -glucosidase is an important enzyme in carbohydrates digestion, that catalysis  
538 the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and converts  
539 them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et al., 2014;  
540 Proenca et al., 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -glucosidase  
541 enzyme is used to determine antidiabetics activity. This is supported by Werdani and  
542 Widyawati (2018), that *Pluchea* infusion has the potential as an antidiabetic agent.  
543 Widyawati et al. (2020) found that brewing fresh *Pluchea* infusion at 95 °C for 5 minutes  
544 has an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857 %.

545 The results showed, that the ability to inhibit the  $\alpha$ -glucosidase enzyme decreased  
546 with increasing steeping temperature and storage time. Steeping at 95 °C for fresh  
547 *Pluchea* infusion (un-stored) obtained the lowest inhibitory ability, i.e.,  $48.32 \pm 1.27$  %,

548 and the highest inhibitory activity was found at 70 °C **steeping** temperature for fresh  
549 ***Pluchea*** infusion, which was  $95.11 \pm 0.70\%$  (Figure 3b). The results of a paired T test  
550 showed that GA of ***Pluchea*** infusion was significantly different at both steeping  
551 temperature and long storage. The antidiabetic activity of ***Pluchea*** infusion showed that  
552 the ability to inhibit the  **$\alpha$ -glucosidase enzyme** tended to be higher than the ability to inhibit  
553 the  **$\alpha$ -amylase enzyme**. Li et al. (2018) informed that flavonoid compounds have the ability  
554 to inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. This is due to the total  
555 flavonoids in steeped ***Pluchea*** infusion which tended to have the same pattern as the  
556 ability to inhibit the activity of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. The statistical  
557 analysis using Pearson correlation showed that GA of ***Pluchea*** infusion was strongly and  
558 negatively correlated with TPC, TTC, DPPH and FRAP, with r was -0.555, -0,715, -0.527  
559 and -0.560, respectively. However, GA was moderately and positively correlated to TFC,  
560 with r was 0.350 and strongly and positively correlated to AA with r was 0.725. Flavonoid  
561 compounds, such as rutin, myricetin, kaempferol, and quercetin which have antioxidant  
562 and antihyperglycemic activities. The ability to inhibit the action of enzymes from flavonoid  
563 compounds is determined by the position and number of hydroxyl groups and the number  
564 of double bonds in rings A and B and the heterocyclic ring in ring C. The ability to inhibit  
565 the  $\alpha$ -glucosidase enzyme from ***Pluchea*** infusion was significantly affected by the  
566 **steeping** temperature and **long storage**. The capability of ***Pluchea*** infusion to obstruct the  
567  $\alpha$ -glucosidase enzyme was greater than the  $\alpha$ -amylase enzyme because the mechanism  
568 of two enzymes was different, according to the opinion of McCue et al. (2005). Widyawati  
569 et al. (2017) informed that phenolic and non-phenolic compounds determine the inhibitory  
570 activity of the  $\alpha$ -glucosidase enzyme. The ability of bound phenolic compounds to inhibit

571  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. The presence of  
572 polymerization and degradation reactions, that may be occurred in **Pluchea** infusion  
573 during storage, affects the structure and profile of phenolic and non-phenolic compounds.  
574 Asriningtyas et al. (2014) claimed that **Pluchea** leaves contain 3,5-di-O-caffeoylquinic  
575 acid, 4,5-di-O-caffeoylquinic acid methyl ester, 3,4,5-tri-O-caffeoylquinic acid methyl  
576 ester, 3,4,5-tri-O-caffeoylquinic acid, and 1,3,4,5-tetra-O-caffeoylquinic acid. Quinic acid  
577 is methyl esterified with the number of caffeic groups in the molecule that determines the  
578 activity of inhibiting the  $\alpha$ -glucosidase enzyme. Analysis of caffeoylquinic acids in  
579 **Pluchea** infusion was obtained that the higher steeping temperature and long storage  
580 caused increased concentration of them, but the  $\alpha$ -glucosidase inhibition activity of them  
581 was reduced. Aleixandre et al. (2022) reported that the simple phenolic acids forming a  
582 dipole-dipole interaction of active site from  $\alpha$ -glucosidase enzyme are effectively inhibiting  
583 the enzyme.

584 This study was obtained information that the increasing of steeping temperature  
585 and storage time caused a degradation reaction of polyphenol compounds to produce  
586 simple phenolic compounds, such as gallic acid, (+)-catechin, myricetin, quercetin,  
587 kaempferol, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-  
588 caffeoylquinic acid, supported the results of total phenolic content and total tannin content  
589 assays. Increased concentration of simple phenolic compounds determined the ability of  
590 these compounds as antioxidant agents, but reduced their capability as antidiabetic  
591 agents.

592

## 593 **CONCLUSION**

594 The steeping temperature and storage time of *Pluchea* infusion significantly  
595 influenced bioactive contents, antioxidant and antidiabetic activities. TPC, TTC, and TFC  
596 were significantly different at various steeping temperature and storage period based on  
597 statistical analysis using a paired T test at  $\alpha \leq 0.05$ . There was the difference of the  
598 phenolic compound profile in fresh and stored of *Pluchea* infusion and various steeping  
599 temperature. The simple phenolic compounds were detected in *Pluchea* infusion  
600 including gallic acid, (+)-catechin, quercetin, myricetin, kaempferol, 3,4-di-O-  
601 caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid and 4,5-di-O-caffeoylquinic acid. The  
602 results of statistical analysis using a paired T test at  $\alpha \leq 0.05$  showed that gallic acid and  
603 kaempferol of *Pluchea* infusion were insignificantly different at various steeping  
604 temperature and long storage. Nevertheless, the concentration of quercetin and 3,5-  
605 dicaffeoylquinic acid of *Pluchea* infusion was significantly different of two treatments  
606 except at 70 °C. The (+)-catechin concentration of *Pluchea* infusion was significantly  
607 different at 95 °C, but the myricetin was different concentration at 80 and 95 °C. The 3,4-  
608 dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid compounds from *Pluchea* infusion  
609 were significantly different at 60 °C, however the concentration of 3,4-dicaffeoylquinic acid  
610 was also significantly different at 80 and 95 °C. TPC, TTC and TFC of *Pluchea* infusion  
611 were significantly different at various steeping temperature and storage period. TPC and  
612 TTC significantly increased with increasing steeping temperature and long storage, but  
613 TFC significantly increased at various steeping temperature and significantly decreased  
614 at long storage. The bioactive compounds of *Pluchea* infusion influenced antioxidant  
615 activities (DPPH and FRAP) and antidiabetic activity (AA and GA). The DPPH was  
616 strongly and positively correlated with TPC and TTC, but it was strongly and negatively

617 correlated with TFC, with coefficient  $r$  0.993, 0.942, and -0.940, respectively. The  
618 correlated pattern between FRAP and bioactive contents of *Pluchea* infusion was similar  
619 to it between DPPH and bioactive contents. The correlated coefficient values ( $r$ ) between  
620 FRAP and TPC, TTC and TFC were 0.953, 0.948 and -0.826, respectively. The AA and  
621 GA were strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was  
622 moderately and negatively significant correlated with TFC. Between the antioxidant  
623 activity of DPPH and FRAP and the antidiabetic activity of AA and GA of *Pluchea* infusion  
624 were strongly and positively correlated with correlation coefficient ( $r$ ) values of 0.956 and  
625 0.725, respectively.

626

#### 627 DATA AVAILABILITY

628 Table and figure used to support of this study were included in the article.

629

#### 630 CONFLICT OF INTEREST

631 The authors declare no conflict of interest.

632

#### 633 ACKNOWLEDGEMENTS

634 The authors would like to thank the he Ministry of Education and Culture of the Republic  
635 of Indonesia for fundamental research grant to higher education institutions in 2022

636

#### 637 REFERENCES



638 ACAR A, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food  
639 wastes:evaluation of temperature and time effects on quality parameters. Applied Food  
640 Res. 2(100087):1-8.

641 ALEIXANDRE A et al. 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -  
642 glucosidase and influence of reaction conditions. Food Chem. 372(131231):1-9.

643 ALI et al. 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile.  
644 Food Chem. 89(2005):27-36.

645 ALI A. et al. 2018. Impact of storage conditions on the stability of predominant phenolic  
646 constituents and antioxidant activity of dried *piper bettle* extracts. Molecules.23(24):1-13.

647 AL-TEMIMI A, CHOUDHARY R. 2013. Determination of antioxidant activity in different  
648 kinds of plants *in vivo* and *in vitro* by using diverse technical methods. J Nut. Food Sci.  
649 3(1):1-6.

650 AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and  
651 methanol extract and fractions of *Gongronema latifolium* (*Asclepidaceae*) leaves in  
652 alloxan diabetic rats. J Applied Pharm. Sci. 01(09): 99-102.

653 ARSININGTYAS IS. et al. 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves  
654 of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product  
655 use. Natural Product Res. 28(17):1350-1353.

656 ARYAL S et al. 2019. Total phenolic content, flavonoid content and antioxidant of wild  
657 vegetables from Western Nepal. Plants. 8(96): 1-12.

658 AYELE DT, AKELE ML, MELESE A.T. 2022. Analysis of total phenolic contents,  
659 favonoids, antioxidant and antibacterial activities of *Croton macrostachyus* root extracts.  
660 BMC Chem. 16(30): 1-9.

661 BALIYAN S et al. 2022. Determination of antioxidants by DPPH radical scavenging  
662 activity and quantitative phytochemical analysis of *Ficus religiosa*. *Molecules*. 27(1326):  
663 1-19.

664 CASTIGLIONI S et al. 2015. Influence of steeping conditions (time, temperature, and  
665 particle size) on antioxidant properties and sensory attributes of some white and green  
666 teas. *Intern. J. Food Sci. Nut.* Online 1-7.

667 Chan EWC et al. 2022. *Pluchea indica*: An updated review of its botany, uses, bioactive  
668 compounds and pharmacological properties. *Pharmaceutical Sciences Asia*. 49(1): 77-  
669 85.

670 CHANDRA S et al. 2014. Assessment of total phenolic and flavonoid content, antioxidant  
671 properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit  
672 crops: A Comparative study. Hindawi Publishing Corporation Evidence-Based  
673 Complementary Alter. Med. 2014:1-9 (2014)

674 CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic, flavonoids,  
675 tannin and chlorophyll content of leaves of *Strobilanthes Kunthiana (Neelakurinji)*. *J. Med.*  
676 *Plants Studies*. 4(4):282-286.

677 DOBRINAS S, SOCEANU A, POPESCU V, POPOVICI IC, JITARIU D. 2021.  
678 Relationship between total phenolic content, antioxidant capacity, Fe and Cu content from  
679 tea plant samples at different brewing times. *Processes*. 9(1311):1-11.c

680 FERRARIS Z. 2023. *Pluchea indica* Less.iNaturalist.org.  
681 <https://www.gbif.org/occurrence/4022273660>.

682 FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional  
683 properties and visual appearance of Dampit Robusta coffee leaves tea. *Advances in Food*  
684 *Sci. Sustain. Agric. Agro-industrial Engineering*. 4(1):25-32.

685 FU L et al. 2017. Total phenolic contents and antioxidant capacities of herbal and tea  
686 infusions. *Intern. J. Molecular Sci.* 12:2112-2124.

687 GAO MR et al. 2019. A theoretical and experimental study: the influence of different  
688 standards on the determination of total phenol content in the Folin–Ciocalteu assay. *J.*  
689 *Food Measurement and Characteristic*. 13:1349–1356.

690 HUANG D, OU B, PRIOR RL. 2005. The Chemistry behind antioxidant capacity assays.  
691 *J. Agric. Food Chem.* 53:1841-1856.

692 ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties  
693 of rosehip tea beverage: study by response surface methodology. *J. Food Sci. Tech.*  
694 54(11):3737–3743.

695 JAYANI NIE et al. 2022. Evaluation of stability and quality characteristics of moringa  
696 (*Moringa oleifera*) herbal tea during storage. *Food Res.* 6(4):399-406.

697 KIM JM et al. 2020. Effect of storage temperature on the antioxidant activity and catechins  
698 stability of Matcha (*Camellia sinensis*). *Food Science and Biotechnology.* 29(9):1261–  
699 1271.

700 KILIC et al. 2017. Antioxidant properties of some herbal teas (green tea, senna, corn silk,  
701 rosmery) brewed at different temperatures. *Intern. J. Secondary Metabolite.* 4(3):142-248  
702 Special Issue 1.

703 KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of  
704 extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves

705 from different provenances in Thailand. *Revista Brasileira de Farmacognosia*. 28(2018):  
706 145–150.

707 KOWALSKA J et al. 2021. Influence of tea brewing parameters on the antioxidant  
708 potential of infusions and extracts depending on the degree of processing of the leaves  
709 of *Camellia sinensis*. *Molecules*.26(4773):1-17.

710 KRUK J, ABOUL-ENAIN BH, DUCHNIK E, MARCHLEWICZ M. 2022. Antioxidative  
711 properties of phenolic compounds and their effect on oxidative stress induced by severe  
712 physical exercise. *J. Physiol. Sci.* 72(19):1-24.

713 KUMAR N, GOEL N. 2019. Phenolic acids: natural versatile molecules with promising  
714 therapeutic applications. *Biotechnology Reports*. 24(e00370):1-10.

715 LI K et al. 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the flavonoids  
716 rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity  
717 relationship of its eight flavonoids by a refined assign score method. *Chem. Central J.*  
718 12(82):1-11.

719 LIN YS. et al. 2020. Effects of storage time and temperature on antioxidants in juice from  
720 *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser. *Molecules*.  
721 25(3614): 1-12.

722 MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content  
723 and antioxidant activity test of avocado seed extract (*Persea Americana* Mill). *Jurnal MIPA*  
724 *Unsrat Online*. 1(1): 5-10. [https://media.neliti.com/media/publications/116214-ID-  
725 penentuan-kandungan-tanin-dan-uji-aktivi.pdf](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf) [In Bahasa Indonesia]

726 MARTINEZ-SOLIS J et al. 2022. Antidiabetic and toxicological effect of the tea infusion  
727 of summer collection from *Annona cherimola* Miller leaves. *Plants*. 11(3224):1-17.

728 MATHUR R, VIJAYVERGIA R. 2017. Determination of total flavonoid and phenol content  
729 in *Mimusops Elengi* Linn. Intern. J. Pharm. Sci. Res. 8(12): 5282-5285.

730 MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of  
731 sprouted and solid-state bioprocessed soybean. Asia Pacific J. Clinical Nut. 14(2): 145-  
732 152.

733 MORAES-DE-SOUZA RA, OLDONI TLC, REGITANO-D'ARCE MAB, ALENCAR SM.  
734 2008. Antioxidant activity and phenolic composition of herbal infusions consumed in  
735 Brazil. Cienc Tecnol. Aliment. 6(1):41-47.

736 MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant  
737 activity: A Review. Intern. J. Molecular Sci. 22(3380):1-30.

738 NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus*  
739 *sabdariffa* L.): Effect of dying temperature and brewing conditions on total soluble solid,  
740 phenolic content, antioxidant capacity and sensory quality. Beverages.6(2):1-11 (2020).

741 NOREEN H. et al. 2017. Measurement of total phenolic content and antioxidant activity  
742 of aerial parts of medicinal plant *Coronopus didymus*. Asian Pacific J. Tropical Med. 10(8):  
743 792–801.

744 NURCHOLIS GW et al. 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity in-  
745 vitro and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum pictum*  
746 (L.). Current Biochem. 1(2): 58-65.

747 OH J et al. 2013. Antioxidant and antimicrobial activities of various leafy herbal teas. Food  
748 Control. 31(2013):403-409.

749 PROENCA C et al. 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an in vitro and in silico  
750 structure–activity relationship study. J. Enzyme Inhibition Med. Chem. 32(1): 1216–1228.

751 PUREWAL SS et al. 2022. Unraveling the effect of storage duration on antioxidant  
752 properties, physicochemical and sensorial parameters of ready to serve Kinnow-aml  
753 beverages. Applied Food Res. 2(1): 1-8.

754 RAHARJO D, HARYOTO. 2019. Antioxidant activity of mangrove *Sonneratia caseolaris*  
755 L. using the FRAP method. ISETH. 2019: 623-629.

756 RAMPHINWA ML et al. 2023. Eco-physiological response of secondary metabolites of  
757 teas: review of quality attributes of herbal tea. Frontiers in Sustain. Food Systems.  
758 7(990334):1-14.

759 REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids.  
760 Czech J. Food Sci. 30(2):171-177.

761 RUAN J et al. 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea*  
762 *indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel  
763 column chromatography fractions. Molecules. 24(2784):1-20.

764 RUSITA YD et al. 2019. Comparison of tannin concentration on fresh and dried tea leaf  
765 (*Camelia sinensis* L.) extract using UV-visible spectrophotometric method. 1st  
766 International Conference of Health, Science & Technology (ICOHETECH) 2019 E-ISBN:  
767 978-623-92207-0-9. November 16<sup>th</sup>, 2019.LPPM Universitas Duta Bangsa Surakarta,  
768 Surakarta.

769 SANGEETHA R, VEDASREE N. 2012. In Vitro  $\alpha$ -Amylase inhibitory activity of the leaves  
770 of *Thespesia populnea*. Intern. Scholarly Res.Network. 2012: 1-4 (2012)

771 SHRAIM AM et al. 2021. Determination of total flavonoid content by aluminum chloride  
772 assay: A critical evaluation. LWT Food Sci. Tech. 150(111932):1-11.

773 SILVA-RAMIREZ KA et al. 2020. Effect of drying and steeping temperature on the

774 phenolic content, antioxidant activity, aromatic compounds and sensory properties of  
775 *Culila polyantha* Benth. Infusions. Processes. 8(1378):2-20.

776 SRISOOK K et al. 2012. Antioxidant and anti-inflammatory activities of hot water extract  
777 from *Pluchea indica* Less. herbal tea. J. Med. Plants Res. 6(23):4077-4081.

778 SURIYAPHAN O. 2014. Nutrition, health benefits and applications of *Pluchea indica* (L)  
779 Less leaves. Pharm. Sci. Asia. 41:1–10.

780 SU D et al. 2019. Impact of thermal processing and storage temperature on phenolic  
781 profile and antioxidant activity of different varieties of lychee juice. LWT-Food Tech.  
782 116(108578):1-9.

783 THANAJIRUSCHAYA P et al. 2010. Effect of storage time and temperature on antioxidant  
784 components and properties of milled rice. Khon Kaen Univ. Res. J.15(9):843-851.

785 VONGSAK B et al. 2018. Comparison of active constituents, antioxidant capacity, and  
786 αglucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. Food  
787 Biosci. 25: 68–73.

788 WANG SC. et al. 2022. Effect of brewing conditions on polyphenols in the dark tea  
789 (*Camellia sinensis* L.) infusions: content, composition and antioxidant activities. Food  
790 Sci.Tech. 42(e36322):1-9.

791 WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica*  
792 Less as functional beverage in diabetic patients. ASSEHR. 98:164-167.

793 WIDYAWATI PS et al. 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract  
794 as antioxidant and antiwarmed over flavor (WOF) of duck meat. International Congress  
795 on Challenges of Biotechnological Research in Food and Health. November 15<sup>th</sup>  
796 2014.Agricultural Technology Faculty, Slamet Riyadi University, Surakarta.

797 WIDYAWATI PS et al. 2016. The physicochemical and antioxidant properties of *Pluchea*  
798 *indica* Less drink in tea bag packaging. IJFNS. 5: 13–120.

799 WIDYAWATI PS et al. 2017. In vitro antioxidant capacities and antidiabetic properties of  
800 *pluchea* leaves. IJPPS. 9(8): 203-208.

801 WIDYAWATI PS et al. 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica*  
802 Less beverage. CJFST.12(4): 125-139.

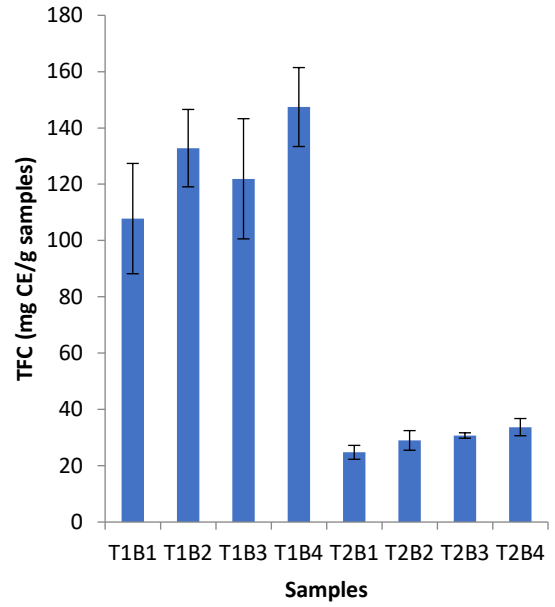
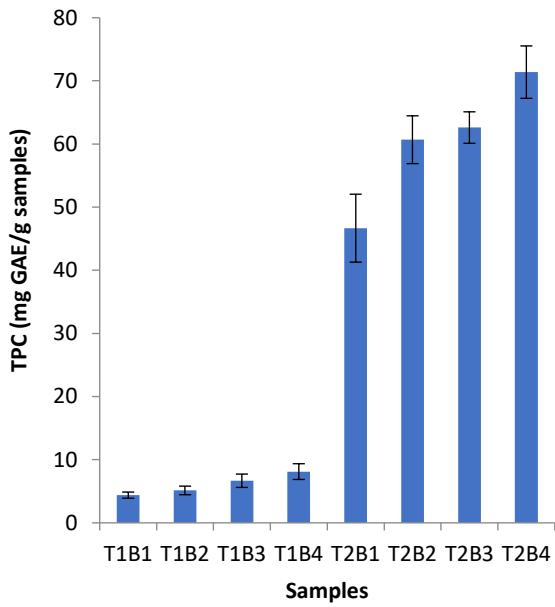
803 WIDYAWATI PS ET AL. 2022. The effect of κ-carrageenan proportion and hot water  
804 extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of stink  
805 lily (*Amorphophallus muelleri*) wet noodles. Molecules. 27(5062):1-16.

806 XU P, CHEN L, WANG Y. 2019. Effect of storage time on antioxidant activity and inhibition  
807 on α-amylase and α-glucosidase of white tea. Food Sci. Nut. 7(2): 636-644.

808 ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction time on caffeine in  
809 coffee. Lantanida J. 6(2):103-202. [https://jurnal.ar-](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811)  
810 [raniry.ac.id/index.php/lantanida/article/view/3811](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811). [In Bahasa Indonesia]

811 ZHANG Y et al. 2021. Effect of storage conditions and time on the polyphenol content of  
812 wheat flours. Processes. 9(248):1-11.



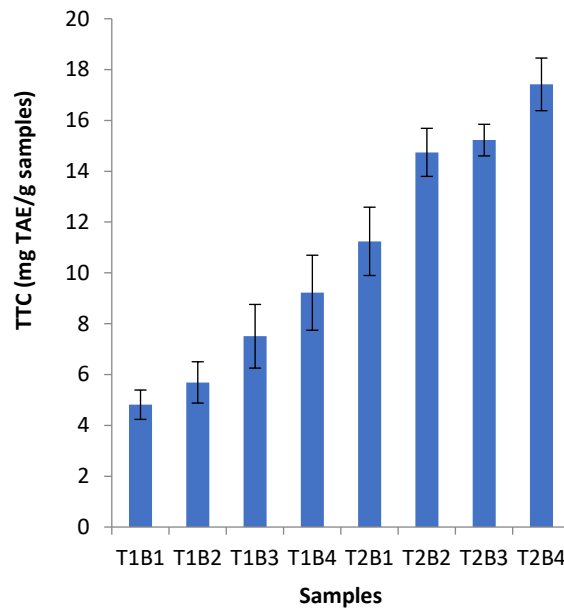


813

814

(a)

(b)



815

816

(c)

817

Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping

818

temperature and storage time (a) Total phenolic content (b) Total flavonoid

819

content (c) Total tannin content. Data were expressed as mean  $\pm$  standard

820 deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at  
821 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-  
822 stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped at 70 °C,  
823 stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped at  
824 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5  
825 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq$   
826 0.05.  
827

Table 1. Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperature and storage time

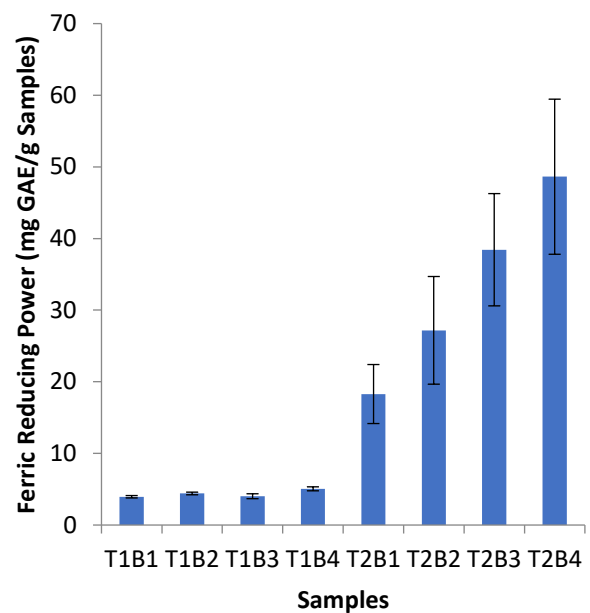
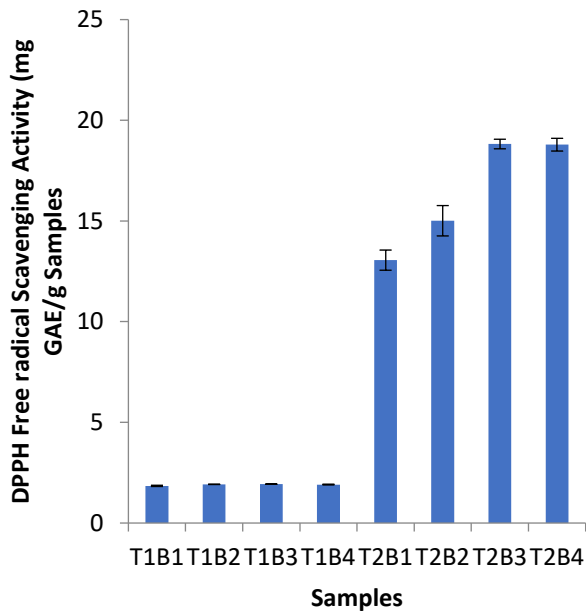
Phenolic Compounds	Steeping Temperature (°C)	Mean±SD Un-stored	Mean±SD Stored	Mean difference ±SD	Sig (2-tailed)
Gallic Acid (µg/g samples)	60	0.2132±0.0027	0.2364±0.0015	0.0375±0.0175	0.2030
	70	0.2157±0.0013	0.2324±0.0214	0.0167±0.0227	0.4870
	80	0.2234±0.0122	0.2347±0.0078	0.0386±0.0264	0.2870
	95	0.2316±0.0104	0.2402±0.0169	0.0086±0.1990	0.8500
(+) -Catechin (µg/g samples)	60	0.3425±0.0110	0.5085±0.0111	-0.1576±0.0885	0.241
	70	0.3260±0.0265	0.5448±0.0006	-0.2188±0.0259	0.053
	80	0.3240±0.0222	0.5023±0.0773	-0.1451±0.0248	0.077
	95	0.4039±0.0320	0.5995±0.0372	-0.2049±0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756±0.1234	1.4762±0.0271	-1.2887±0.3222	0.111
	70	0.2587±0.0160	1.4245±0.2526	-1.1657±0.2695	0.103
	80	0.4175±0.0104	1.4570±0.0925	-1.0391±0.0841	0.036*
	95	0.8786±0.0434	2.6138±0.0695	-1.1735±0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220±0.0268	0.6220±0.0706	-0.5999±0.9733	0.544
	70	0.1530±0.0511	1.0708±0.0289	-0.9177±0.0222	0.011*
	80	0.3666±0.0103	0.8629±0.0815	-0.1082±0.4462	0.790
	95	0.6559±0.0570	2.0230±0.0573	-1.4123±0.3203	0.101
Kaempferol (µg/g samples)	60	0.1394±0.0202	0.3675±0.0183	-0.3207±0.1122	0.154
	70	0.0514±0.0037	0.3726±0.0944	0.3213±0.0907	0.125
	80	0.3699±0.0924	0.7966±0.0366	-0.4267±0.2727	0.271
	95	0.5913±0.0239	0.9478±0.0287	-0.3565±0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103±0.0628	2.4863±0.0270	-1.8760±0.2074	0.050*
	70	0.6271±0.0099	2.3403±0.0325	-1.7131±0.3152	0.082
	80	0.7967±0.03060	2.6278±0.0211	-1.8311±0.0095	0.002*
	95	1.5386±0.0668	4.0211±0.0851	-2.4825±0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635±0.0628	0.9449±0.0501	-0.2814±0.4458	0.536
	70	0.6162±0.0099	0.9485±0.0794	-0.3323±0.0301	0.041*
	80	0.6601±0.0306	0.9099±0.0387	-0.2498±0.3127	0.461
	95	0.6642±0.0668	1.3156±0.0166	-0.6514±0.2666	0.179

4,5-di-O-Caffeoylquinic acid ( $\mu\text{g/g}$ samples)	60	0.4906 $\pm$ 0.0060	1.1842 $\pm$ 0.0120	-0.6886 $\pm$ 0.2723	0.018*
	70	0.4807 $\pm$ 0.0034	1.0089 $\pm$ 0.0736	-0.5281 $\pm$ 0.0702	0.060
	80	0.5299 $\pm$ 0.0053	1.2382 $\pm$ 0.1435	-0.7082 $\pm$ 0.1489	0.094
	95	1.0018 $\pm$ 0.0526	1.3797 $\pm$ 0.2170	-0.3086 $\pm$ 0.3086	0.333

829 Note : Data were expressed as mean  $\pm$ standard deviation (n=2). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-  
830 steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C,  
831 stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped  
832 at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature,  
833 calculated using a paired T test at  $\alpha \leq 0.05$ . \*  $\alpha \leq 0.05$ .

834

835

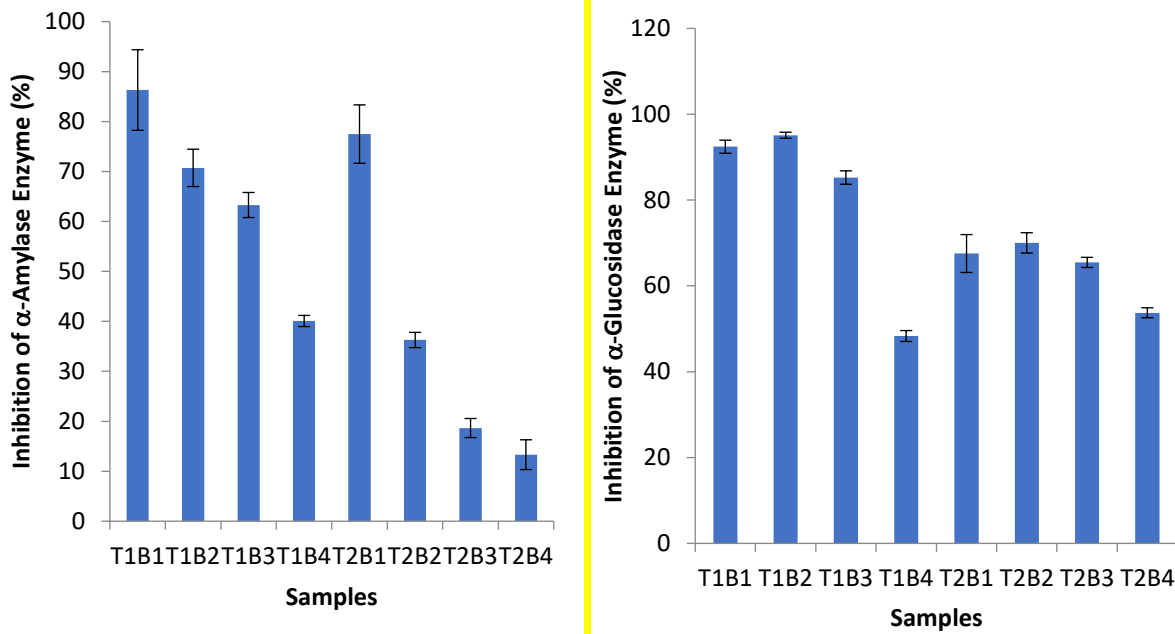


(a)

(b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage time (a) DPPH (b) FRAP. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T2B2-steeped at 70 °C, stored for 5 years; T2B3-steeped at 80 °C, stored for 5 years; T2B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq$

0.05



(a)

(b)

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage time (a)  $\alpha$ -amylase (b)  $\alpha$ -glucosidase. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T2B2-steeped at 70 °C, stored for 5 years; T2B3-steeped at 80 °C, stored for 5 years; T2B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$

860 Table 2. Pearson correlation coefficients between bioactive contents (TPC, TFC and TAC), antioxidant activity (DPPH and  
 861 FRAP) and antidiabetic activity (AA and GA)

	TPC	TFC	TTC	DPPH	FRAP	Alpha Glucosidase	Alpha Amylase
TPC	1						
TFC	-0.93589	1					
TTC	0.960028	-0.81321	1				
DPPH	0.992776	-0.93992	0.942273	1			
FRAP	0.953366	-0.82636	0.947778	0.956242	1		
Alpha Glucosidase	-0.55512	0.349873	-0.71534	-0.5272	-0.55947	1	
Alpha Amylase	-0.70842	0.429393	-0.8569	-0.69579	-0.80548	0.725161631	1

862 Note: Correlation significant at the 0.05 level (2-tailed)

863



Paini Sri Widyawati <paini@ukwms.ac.id>

---

## Fwd: Comments on PJS Paper Ms 23-158

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Thu, Nov 23, 2023 at 7:56 AM

Dear Dr. Widyawati,

We confirm the receipt of your revised Ms 23-158 paper, as well as your point-for-point response to the reviewers' comments. These will be relayed to the reviewers for another round of evaluation.

Thank you for your sustained contribution to PJS!

Sincerely,  
Ms. CARYL MARIA MINETTE I. ULAY  
Editorial Assistant

For Dr. CAESAR A. SALOMA  
Editor-in-Chief  
[Quoted text hidden]



5. Third Revision: Major Revision (15-1-2024)
  - Correspondence
  - Review Note
  - Document



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**From Caesar Saloma/15 January 2024/ Editorial Decision/ MS 23-158R**

---

Caesar Saloma &lt;caesar.saloma@gmail.com&gt;

Mon, Jan 15, 2024 at 4:16 PM

To: paini@ukwms.ac.id

Cc: DOST STII PJS &lt;pjs@stii.dost.gov.ph&gt;, Philippine Journal of Science &lt;philjournsci@gmail.com&gt;

15 January 2024

DR. PAINI SRI WIDYAWATI  
Food Technology Study Program  
Agricultural Technology Faculty  
Widya Mandala Surabaya Catholic University  
Surabaya, Indonesia  
[paini@ukwms.ac.id](mailto:paini@ukwms.ac.id)

Subject: MS 23-158R

Title: Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea indica* Less Tea

Authors Paini Sri Widyawati and Yufita Ratnasari Wilianto

Dear Dr Widyawati:

I would like to provide you with an opportunity to respond to the latest comments of Reviewers 2 and 3 before deciding on the publication suitability of your manuscript submission in the Philippine Journal of Science. The comments are sent herewith.

My recommendation is for you to address carefully and substantially in your revised manuscript should you decide to submit it, all the points that were raised.

Please provide me with a point-by-point response together with the corresponding action taken in the revised manuscript.

Submit the pertinent documents not later than 22 January 2024 and only to the PJS Editorial Office at: [philjournsci@gmail.com](mailto:philjournsci@gmail.com); [pjs@stii.dost.gov.ph](mailto:pjs@stii.dost.gov.ph)).

Thank you.

Sincerely yours,

Caesar Saloma (signed)

Editor-in-Chief

The Philippine Journal of Science

## COMMENTS OF REVIEWERS

Reviewer 1

[1st evaluation] Paper secured no affirming commitment from experts.

Reviewer 2

[1st evaluation] Paper as presently written is unacceptable for publication; needs extensive revision.

[2nd evaluation] Reconsider only after the comments/recommendations are clarified and/or complied with. Paper should be published as a research note/short communication.

Please find attached the second revision of the Ms 23-158 article. My comments are found in the paper. Do I have to fill up another evaluation form? My general recommendation is to reconsider only after the comments/recommendations are clarified and/or complied with as a research note.

See attachment.

Reviewer 3

[1st evaluation] Reconsider only after the comments/recommendations are clarified and/or complied with. Paper should be published as a research note/short communication.

[2nd evaluation] Accept paper for publication.

#### Specific Comments and Recommendations

##### Page Line Comments and Recommendations

12-13 261-286

These paragraphs are more appropriate to be part of the introduction rather than the results and discussion. It does not present any result or discussion of the result of the current study.

14 297- As shown in which table of figures? Moreover, is it only T-test used for the determination of

299 significant differences among treatments. It may be true for comparing the stored and fresh Pluchea tea but not for the steeping temperature with 4 treatments.

14 301-307

These significant differences should be reflected in Figure 1a using different letters.

15 336-349

These significant differences are not reflected in table 1. Table 1 shows the significant differences between fresh and stored teas but not the effect of steeping temperature. Authors can show the significant differences within row and column to reflect the effect of storage and steeping temperature, respectively. After reflecting the result of the significant differences among teas steeped at different temperatures the author should revise the discussion.

17 366-375

Is it the only T-test used for the determination of significant differences among treatments. It may be true for comparing the stored and fresh Pluchea tea but not for the steeping temperature with 4 treatments. These significant differences should be reflected in Figure 1b using different letters.

17 377-387

Is it the only T-test used for the determination of significant differences among treatments. It may be true for comparing the stored and fresh Pluchea tea but not for the steeping temperature with 4 treatments. These significant differences should be reflected in Figure 1c using different letters.

19 429-432

These significant differences should be reflected in Figure 2a using different letters. Then it should be properly discussed in L429-432

21 465-472

These significant differences should be reflected in Figure 2b using different letters. Then it should be properly discussed in L465-472

23 503-510

These significant differences should be reflected in Figure 3a using different letters. Then it should be properly discussed in L503-510

24 545-551

These significant differences should be reflected in Figure 3b using different letters. Then it should be properly discussed in L545-551

37-38 These figures (Figure 1) should reflect the result of the statistical analysis ie. letters indicating significant differences among treatments

39-40 Table 1 shows that there are significant differences between fresh and stored teas but not the effect of steeping temperature. Authors can show the significant differences within row and column to reflect the effect of storage and steeping temperature, respectively.

41 These figures (Figure 2) should reflect the result of the statistical analysis ie. letters indicating significant differences among treatments.

42 These figures (Figure 3) should reflect the result of the statistical analysis ie. letters indicating significant differences among treatments.

Initial manuscript submitted to PJS: 05 May 2023

Reviewers' comments sent to authors: 20 Sep 2023

Revised manuscript sent to PJS: 22 Nov 2023

END.

--

Caesar Saloma, PhD NAST FSPP SMOPTICA

Professor

National Institute of Physics

University of the Philippines Diliman

Quezon City 1101, Philippines

Voice/Fax: +632 920 9749 VOIP Trunkline: +632 981 8500 loc 3701

E-mail: [caesar.saloma@gmail.com](mailto:caesar.saloma@gmail.com)

[http://en.wikipedia.org/wiki/Caesar\\_Saloma](http://en.wikipedia.org/wiki/Caesar_Saloma)



**R2 Ms 23-158 Reviewer 2 Comments on Revised Manuscript.docx**

140K

1 **Effect of Steeping Temperature and Storage [TimePeriod](#) on the Bioactive**  
2 **Compounds, Antioxidant and Antidiabetic Activities of Infusion from Powdered**  
3 ***Pluchea Indica Less***

4 Painsi Sri Widyawati<sup>1\*)</sup>, Yufita Ratnasari Wilianto<sup>2)</sup>

5 <sup>1)</sup>Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala  
6 Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

7 <sup>2)</sup>Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic  
8 University, Kalisari Street Number 1, Surabaya 60272, Indonesia

9 Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea*  
10 *indica Less*, storage [timeperiod](#)

11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
Corresponding Author: [painsi@ukwms.ac.id](mailto:painsi@ukwms.ac.id)

21 ABSTRACT

22 This study was done to determine the effects of steeping temperature and storage  
23 ~~timeperiod~~ on the bioactive contents, antioxidant and antidiabetic activities of *Pluchea*  
24 ~~leaf infusion~~. The research used a randomized block design with two factors, i.e., steeping  
25 temperature (T) and storage ~~timeperiod~~ (B). The ~~variety of the Pluchea leaf blades were~~  
26 ~~exposed to 4~~ steeping temperatures ~~included of~~ 60 (T1), 70 (T2), 80 (T3), and 95 (T4)  
27 ( $^{\circ}\text{C}$ ) with the storage ~~timeperiod-period~~ of 0 (B1) and 5 (B2) ~~(year)~~. ~~The research~~  
28 ~~resultedresulting in~~ 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2,  
29 T4B1, T4B2). Statistical analysis using a paired ~~t-T~~ test at  $\alpha \leq 0.05$  showed that  
30 treatments significantly ~~affected influenced~~ the bioactive contents (total phenol (TPC),  
31 total tannin (TTC), total flavonoid (TFC)), antioxidant [(DPPH scavenging activity (DPPH)  
32 and ferric reducing antioxidant power (FRAP)] ~~potential~~ and antidiabetic [( $\alpha$ -amylase  
33 (AA) and  $\alpha$ -glucosidase (GA) ~~inhibitorsinhibition~~] ~~activities-properties~~ of the *Pluchea* leaf  
34 ~~infusionsamples~~. TFC decreased during storage period but significantly increased at  
35 higher steeping temperature. The AA and GA of *Pluchea* infusion increased until 70  $^{\circ}\text{C}$   
36 ~~of the steeping temperature, but decreased until 95  $^{\circ}\text{C}$ . The bioactive contents influenced~~  
37 ~~antioxidant and antidiabetic activities. TFC was decreased for storage time and significant~~  
38 ~~increased at higher steeping temperature. The AA and GA of Pluchea infusion increased~~  
39 ~~until 70  $^{\circ}\text{C}$  of the steeping temperature, but decreased until 95  $^{\circ}\text{C}$ . The AA and GA were~~  
40 strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was  
41 moderately and negatively correlated with TFC. ~~Between-T~~ the antioxidant activity of  
42 ~~DPPH and FRAP~~ and the antidiabetic activity of AA and GA of *Pluchea* infusion were  
43 strongly and positively correlated. ~~with correlation coefficient (r) values of 0.956 and~~

**Commented [A1]:** Describe treatment effects on total phenolics, tannins, antioxidant, and antidiabetic in one brief sentence each and indicate statistical significance.

**Commented [A2]:** State briefly results of the correlation analysis.

44 0.725, respectively. The treatments gave different effect of simple phenolic compounds  
45 derived from *Pluchea* leaf infusion at different steeping temperatures and storage  
46 included, such as gallic acid, kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-  
47 caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid of  
48 *Pluchea* infusion at different steeping temperature and long storage. To obtain high  
49 antioxidant activity, *Pluchea* infusion selected was stored and steeped at high  
50 temperature, however high antidiabetic activity obtained was fresh *Pluchea* infusion and  
51 steeped at low temperature.

Formatted: Font: Italic

## 52

### 53 INTRODUCTION

54 *Pluchea* herbal tea is a product of dried *Pluchea* leaf processing introduced by  
55 world people (Srisook et al., 2012; Widyawati et al., 2016) because of the efficacy of the  
56 active components in *Pluchea* leaves, as a herbal plant that has been widely used for  
57 traditional medicine and food (Chan et al., 2022). *Pluchea* leaves are composed many  
58 nutrients and bioactive compounds useful to body health. The nutrient compositions in  
59 the *Pluchea* leaves include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates,  
60 calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive compounds is comprised, i.e.,  
61 chlorogenic acid, caffeic acid, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-  
62 caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-  
63 caffeoylquinic acid, quercetin, myricetin, kaempferol, total anthocyanin,  $\beta$ -carotene, and  
64 total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et  
65 al., 2022, Chan et al., 2022).

Corresponding Author: paini@ukwms.ac.id

66 Steeping process of *Pluchea leaves* can be performed with fresh or dry leaves  
67 ~~infusion by~~ in hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et  
68 al., 2020; Jayani et al., 2022). In Asia ~~area~~, especially in Indonesia, people usually  
69 consume the *Pluchea infusion* ~~with brewing of~~ steeping 2 g of powdered *Pluchea*  
70 leaves in tea bag ~~by~~ in 100 mL of hot ~~water~~ or boiling water. ~~Each tea bag contained 2 g~~  
71 ~~of *Pluchea* leaf powder is steeped with 100 mL hot water or boiling water.~~ Widyawati et  
72 al. (2016) claimed that steeping of 2 g of *Pluchea leaf powder* at 95 °C for 5 minutes  
73 ~~results exhibits~~ total phenolic ~~content, and~~ total flavonoid contents, the ability to scavenge  
74 DPPH free radicals, and the capability ~~of to~~ reduce ferric ions ~~at~~ 9.3 mg gallic acid  
75 equivalent (GAE)/g samples, 22.0 mg gallic acid equivalent (GAE)/g samples, 27.2 mg  
76 gallic acid equivalent (GAE)/g samples, and 10.2 mg gallic acid equivalent (GAE)/g  
77 samples, respectively. Werdani and Widyawati (2018) reported that drinking of *Pluchea*  
78 *leaf powder infusion* in the morning and evening regularly (2 g/100 mL) can decline blood  
79 sugar levels.

80 The steeping of *Pluchea herbal tea* with hot water at 95 °C for 5 minutes certainly  
81 determines the stability and amount of extracted bioactive compounds, that influences  
82 the biological activity, especially antioxidant and antidiabetic activities. Silva-Ramirez et  
83 al. (2020) reported that the infusion process can influence the ~~if~~ content and composition  
84 of the bioactive compounds and antioxidant activity of tea. Acar et al. (2022) informed  
85 that infusion quality of *herbal* tea extract depends on several factors, i.e., ~~time~~ ~~storage~~  
86 and temperature. Polyphenol profile and antioxidant properties of *herbal* tea infusion  
87 decline with an increase in steeping/brewing and storage temperatures, and longer  
88 exposure ~~time~~ ~~periods~~.



89 Several studies have mentioned the effect of steeping temperature ~~to on the~~  
90 bioactive compound contents and antioxidant activity, such as some white and green teas  
91 are effective with hot water at 90 °C for 7 min (Castiglioni et al., 2015), ~~on~~ roseship tea is  
92 effectively ~~at infusion~~ ~~timeperiod~~ around 6-8 min at temperatures of 84-86 °C (Ilyasoglu  
93 and Arpa, 2017), ~~on the caffeine content extracted the coffee at the~~ brewing temperature  
94 ~~of coffee influences the caffeine content extracted~~ (Zarwinda and Sartika, 2018), ~~and the~~  
95 ~~steeping the high total phenol content and antioxidant activity~~ of dark tea at 92 °C for 27  
96 min ~~results the highest total phenol content and antioxidant activity~~ (Wang et al., 2022).  
97 The study of the effect of steeping temperature to *Pluchea* infusion was carried out to  
98 afford information about ~~the most efficient~~ preparation of *powdered Pluchea leaves* ~~most~~  
99 ~~efficiently~~ to get higher ~~the~~ bioactive compounds, antioxidant and antidiabetic activities.

100 ~~On the other hand, storage~~ ~~Storage~~ ~~timeperiod~~ *tea* usually for several months until  
101 ~~years~~ of *Pluchea* herbal tea also affects the levels of the bioactive compounds and  
102 biological activity ~~because this herbal tea usually is stored for a several months until years~~  
103 (Jayani et al., 2022). Tea or herbal tea is generally stored in ambient temperature and  
104 packed in tea bag or ~~Alu~~ foil standing proud or a combination of both. Many researchers  
105 ~~informed~~ ~~reported~~ that storage ~~timeperiod~~ decreases the bioactive compounds,  
106 antioxidant and antidiabetic activities, i.e., juice from *Momordica charantia* L. (Lin et al.,  
107 2020), dried *Piper bettle* extracts (Ali et al., 2018), white tea (Xu et al., 2019), kinnow-  
108 amla beverages (Purewal et al., 2022), whole wheat flour (Zhang et al., 2021).

109 Therefore, *this research studied the effect of steeping temperature and storage*  
110 *timeperiod* on the bioactive compounds ~~[(total phenolic content (TPC), total flavonoid~~  
111 ~~content (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging~~

Commented [A3]: IS THIS ALUMINUM? If so, replace Alu with aluminum

Commented [A4]: Do you mean standing pouch?

112 activity (DPPH), ferric reducing antioxidant power (FRAP)] and antidiabetic activities [( $\alpha$ -  
113 amylase (AA) and  $\alpha$ -glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea*  
114 leaves. The study was done to determine total phenolic content (TPC), total flavonoid  
115 content (TFC), total tannin content (TTC), DPPH free radical scavenging activity (DPPH),  
116 ferric reducing antioxidant power (FRAP),  $\alpha$ -amylase (AA) and  $\alpha$ -glycosidase (GA)  
117 inhibition activities, and on the phenolic compound profile.

## 119 MATERIALS AND METHODS

### 120 RAW MATERIALS AND PREPARATION

121 The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya,  
122 East Java, Indonesia. The *Pluchea* plants were included in Asteraceae family with  
123 specification according to the GBIF taxon ID number database:3132728 (Ferraris, 2023).  
124 *Pluchea* leaves at 1-6 level of each branch ~~offrom~~ the shoot were collected, sorted,  
125 washed and dried to ~~get a~~ moisture content ~~of~~ around  $11.16 \pm 0.09$  % dry basise  
126 (Widyawati et al., 2022). The ~~powdoring of~~ dried *Pluchea* leaves was ~~done~~ pulverized to  
127 ~~get a~~ 45-mesh size powder. ~~And then, the heating of T~~the *Pluchea* leaf powder was ~~done~~  
128 ~~using a drying~~dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 °C for  
129 10 min to reduce microbial organisms. ~~and Then, 2 g of the powder were~~ packed using  
130 ~~into a paper filter~~ infusion bag, ~~that made from paper filter around 2 g/bag. And then all~~  
131 ~~of samples called~~Packed samples were *Pluchea* herbal tea was stored for 0 (un-stored)  
132 and 5 (stored)years in standing pouch before analysis.

133 In the research, the one tea bag of *Pluchea* herbal tea that stored 0 (B1) and 5  
134 (B2) year, was steeped with 100 mL hot water at various temperatures, including 60 (T1),

135 70 (T2), 80 (T3), and 95 (T4) °C for 5 min with infusion method ~~that obtained~~obtaining 8  
136 treatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2.  
137 After the temperature of *Pluchea* infusion similar to ambient temperature was analyzed  
138 further.

## 140 REAGENTS

141 The ~~compounds~~reagents used ~~to analyze~~in the analyses including include 2,2-  
142 diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid, α-amylase, α-  
143 glucosidase, pNPG (p-nitrophenyl-α-glucopyranoside), (+)-catechin, kaempferol,  
144 myricetin, quercetin, 3,4-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, 3,5-di-O-  
145 caffeoylquinic acid, and (+)-catechin were purchased from Sigma-Aldrich (St. Louis, MO,  
146 USA). Methanol, Folin–Ciocalteu’s Phenol, sodium nitric, aluminum chloride, ferric  
147 chloride, sodium dihydrogen phosphate, sodium phosphate, potassium ferricyanide,  
148 starch, acetic acid, and sodium hydroxide were purchased from Merck (Kenilworth, NJ,  
149 USA). All reagents used were of analytical grade except for distilled water which was  
150 purchased from PT Aqua Industry Surabaya.

## 152 METHODOLOGY

### 153 ANALYSIS OF THE BIOACTIVE COMPOUNDS

#### 154 TOTAL PHENOLIC CONTENT ANALYSIS

155 Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the  
156 technique by Gao et al. (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu’s  
157 phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. And

**Commented [A5]:** Confusing, needs to be re-written eg The unstored samples were steeped in 100 mL distilled water at 60, 70, 80, and 95 °C for 5 min, then immediately were analyzed for the bioactive compounds [(total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC)], antioxidant potential [(DPPH free radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP)] and antidiabetic activities [(α-amylase (AA) and α-glycosidase (GA) inhibition)]. The rest of the samples were stored at (describe storage conditions) and analyze after 5 years..

**Commented [A6]:** Confusing

**Formatted:** Centered

158 then 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5 % was ~~entered added and filled up to 10 mL volume with distilled~~  
159 ~~water and distilled water was added until 10 mL volume.~~ The color intensity of solution  
160 was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at  $\lambda$  760 nm  
161 with gallic acid as the reference standard. The total phenolic content was calculated using  
162 the formula:  $y=0.00009x+0.008$  with  $R^2=0.9941$ . The results were expressed as mg gallic  
163 acid equivalent (GAE)/g samples.

Commented [A7]: Specify what color

164

#### 165 TOTAL FLAVONOID CONTENT ASSAY

166 Total flavonoid content (TFC) of the samples was measured based on the reaction  
167 between AlCl<sub>3</sub> and NaNO<sub>2</sub> with ~~an the~~ aromatic ring of flavonoid compounds, especially  
168 flavonol and flavon (Shraim et al., 2021). The reaction between AlCl<sub>3</sub> and flavonoid  
169 compounds resulted ~~in~~ a yellow solution. About 30  $\mu$ L *Pluchea* infusion was mixed with  
170 0.3 mL NaNO<sub>2</sub> 5 % in 10 mL volumetric flask and incubated for 5 min. The mixture was  
171 added with 0.3 mL AlCl<sub>3</sub> 10 % for 5 min. And then, 2 mL NaOH 1 M and distilled water  
172 were added until 10 mL volume. Then, the red solution was produced after NaOH solution  
173 addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800,  
174 Shimadzu, Japan) at  $\lambda$  510 nm with (+)-catechin as the reference standard compound,  
175 and the results were expressed as mg catechin equivalents (CE)/g samples using the  
176 formula:  $y=0.00008x-0.0023$  with  $R^2= 0.9980$ .

177

#### 178 TOTAL TANNIN CONTENT ANALYSIS

179 Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method  
180 (Chandran and Indira, 2016). Approximately 10  $\mu$ L *Pluchea* infusion was added ~~with~~ 1 mL

181 Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flask and incubated for 5 min.  
182 Then, the mixture was added with 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5 % and filled up to 10 mL volume with  
183 distilled water. ~~was added until 10 mL volume.~~ The blue dark color solution ~~that was~~  
184 measured in UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at λ 760 nm with tannic  
185 acid as the reference standard. Calculation of TTC was expressed as mg tannic acid  
186 equivalents (TAE)/g samples used the formula:  $y=0.00009x+0.0021$  with  $R^2=0.9993$

## 188 ANALYSIS OF THE ANTIOXIDANT POTENTIAL

Formatted: Centered

### 189 DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

190 The DPPH free radical scavenging activity (DPPH) was measured by the  
191 spectrophotometric method (Widyawati et al., 2017) to determine ~~the ability of the~~  
192 ~~phytochemicals antioxidant activity of in~~ the *Pluchea* leaf infusion to ~~doner donate~~  
193 hydrogen atom to the nitrogen atom in DPPH resulting in the formation of ~~-DPPH-H~~  
194 compound with exhibiting a yellow-colored solution. About 25 μL *Pluchea* leaf infusion  
195 was ~~entered poured~~ into reaction tube ~~and into which was added~~ added 3 mL DPPH  
196 solution (4 mg/100 mL). ~~And then the solution was~~After incubationed for 15 min in a dark  
197 room, ~~the and~~ absorbance was measured by a spectrophotometer (Spectrophotometer  
198 UV-Vis 1800, Shimadzu, Japan) at λ. 517 nm. The reference standard compound was  
199 gallic acid and the results of analysis were expressed as mg gallic acid equivalents  
200 (GAE)/g samples that calculated using formula:  $y=0.146x+1.7896$  with  $R^2=0.9975$ .

### 202 FERRIC REDUCING POWER ANALYSIS

203 Ferric reducing power (FRAP) was determined following the method used by  
204 Widyawati et al. (2014) method. Approximately 10  $\mu$ L of samples were added 2.5 mL  
205 phosphate buffer pH 6.6 and 2.5 mL and 1% potassium ferricyanide 1% in the reaction  
206 tube. And then mixture was shaken and ~~incubation~~ incubated for 20 min at 50 °C. Finally,  
207 2.5 mL chloroacetic acid 10% (w/v) was added. Into the 2.5 mL supernatant was added  
208 2.5 mL distilled water, 0.5 mL ferric chloride 0.1% (w/v) and incubated for 10 min.  
209 Potency of the samples reducing iron (III) to iron (II) ion was ~~signed~~ indicated by the  
210 intensity of blue color formed that was measured using UV-Vis spectrophotometer  
211 (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  700 nm. Intensity of the blue  
212 color indicated higher reducing capacity. The reducing power expressed as mg gallic acid  
213 equivalent (GAE)/g samples was calculated using the formula:  $y=0.0002x+0,0256$  with  
214  $R^2=0,9906$ .

215

#### 216 $\alpha$ -AMYLASE ENZYME INHIBITION CAPACITY ASSAY

217 In vitro inhibition of  $\alpha$ -amylase enzyme (AA) followed the procedure as described  
218 by Widyawati et al. (2020). Each 500  $\mu$ L of samples, was mixed with starch 1 % (w/v) and  
219 sodium acetate buffer pH 5, ~~were mixed. Then, into each~~ 250  $\mu$ L of the mixture and was  
220 added an  $\alpha$ -amylase solution (0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in  
221 50 mL of 0.2 M sodium acetate pH 5). Mixture was shaken and into which was added 2  
222 mL sodium hydroxide 1M. Before the analysis, this mixture was incubated at 37 °C for 10  
223 min. Then, the capacity of the  $\alpha$ -amylase enzyme to hydrolyzed the starch to release  
224 glucose was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800,  
225 Shimadzu, Japan) that could be analyzed based on absorbance at  $\lambda$  540 nm. The

226 inhibition percentage of  $\alpha$ -amylase was assessed using the formula:  $(ACb - ACa) - (As$   
227  $- Ab) (ACb - ACa) \times 100 \%$ . Where, ACb is the absorbance of 100 % enzyme activity  
228 (solvent with the enzyme), ACa is the absorbance of 0 % enzyme activity (solvent without  
229 the enzyme), As is the absorbance of test sample with enzyme, Ab is absorbance of test  
230 sample without enzyme.

231

### 232 $\alpha$ -GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

233 The analysis of the  $\alpha$ -glycosidase inhibitor activity (GA) was done by Widyawati et  
234 al. (2020) method with slight modification. About 150  $\mu$ L samples ~~contained~~ containing  
235 100  $\mu$ L *Pluchea* infusion and 50  $\mu$ L pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M  
236 at pH 7) were reacted with 50  $\mu$ L  $\alpha$ -glycosidase 2 mM (0.0833 unit/mL), and then the  
237 mixture was incubated at 37 °C for 15 min. ~~Finally, the~~The reaction was stopped with the  
238 addition of 1000  $\mu$ L sodium carbonate 0.2 M. The residue of this enzyme hydrolyzed p-  
239 nitrophenyl- $\alpha$ -D-glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The

Commented [A8]: Confusing. Rewrite

240 ~~inhibition~~ activity of ~~steeping the~~ *Pluchea* ~~tea-infusion to enzyme~~ was measured by UV-  
241 vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at  $\lambda$  405 nm.

242 The inhibition percentage of  $\alpha$ -glycosidase was calculated using formula:  $(ACb - ACa) -$   
243  $(As - Ab) (ACb - ACa) \times 100 \%$ . Where, ACb is the absorbance of 100 % enzyme activity  
244 (solvent with enzyme), ACa is the absorbance of 0 % enzyme activity (solvent without  
245 enzyme), As is the absorbance of test sample with enzyme, Ab is the absorbance of test  
246 sample without enzyme.

247

### 248 HPLC ANALYSIS OF PHENOLICS

Corresponding Author: paini@ukwms.ac.id

249 The phenolic compounds of the samples were analyzed by HPLC based on  
250 Kongkiatpaiboon et al. (2018) method with modifications. Each *Pluchea* infusion was  
251 sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter  
252 syringe (Whatmann, 0.2 µm, NYL). About 20 µL of sample was injected in an HPLC  
253 (LC20AD series, Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC  
254 LC-20AD pump, CTO-30A column oven, CBM-20A/20 Alite system controller, and SPD-  
255 20A/20 AV UV-Vis detector. Separation of phenolic compounds in samples was carried  
256 out using a Shim-pack VP-ODS C18 column (ID 5 µm × 50 mm × 4.6 mm) with a GVP-  
257 ODS Cartridge guard column (2 pieces) (ID 10 mm × 4.6 mm). ~~Analytical conditions:~~ The  
258 mobile phase used consisted of a solution of (A) 0.5 % acetic acid in water and (B)  
259 absolute methanol. Analysis was carried out using a gradient system in the following  
260 order: initial conditions of 10 % B in A to 50 % B in A were maintained for 40 minutes;  
261 then 100 % B was maintained for 20 minutes. Next the column was re-equilibrated with  
262 10 % B in A maintained for 10 minutes before analysis of the next sample. The sample  
263 flow rate was set at 1.0 ml/min with a controlled temperature at 40 °C. Detection was used  
264 at a wavelength of 280 nm. The reference standard used were gallic acid, (+)-catechin,  
265 myricetin, quercetin, kaempferol, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and  
266 4,5-dicaffeoylquinic acid. All of the reference standard was dissolved in distilled water  
267 and prepared similar to the samples before injected in HPLC.

268

## 269 EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

270 The research design used a randomized block design with two factors, i.e., the  
271 steeping temperature (T) and the storage ~~time~~ timeperiod (B). *Pluchea* leaf blades were



272 subjected to 4 steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95  
273 °C (T4), and the storage ~~timeperiod~~ of 0 year /~~fresh-un-stored~~ (B1), and 5 year/stored  
274 (B2). ~~The research resultedresulting in~~ 8 treatment combinations (T1B1, T1B2, T2B1,  
275 T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic was repeated two  
276 ~~timeperiods~~. The data of samples were analyzed by ANOVA at  $\alpha \leq 0.05$ , and continued  
277 analysis using a paired T test at  $\alpha \leq 0.05$ . treatment means of specific phenolic  
278 compounds that were identified were expressed as the mean  $\pm$  SD. The analysis used  
279 SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

**Commented [A9]:** Were all analyses replicated 2 periods only? What do you mean by 'continued analysis using a paired t-test at  $\alpha \leq 0.05$ '? Rewrite this part of the paragraph.

## 281 RESULTS AND DISCUSSIONS

282 *Pluchea* leaf infusion is produced by young *Pluchea* leaf from 1-6 level on each  
283 branch the shoot (Widyawati et al., 2022), that is steeped at 95 °C for 5 min, has many  
284 biological activities, such as antioxidant activity (Widyawati et al., 2016), antidiabetic  
285 activity (Werdani and Widyawati, 2018), anti-inflammatory (Srisook et al., 2015). The  
286 chemical constituents in *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols,  
287 cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2  
288 g/100 mL steeping *Pluchea* tea has total phenolic content 9.3 mg gallic acid equivalents  
289 (GAE)/g samples, total flavonoid content 22.0 mg catechin equivalents (CE)/g samples,  
290 DPPH free radical scavenging activity 27.2 mg gallic acid equivalents (GAE)/g samples,  
291 and ferric reducing power 10.2 mg gallic acid equivalents (GAE)/g samples (Widyawati et  
292 al., 2016). Previous research has informed related to the composition of phytochemical  
293 compounds in *Pluchea* leaves, such as phenolic acids such as chlorogenic acids, caffeic  
294 acids, 3-O-caffeoylquinic acids, 4-O-caffeoylquinic acids, 5-O-caffeoylquinic acids, 3,4-

295 di-O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic  
296 acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin;  $\beta$ -  
297 carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al.,  
298 2019; Chan et al., 2022; Widyawati et al., 2022). Presence of phytochemical compounds  
299 in herbal product were influenced by environmental factors, i.e., temperature, light  
300 exposure, oxygen level, pH and moisture. The structure of phytochemical compounds in  
301 herbal tea is very sensitive of the surrounding changes. The effect arising from these  
302 changes causes the structure of the phytochemical molecule to be degraded to produce  
303 smaller size molecules or to combine to produce larger size molecules (Ali et al., 2018;  
304 Jayani et al. 2022, Ramphinwa et al., 2023). Therefore, this study was conducted to  
305 determine the effect of steeping temperature and storage ~~time~~period of *Pluchea* tea on  
306 levels of the bioactive compounds, antioxidant and antidiabetic properties and phenolic  
307 compound profile.

**Commented [A10]:** Delete this part. Information in here are already found in the Introduction section.

## 309 BIOACTIVE COMPOUNDS

### 310 Phenolics Compounds

**Formatted:** Centered

311 The bioactive compounds are active compounds in plants that are essential to  
312 protect a body health (Nguyen and Chuyen, 2020). These compounds usually have many  
313 biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer,  
314 antimicrobial, antibacterial, anti-cholesterol and so on (Suriyaphan, 2014; Acar et al.,  
315 2022). Phenolic compounds have potential redox properties that can scavenge free  
316 radicals that can cause a number of chronic diseases (Noreen et al., 2017; Aryal et al.,  
317 2019; Acar et al., 2022).

318 The total phenolic content (TPC) of *Pluchea* infusion at different steeping  
319 temperature and storage period generally significantly increased with increasing steeping  
320 temperature and storage period based on paired ~~T-t~~ test at  $\alpha \leq 0.05$  (Figure 1a). Steeped  
321 and stored infusion had significantly higher amounts of phenolic compounds than the  
322 samples that were steeped and un-stored. Further, the highest total phenolic content was  
323 observed in samples infused at 95 °C and stored for 5 years (at 71.38±4.14 mg GAE/g  
324 samples) while the lowest was measured in the un-stored samples and infused at 60 °C  
325 (at mg GAE/g sample). Phenolic content of stored samples that were infused at  
326 different temperatures that then stored were steeped only at 60 and 95 °C also showed a  
327 significant increase in their phenolic content. This implies that the steeping temperature  
328 and the storage periods significantly resulted in the high amounts of the phenolic  
329 compounds of the infusions. Results also indicated that phenolic compounds were  
330 generally greater in the infusion at high steeping temperatures and long storage period  
331 (Figure 1a). This could have been due to that fact that during steeping fresh *Pluchea* tea  
332 had a lower total phenolic content than stored *Pluchea* tea for 5 years, besides that the  
333 higher the steeping temperature also caused the greater the extracted total phenolic  
334 content. The temperature of infusion influenced total phenolic content, it could relate to  
335 This could have been due to the fact that the steeping temperature and storage period  
336 can cause the process of degradation, oxidation, and leaching/release of phenolic  
337 compounds. Phenolic compounds are water soluble and thus soaking in hot water for a  
338 certain period of period as in steeping causes the migration process of more phenolic  
339 compounds to the water because of longer increasing contact exposure between of  
340 phenolic compounds to water (Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al.

341 (2022). Su et al. (2019) reported that temperature treatment can stimulate the release  
342 of phenolic compounds of lychee juice stored at different temperatures of 4 and 45 °C  
343 and different long storage (fresh and 72 hours).  
344 this compounds and water. The same phenomena also occurred in Castiglioni  
345 et al. (2015); Kilic et al. (2017), and Acar et al. (2022).  
346 This occurrence showed that steeping temperature and storage period caused the  
347 process of degradation and oxidation of phenolic compounds. Su et al. (2019) reported  
348 that temperature treatment can stimulate the release of phenolic compounds and  
349 increase antioxidant activity of lychee juice stored at different temperatures of 4 and 45  
350 °C and different long storage (fresh and 72 hours). Hydrogen bonding is affected by  
351 Temperature treatment because the degrades (or hydrolyzes) the hydrogen bond  
352 between phenolic compounds and proteins can be degraded that the measured levels  
353 resulting in an increase of phenolic compounds when exposed to are higher  
354 temperatures. The phenomena were supported by (Ali et al. (2018); Jayani et al. (2022),  
355 and Ramphinwa et al. (2023). Zhang et al. (2021) reported that phenolic compounds  
356 present in plants are not completely stable, but are easily degraded during storage after  
357 harvest. Reblova (2012) claimed that antioxidant compounds can be slowly degraded  
358 with increasing temperature. Besides that, Fibrianto et al. (2021) also stated that the  
359 brewing temperature has an effect on the extracted antioxidant compounds, such as  
360 alkaloids, catechins and tannins. Thus, there is an assumption that temperature and  
361 storage caused the degradation, oxidation and hydrolysis of the phenolic compounds that  
362 the phenolic compounds in *Pluchea* infusion are degraded due to oxidation and hydrolysis  
363 because of temperature and storage time period and can be easily extracted during

364 steeping, thus resulting in the increased amount of the phenolic content  
365 compounds as the at higher steeping temperature and longer storage increase period.

366 Based on using of a reference standard could be informed that Simple phenolic  
367 compounds identified in steeped and stored in *Pluchea leaf* infusion, including gallic  
368 acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-O-caffeoylquinic acids,  
369 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic acids was showed in Table 1.

370 The treatment effects results of statistical analysis using a paired T test at  $\alpha \leq 0.05$  showed  
371 that gallic acid and kaempferol contents of *Pluchea* infusion were insignificantly different  
372 at various steeping temperature and long storage periods. Nevertheless, the The  
373 concentration of quercetin and 3,5-di-O-caffeoylquinic acid of the un-stored and stored  
374 *Pluchea* infusion was significantly different from the rest of the samples between of two  
375 treatments except at 70 °C. The while (+)-catechin concentration of *Pluchea* infusion was  
376 only significantly different at 95 °C, but the myricetin content was significantly different  
377 different concentration at 80 and 95 °C. The 3,4-di-O-caffeoylquinic acid content showed  
378 significace difference at 60, 80 and 95 °C and while 4,5-O-dicaffeoylquinic acid  
379 compounds content from *Pluchea* infusion were was only significantly different at 60 °C,  
380 however the concentration of 3,4 dicaffeoylquinic acid was also significantly different at  
381 80 and 95 °C.

382 Based on the analysis of concentration of Results further showed simple phenolic  
383 compounds showed that gallic acids and kaempferol were relatively stable phenolic acid  
384 because of as reflected by the insignificant changes when exposed no changes at to the  
385 different steeping temperature and storage time period, with concentration about 0.24 ±  
386 0.00 to 0.24 ± 0.02 µg/g samples and 0.14 ± 0.02 to 0.95 ± 0.03 µg/g samples, respectively.

387 ~~However, myricetin~~Myricetin, (+)-catechin and 3,4-di-O-caffeoylquinic acid showed a  
388 drastic ~~increasing~~ increase at higher steeping temperature and longer storage period  
389 ~~implying -It's meant~~ that these compounds tended to be relatively labile. Quercetin, 3,5-  
390 di-O-caffeoylquinic acid and 4,5-di-O-caffeoylquinic acid underwent moderate changes  
391 ~~compared to the other two groups of phenolic acids.~~ Therefore, myricetin, (+)-catechin  
392 and 3,4-di-O-caffeoylquinic acid were easier to dissolve or degraded to form simple  
393 phenolic compounds at higher steeping temperature and storage ~~time~~period. ~~can cause~~  
394 ~~macromolecules of three phenolic acids in herbal tea convenient degradable to form~~  
395 simple phenolic compounds for storage, as explained by (Su et al. (2019), Ali et al. (2018);  
396 Jayani et al. (2022); Ramphinwa et al. (2023), and Zhang et al. (2021). Degradable  
397 polyphenol compounds have a simple structure and free hydroxyl groups that can react  
398 with Folin-Ciocalteu's Phenol reagent, resulting complex blue solution that can detected  
399 as total phenolic content.

**Commented [A11]:** Does the negative sign mean an increase or decrease

400 **Flavonoid Content (TFC)**

401 **Flavonoids are the major phenolic compounds that have potential chemical and**  
402 **biological activities, such as** radical scavenging and antimicrobial activities (Ayele et al.,  
403 2022; Chandra et al., 2014) **that can** protect the human body from the oxidative stress  
404 caused many degenerative diseases, especially cancer, cardiovascular problems and  
405 ageing (Mathur and Vijayvergia, 2017). **The total flavonoid content of steeped *Pluchea***  
406 **infusion decreased with longer storage period. Un-stored samples exhibited higher**  
407 **flavonoid content than the stored samples. The statistical analysis using a paired T test**  
408 **at  $\alpha= 0.05$  showed that total flavonoid content of *Pluchea* infusion was significantly**  
409 **different between ~~two treatments~~the steeped un-stored and steeped stored samples**

**Commented [A12]:** What does the negative (-) sign implies? What is your basis of classifying the simple phenolic compounds as relatively labile, moderate?

**Formatted:** Centered

410 (Figure 1b). The highest total flavonoid content was exhibited by ~~fresh the un-stored~~  
411 ~~samples steeped at 95 °C at~~ about 147.42±14.03 mg CE/g samples. Total flavonoid  
412 content was significantly lower in the stored ~~samples regardless of steeping temperature~~  
413 ~~than those of the un-stored around 24.75±2.47 to 33.71±3.06 mg CE/g samples~~ implying  
414 that the increase in the flavonoid content of the infusion was affected primarily by the  
415 steeping temperature.

Commented [A13]: cite similar studies to support your findings

#### 416 Tannin Content (TTC)

Formatted: Centered

417 Tannins are bioactive compounds that provide properties, such as astringent, anti-  
418 diarrheal, antibacterial and antioxidant (Malangngi et al., 2012). Generally, results  
419 indicated that the total tannin content of *Pluchea* infusion significantly increased with  
420 increasing steeping temperature and storage period (Figure 1c). Among, the un-stored  
421 steeped samples, the tannin content was significantly lowest in ~~the~~ samples infused at 60  
422 °C ~~at~~ about 4.81±0.58 to 17.42±1.04 mg TAE/g samples, ~~which is was~~ significantly  
423 different ~~lower~~ from ~~that of~~ the lowest tannin content of the stored samples. Among the  
424 stored and steeped samples, the highest tannin content was observed at samples  
425 steeped at 95 °C about 17.42 ± 1.04 mg TAE/g samples and was significantly different  
426 from that of the highest tannin content of the un-stored steeped samples at 95 °C about  
427 9.22 ± 1.48 mg TAE/g samples. ~~Indicating that the tannin content was primarily affected~~  
428 ~~by both high steeping temperature and long storage period than high steeping~~  
429 ~~temperature and that the presence of high tannin content was primarily brought about by~~  
430 ~~long storage period. Kowalska et al. (2021) informed that~~ the condensation of catechins  
431 to tannins ~~of polyphenolic compounds~~ is a dominant process ~~occurred-occurring~~ in tea  
432 leaves that is accelerated during maceration of raw ~~material~~ tea leaves (Kowalska et al.

433 (2021) could have had contributed to the observed increase in the tannin content in the  
434 treated samples. However, the high temperature can degrade polyphenolic compounds  
435 to form simple phenolic compounds that is essential to body health. The results showed,  
436 that the higher the brewing temperature and the longer the storage time caused the tannin  
437 compound to degrade to result catechin compounds. This phenomenon is in line with the  
438 increase in total phenol levels and the concentration of (+) catechin compounds. Ali et al.  
439 (2018) said that pH, storage temperature, chemical structure and concentration, light,  
440 oxygen, enzymes and metal ions affect the presence of bioactive compounds in the  
441 material. Nevertheless,

442 Although, high temperature and long storage period can cause the degradation of  
443 tannins to catechins, Rusita et al. (2019) emphasized that tannins are a polar  
444 thermostable complex compounds, that is are resistant to heating, indicating that even  
445 with the exposure to high temperature, the tannins still remained high in the treated  
446 samples as a result the tannin content in *Pluchea* tea increases with increasing steeping  
447 temperature and storage time period, this is caused tannins are thermostable complex  
448 compounds.

449

#### 450 ANTIOXIDANT ACTIVITY

451 Antioxidant activity is capability of compounds to inhibit the oxidation of  
452 macromolecules from biological target that involve in oxidative chain reactions (Ali et al.,  
453 2005; Oh et al., 2013). The antioxidant activity assay was done in this research using  
454 DPPH Free Radical Scavenging Activity (DPPH) and ferric reducing power (FRAP)  
455 methods. The phenolic compounds are an active antioxidant that have antioxidant

Formatted: Centered



456 capability ~~that depends~~ on their redox properties. The structure of phenolic compounds  
457 determine ~~the~~ effectivity to ~~doner donate~~ hydrogen atom which is negatively correlated  
458 with the O-H phenolic bond strength. The higher antioxidant power of phenolic  
459 compounds is caused ~~by~~ the weaker O-H phenolic bond (Kruk et al., 2022). The  
460 mechanism of phenolic compounds ~~is involved~~ as antioxidants ~~through depends on their~~  
461 the ability to donate hydrogen atom ~~ands~~, transfer electrons, ~~and as~~ reducing agents and  
462 singlet oxygen quenchers (Ali et al., 2005; Huang et al. 2005).

Commented [A14]: what do you mean? rewrite

#### 464 DPPH Free Radical Scavenging Activity

Formatted: Centered

465 DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate  
466 antioxidant activity because this method ~~is simple~~ that is suitable to measure the donating  
467 hydrogen atoms capability of ~~herbal infusion~~. This reaction can cause the purple color of  
468 ~~DPPH to change to yellow color~~ (Munteanu and Apetrei, 2021; Baliyan et al., 2022).  
469 ~~Figure 2a shows that the free radical scavenging property of the stored and steeped~~  
470 ~~samples were significantly higher than the un-stored steeped samples. The result of~~  
471 ~~DPPH assay~~ It can also be observed ~~indicates~~ that the ~~free radical scavenging property~~  
472 ~~DPPH values accrued~~ was significantly different among the stored and steeped samples  
473 ~~but insignificant among the un-stored and steeped samples at higher steeping~~  
474 ~~temperature and longer storage timeperiod. Statistical analysis by ANOVA using a paired~~  
475 ~~T test at  $\alpha \leq 0.05$  proven that the higher the steeping temperature of fresh *Pluchea*~~  
476 ~~infusion (T1B1, T2B1, T3B1, and T4B1) was consistent the ability to DPPH free radicals~~  
477 ~~scavenging activity, whereas the stored *Pluchea* infusion resulted in the higher activity~~  
478 ~~and the values went up as rising of the infusion temperature. *Pluchea* infusion stored at~~

479 room temperature for 5 years resulted in the high DPPH-free radical scavenging activity  
480 by more than 100 %. Steeping at higher temperatures significantly increased the DPPH  
481 free radical scavenging activity in stored *Pluchea* infusion by around 15 to 25 %. Steeping  
482 at 80-95 °C in stored *Pluchea* infusion insignificantly affected the free radical scavenging  
483 property of the bioactive compounds (Figure 2a). This implies that that the higher free  
484 radical scavenging property was primarily affected by the storage period than steeping  
485 temperature. During the storage process it is possible to form complex phenolic  
486 compounds which provide a high ability to scavenge DPPH-free radicals  
487 (Thanajiruschaya et al., 2010)

488 Scavenging The scavenging activity of DPPH free radicals of the the samples was  
489 strongly and positively correlated with total with total phenolic and tannin contents levels,  
490 but inversely to with total flavonoid levels. Based on Pearson correlation at Table 2, the  
491 correlated coefficient values (r) between DPPH and TPC, TTC and TFC were 0.993,  
492 0.942, and 0.940, respectively. During the storage process it is possible to form complex  
493 phenolic compounds which provide a high ability to scavenge DPPH free radicals  
494 (Thanajiruschaya et al., 2010). This research study also demonstrated that longer storage  
495 time period and higher infusion temperature produced many simple phenolic compounds  
496 with free hydroxyl groups capable to donor hydrogen atom to DPPH free radical. Many  
497 phenolic acids, such as gallic acids, (+)-catechins, myricetins, kaempferols, quercetins,  
498 3,5-di-O-caffeoylquinic acids, 3,4-di-O-caffeoylquinic acids, 4,5-di-O-caffeoylquinic acids  
499 have established potential antioxidant activity (Kumar and Goel, 2019) (Table 1). Kruk  
500 et al (2022) informed that the capability of phenolic compounds to donor hydrogen atom  
501 depends on chemical structure, number and position of hydroxyl groups attached to a

Commented [A15]:

Commented [A16R15]: Clarify on how you were able to come up with free radical scavenging activity by more than 100 %. Steeping temperatures significantly increased the free radical scavenging activity in stored *Pluchea* infusion by around 15 to 25 %

Commented [A17]: Explain/interpret this observation based on the data that you were able to obtain.

502 benzene ring, a double bond between C2 and C3 rings and a carbonyl group (C=O) on  
503 the C ring at C4. The effectivity of antioxidant compounds donor hydrogen atom is  
504 determined by O-H bond dissociation energy.

505 The DPPH-free radical scavenging property observed in the study was not in  
506 consistent with the results of the study by Moraes-de-Souza et al. (2008). The research  
507 shows that total phenolic content of herbal infusion is low correlated with DPPH-free  
508 radical scavenging activity. However, Dobrinas et al. (2021) informed that total phenolic  
509 content of tea infusion is positively and significantly correlated with the free radical  
510 scavenging property/inhibitor activity of DPPH of tea infusion.

511

#### Ferric Reducing Antioxidant Power (FRAP)

Formatted: Centered

512 FRAP is an analysis of antioxidant power of the phytochemical compounds based  
513 on the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic  
514 acid, and ferric chloride to produce a color complex, that can be measured at  $\lambda$  700 nm  
515 (Fu et al., 2011; Al-Temimi and Choudhary, 2013). The principle of the assay measures  
516 that is based of the ability of antioxidant compounds to reduce iron ions of potassium  
517 ferrocyanide ( $\text{Fe}^{3+}$ ) to be potassium ferrocyanide ( $\text{Fe}^{2+}$ ). Potassium ferrocyanide reacts  
518 with ferric chloride to form a ferric-ferrous complex and results green color solution  
519 (Widyawati et al., 2017; Raharjo and Haryoto, 2019).

520 The results showed that the ferric reducing antioxidant power (FRAP) increased  
521 with at higher steeping temperature and longer storage time period. The lowest FRAP was  
522 observed in the un-stored samples which was steeped at 60 °C at  $3.95 \pm 0.17$  mg gallic  
523 acid equivalents (GAE)/g samples, and the highest was owned exhibited by in *Pluchea*

525 infusion which was stored for 5 years at 95 °C at 48.63 ±10.83 mg gallic acid equivalents  
526 (GAE)/g samples (Figure 2b). FRAP increased significantly as steeping temperature was  
527 increased. FRAP of the samples stored for 5 years was also significantly higher than the  
528 un-stored samples at  $\alpha \leq 0.05$ . Based on Pearson correlation, the FRAP of *Pluchea*  
529 infusion was strongly and positively significant correlated with the DPPH, TPC and TTC,  
530 but inversely to TFC. The correlated coefficient values (r) between FRAP and DPPH,  
531 TPC, TTC and TFC were 0.956, 0.953, 0.948 and -0.826, respectively.

532 This case was is in contrast to with the study on the antioxidant activity of DPPH  
533 and FRAP on of matcha, because The the longer storage time period reduces the levels  
534 of catechin content due to the catechins, such as epigallocatechin gallat (EGCG),  
535 epicatechin gallat (ECG), epigallocatechin (EGC), and epicatechin (EC) which are  
536 bioactive compounds that have high antioxidant activity (Kim et al. 2020), and also the  
537 case of the effect of temperature and storage time in betel (*Piper bettle* L.) extract. Light  
538 and temperature influence degradation of phenolic compounds of betel that determine  
539 antioxidant activity. Different structure of phenolic compounds determines their stability  
540 to degrade accelerating of light and temperature. Hydroxychavicol is the best stability of  
541 phenolic compounds of betel compared with eugenol, isoeugenol and allyl pyrocatechol  
542 (Ali et al., 2018). Thanajiruschaya et al. (2010) revealed that the antioxidant activity of  
543 rice stored at high temperatures is greater than that stored at low temperatures. The ferric  
544 reducing capability of *Pluchea* could have due infusion corresponded to the presence to  
545 of simple phenolic acid values that have the ability to transfer electron from their free  
546 hydroxyl groups of, presence of them in samples could accrue antioxidant activity  
547 because of ability of the electron transfer from free hydroxyl groups of phenolic acids.

Commented [A18]: Relate these with Figure 2b. Rewrite

548 [The FRAP of \*Pluchea\* infusion was strongly and positively significant correlated with the](#)  
549 [DPPH, TPC and TTC, but inversely to TFC.](#)

550 ANTIDIABETIC ACTIVITY

551 [α-Amylase enzyme inhibition activity](#) (AA)

Formatted: Centered

552 [Antidiabetic activity is a measure of the potency of phenolic compounds to regulate](#)  
553 [the uptake of glucose by the cells from the blood through the mediation of 2-degestive](#)  
554 [enzymes i.e., α-amylase and α-glucosidase, which are involved the control of dietary](#)  
555 [carbohydrate digestion and release in the postprandial blood glucose in human body \(Fu](#)  
556 [et al., 2017\). The phenolic compounds have the capability to bind with the protein](#)  
557 [component of α-amylase and α-glucosidase enzymes \(Martinez-Solis et al., 2022\)](#)  
558 [resulting in the reduced activity of the enzymes. The results showed, that the lower](#)  
559 [steeping \*Pluchea\* leaf infusion was able to inhibit the action of the α-amylase enzymes](#)  
560 [\(Figure 3a\). The \*Pluchea\* infusion had very good activity, exhibited a good α-amylase](#)  
561 [enzyme inhibition activity of more than 50 % and even almost 100 % for fresh in the un-](#)  
562 [stored \*Pluchea\* infusion which steeped was brewed at 60, 70 and 80 °C with highest at](#)  
563 [60 °C, and in stored \*Pluchea\* leaf infusion which was steeped at 60 °C. Whereas The](#)  
564 [stored fresh \*Pluchea\* leaf infusion steeped at 70, 80 and 95 °C for 5 minutes had lower](#)  
565 [enzyme inhibition activity an activity of of less than 50 % with lowest at 95 °C inhibiting the](#)  
566 [α-amylase enzyme of less than 50 %, which was equal to 40.08±1.12 %. Widyawati et al.](#)  
567 [\(2017\) detected found that the ability to inhibit the α-amylase enzyme from in fresh un-](#)  
568 [stored \*Pluchea\* infusion steeped at 95 °C for 5 minutes by was also low at 28.79 %.](#)  
569 [Increasing the steeping temperature and storage time period reduced the ability to of the](#)  
570 [phytochemicals in the \*Pluchea\* infusions to inhibit the α-amylase enzyme activity. The](#)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Commented [A19]: Why?/Explain

571 results of the analysis based on a paired T test at  $\alpha \leq 0.05$  showed, that the steeping  
572 temperature and storage timeperiod had a significant effect on the ability to inhibit the  $\alpha$ -  
573 amylase enzyme. Based on Pearson correlation, the Table 2 further shows that the AA of  
574 *Pluchea* infusion was strongly and negatively significant correlated with TPC, TTC, DPPH  
575 and FRAP, but it was moderately and negatively significant correlated with TFC. The  
576 correlated coefficient values ( $r$ ) between AA and TPC, TTC, DPPH, FRAP and TFC were  
577 0.708, -0.857, -0.696, -0.806 and 0.429, respectively.

578 This inhibitory activity was thought to be contributed by other bioactive compounds,  
579 besides phenolics which are sensitive to steeping temperature and storage timeperiod. Li  
580 et al. (2018) stated that there are flavonoid compounds that contribute to the ability to  
581 inhibit the  $\alpha$ -amylase enzyme. Flavonoid compounds with a hydroxyl structure at C-4' in  
582 ring B are more effective than C-6 in ring A. Akah et al. (2011) informed reported that the  
583 phytochemical compounds, such as terpenoids, saponins, flavonoids, glycosides and  
584 carbohydrate, and alkaloids are good antidiabetic metabolites or  $\alpha$ -amylase enzyme  
585 activity inhibitor. Sangeetha and Vedesree (2012) explained, that the ability to inhibit the  
586  $\alpha$ -amylase enzyme was determined by the content of the phenolic compound and protein.  
587 The  $\alpha$ -amylase inhibitor enzyme present in *Pluchea* infusion may be proteinaceous in  
588 nature. Alexandre et al. (2022) informed that phenolic acids have inhibition activity to  $\alpha$ -  
589 amylase enzyme depending their structures. Besides that, capability of phenolic acids to  
590 inhibit  $\alpha$ -amylase enzyme was determined by low half-maximum inhibitory concentration  
591 ( $IC_{50}$ ). There are C=C double bond conjugated with a carbonyl group of phenolic  
592 structures that stabilizes the binding forces to the active site of the  $\alpha$ -amylase. The  
593 hydroxyl groups of them are able to bind by non-covalent interaction, such as hydrogen

Commented [A20]: Implications? Explain

Commented [A21]: What content or what is in the content the influenced the ability to inhibit the enzyme?

Commented [A22]: How will this affect the ability to inhibit the enzyme?

594 binding, cation- $\pi$  interactions, salt bridge interactions, ionic interactions or electrostatic  
595 forces with amino acid residue at the active site in  $\alpha$ -amylase enzyme. ~~Elevated steeping~~  
596 ~~temperature and longer storage period~~ ~~The steeping temperature and storage time can~~  
597 ~~easily cause the~~ removal of the ~~e~~ hydroxyl groups of phenolic compounds that can reduce  
598 ~~their~~ ability of enzyme inhibition. The phenolic acids with a greater number of hydroxyl  
599 groups ~~are exhibits~~ stronger capability to obstruct the  $\alpha$ -amylase enzyme.

**Commented [A23]:** Lines 585 to 595, Either delete or rewrite for better readability and understanding referring to enzyme activity inhibition

#### 600 $\alpha$ -Glucosidase enzyme inhibition activity (GA)

**Commented [A24]:** This part is disorganized. Avoid duplicating statements, observation facts etc

**Formatted:** Centered

601 ~~Alpha~~ $\alpha$ -glucosidase is an important enzyme in carbohydrates digestion, that  
602 catalysis the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and  
603 converts them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et  
604 al., 2014; Proenca et al., 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -  
605 glucosidase enzyme is used to determine ~~their~~ antidiabetics activity. ~~This is supported~~  
606 ~~by~~ Werdani and Widyawati (2018) ~~stated~~, that **Pluchea infusion** has the potential as an  
607 antidiabetic agent. Widyawati et al. (2020) found that brewing fresh **Pluchea infusion** at  
608 95 °C for 5 minutes has an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857 %.

609 ~~The results showed~~, ~~Figure 3b shows~~ that the ability of the **Pluchea leaf infusion**  
610 to inhibit the  $\alpha$ -glucosidase enzyme decreased with increasing steeping temperature and  
611 storage ~~time~~period. Steeping at 95 °C ~~for fresh~~of the un-stored **Pluchea leaf** infusion (~~un-~~  
612 ~~stored~~) obtained the lowest inhibitory ability, i.e.,  $48.32 \pm 1.27$  %, and the highest inhibitory  
613 activity was found at 70 °C ~~steeping temperature for fresh~~ **Pluchea** infusion, which was at  
614  $95.11 \pm 0.70$ % ~~(Figure 3b)~~. The results of a paired T test showed that GA of **Pluchea**  
615 **infusion** was significantly different ~~at both~~between steeping temperature and long storage.  
616 ~~The antidiabetic activity of~~ **Pluchea infusion** ~~Figure 3 further~~ ~~showed~~ shows that the ability

**Formatted:** Font: Not Italic

**Commented [A25]:** Explain

617 of *Pulchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher than the  
618 ability to inhibit the  $\alpha$ -amylase enzyme. Li et al. (2018) informed that flavonoid compounds  
619 have the ability to inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. This is  
620 due to the total flavonoids in steeped *Pluchea* infusion which tended to have the same  
621 pattern as the ability to inhibit the activity of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes.  
622 The statistical analysis using Pearson correlation showed that GA of *Pluchea* infusion  
623 was strongly and negatively correlated with TPC, TTC, DPPH and FRAP  
624 ~~with r was -0.555, -0.715, -0.527 and -0.560, respectively.~~ However, GA was  
625 moderately and positively correlated to TFC, ~~with r was 0.350 and strongly and positively~~  
626 ~~correlated to AA, with r was 0.725.~~ Flavonoid compounds, such as rutin, myricetin,  
627 kaempferol, and quercetin ~~which~~ have antioxidant and antihyperglycemic activities. The  
628 ability to inhibit the action of enzymes from flavonoid compounds is determined by the  
629 position and number of hydroxyl groups and the number of double bonds in rings A and  
630 B and the heterocyclic ring in ring C. The ability to inhibit the  $\alpha$ -glucosidase enzyme from  
631 *Pluchea* infusion was significantly affected by the steeping temperature and long storage.  
632 The capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater than  
633 the  $\alpha$ -amylase enzyme because the mechanism of two enzymes was different, according  
634 to the opinion of McCue et al. (2005). Widyawati et al. (2017) informed that phenolic and  
635 non-phenolic compounds determine the inhibitory activity of the  $\alpha$ -glucosidase enzyme.  
636 The ability of bound phenolic compounds to inhibit  $\alpha$ -glucosidase enzymes was higher  
637 than free phenolic compounds. The presence of polymerization and degradation  
638 reactions, that may be occurred in *Pluchea* infusion during storage, affects the structure  
639 and profile of phenolic and non-phenolic compounds. Asriningtyas et al. (2014) claimed

Formatted: Font: Italic

Commented [A26]: This does not explain explain lines 597-599 with the manner it is written. Include statements that connect the explanation with the observation. Having 'same pattern' is not observed in the figure/graph

Commented [A27]: Interpret/Implications

Commented [A28]: Delete literature citations that are unnecessary to explain the findings



640 that *Pluchea* leaves contain 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid  
641 methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid,  
642 and 1,3,4,5-tetra-*O*-caffeoylquinic acid. Quinic acid is methyl esterified with the number  
643 of caffeic groups in the molecule that determines the activity of inhibiting the  $\alpha$ -  
644 glucosidase enzyme. Analysis of caffeoylquinic acids in *Pluchea* infusion was obtained  
645 that the higher steeping temperature and long storage caused increased concentration  
646 of them, but the  $\alpha$ -glucosidase inhibition activity of them was reduced. Aleixandre et al.  
647 (2022) reported that the simple phenolic acids forming a dipole-dipole interaction of active  
648 site from  $\alpha$ -glucosidase enzyme are effectively inhibiting the enzyme.

649 This study ~~was obtained information~~ showed that the increasing of steeping  
650 temperature and storage ~~time period~~ caused a degradation reaction of polyphenol  
651 compounds to produce simple phenolic compounds, such as gallic acid, (+)-catechin,  
652 myricetin, quercetin, kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic  
653 acid, and 4,5-di-*O*-caffeoylquinic acid, supported the results of total phenolic content and  
654 total tannin content assays. Increased concentration of simple phenolic compounds  
655 determined the ability of these compounds as antioxidant agents, but reduced their  
656 capability as antidiabetic agents.

## 658 CONCLUSION

659 The steeping temperature and storage ~~time period~~ of *Pluchea* infusion significantly  
660 influenced bioactive contents, antioxidant and antidiabetic activities. TPC, TTC, and TFC  
661 were significantly different at various steeping temperature and storage period based on  
662 statistical analysis using a paired ~~T-t~~ test at  $\alpha \leq 0.05$ . ~~There was the difference of t~~The

Corresponding Author: paini@ukwms.ac.id

**Commented [A29]:** Unnecessary because this is not included as one of the derived simple phenolic acids

**Commented [A30]:** Not clear, re-write

**Commented [A31]:** Organize the discussion to explain the observation one at a period. ex:

1) 'Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme decreased with increasing steeping temperature and storage period.' (This must be followed by an explanation or support.)

2) However, GA was moderately and positively correlated to TFC and positively correlated to AA..(This must be followed by implications/support/explanation.)

The ability to inhibit the  $\alpha$ -glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping temperature and long storage.(This can be integrated in 1)

4) The capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater than the  $\alpha$ -amylase enzyme because the mechanism of two enzymes was different, according to the opinion of McCue et al. (2005). (This can be integrated in 1)

The mechanism must be explained -the mechanism of two enzymes was different,

5) Flavonoid compounds, such as rutin, myricetin, kaempferol, and quercetin which have antioxidant and antihyperglycemic activities

6) . Li et al. (2018) informed that flavonoid compounds have the ability to inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. ( May also be integrated in 1)

7) Widyawati et al. (2017) informed that phenolic and non-phenolic compounds determine the inhibitory activity of the  $\alpha$ -glucosidase enzyme. The ability of bound phenolic compounds to inhibit  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. (May also be incorporated in 1).

8) Lines 618 to 629 into 1)

**Commented [A32]:** Reconcile with your discussion

**Commented [A33]:** Suggested conclusion

## CONCLUSION

The total phenolic content (TPC) of *Pluchea* infusion at different steeping temperature and storage period generally significantly increased with increasing steeping temperature and storage period. Steeped and stored infusion had significantly higher amounts of phenolic compounds than the samples that were steeped and un-stored. TPC was highest in the store ...

663 phenolic compound profile in ~~fresh the unstored~~ and stored ~~of *Pluchea*~~ infusion ~~and at~~  
664 various steeping temperature. ~~The included~~ simple phenolic compounds ~~were detected~~  
665 ~~in *Pluchea* infusion includingsuch as~~ gallic acid, (+)-catechin, quercetin, myricetin,  
666 kaempferol, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-  
667 caffeoylquinic acid. The results of statistical analysis using a paired ~~T-t~~ test at  $\alpha \leq 0.05$   
668 showed that gallic acid and kaempferol of *Pluchea* infusion were insignificantly different  
669 at various steeping temperature and long storage. ~~Nevertheless, T~~he concentration of  
670 quercetin and 3,5-dicaffeoylquinic acid of *Pluchea* infusion was significantly different of  
671 two treatments except at 70 °C. The (+)-catechin concentration of *Pluchea* infusion was  
672 significantly different at 95 °C, but the myricetin was different concentration at 80 and 95  
673 °C. The 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid compounds from *Pluchea*  
674 infusion were significantly different at 60 °C, however the concentration of 3,4-  
675 dicaffeoylquinic acid was also significantly different at 80 and 95 °C. TPC, TTC and TFC  
676 of *Pluchea* infusion were significantly different at various steeping temperature and  
677 storage period. TPC and TTC significantly increased with increasing steeping  
678 temperature and long storage, but TFC significantly increased at various steeping  
679 temperature and significantly decreased at long storage. The bioactive compounds of  
680 *Pluchea* infusion influenced antioxidant activities (DPPH and FRAP) and antidiabetic  
681 activity (AA and GA). The DPPH was strongly and positively correlated with TPC and  
682 TTC, but it was strongly and negatively correlated with TFC, with coefficient  $r$  0.993,  
683 0.942, and -0.940, respectively. The correlated pattern between FRAP and bioactive  
684 contents of *Pluchea* infusion was similar to it between DPPH and bioactive contents. The  
685 correlated coefficient values ( $r$ ) between FRAP and TPC, TTC and TFC were 0.953, 0.948

686 and -0.826, respectively. The AA and GA were strongly and negatively correlated with  
687 TPC, TTC, DPPH and FRAP, but it was moderately and negatively significant correlated  
688 with TFC. Between the antioxidant activity of DPPH and FRAP and the antidiabetic  
689 activity of AA and GA of Pluchea infusion were strongly and positively correlated with  
690 correlation coefficient (r) values of 0.956 and 0.725, respectively.

691

#### 692 DATA AVAILABILITY

693 Table and figure used to support of this study were included in the article.

694

#### 695 CONFLICT OF INTEREST

696 The authors declare no conflict of interest.

697

#### 698 ACKNOWLEDGEMENTS

699 The authors would like to thank the he Ministry of Education and Culture of the Republic  
700 of Indonesia for fundamental research grant to higher education institutions in 2022

701

#### 702 REFERENCES

- 703 ACAR A, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food  
704 wastes:evaluation of temperature and time effects on quality parameters. Applied Food  
705 Res. 2(100087):1-8.
- 706 ALEIXANDRE A et al. 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -  
707 glucosidase and influence of reaction conditions. Food Chem. 372(131231):1-9.

**Commented [A34]:** Check for authors that may had been deleted in the paper due to revisions done.

708 ALI et al. 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile.  
709 Food Chem. 89(2005):27-36.

710 ALI A. et al. 2018. Impact of storage conditions on the stability of predominant phenolic  
711 constituents and antioxidant activity of dried *piper betle* extracts. Molecules.23(24):1-13.

712 AL-TEMIMI A, CHOUDHARY R. 2013. Determination of antioxidant activity in different  
713 kinds of plants *in vivo* and *in vitro* by using diverse technical methods. J Nut. Food Sci.  
714 3(1):1-6.

715 AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and  
716 methanol extract and fractions of *Gongronema latifolium* (Asclepidaceae) leaves in  
717 alloxan diabetic rats. J Applied Pharm. Sci. 01(09): 99-102.

718 ARSININGTYAS IS. et al. 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves  
719 of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product  
720 use. Natural Product Res. 28(17):1350-1353.

721 ARYAL S et al. 2019. Total phenolic content, flavonoid content and antioxidant of wild  
722 vegetables from Western Nepal. Plants. 8(96): 1-12.

723 AYELE DT, AKELE ML, MELESE A.T. 2022. Analysis of total phenolic contents,  
724 favonoids, antioxidant and antibacterial activities of *Croton macrostachyus* root extracts.  
725 BMC Chem. 16(30): 1-9.

726 BALIYAN S et al. 2022. Determination of antioxidants by DPPH radical scavenging  
727 activity and quantitative phytochemical analysis of *Ficus religiosa*. Molecules. 27(1326):  
728 1-19.

729 CASTIGLIONI S et al. 2015. Influence of steeping conditions (time, temperature, and  
730 particle size) on antioxidant properties and sensory attributes of some white and green  
731 teas. Intern. J. Food Sci. Nut. Online 1-7.

732 Chan EWC et al. 2022. *Pluchea indica*: An updated review of its botany, uses, bioactive  
733 compounds and pharmacological properties. Pharmaceutical Sciences Asia. 49(1): 77-  
734 85.

735 CHANDRA S et al. 2014. Assessment of total phenolic and flavonoid content, antioxidant  
736 properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit  
737 crops: A Comparative study. Hindawi Publishing Corporation Evidence-Based  
738 Complementary Alter. Med. 2014:1-9 (2014)

739 CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic, flavonoids,  
740 tannin and chlorophyll content of leaves of *Strobilanthes Kunthiana (Neelakurinji)*. J.Med.  
741 Plants Studies. 4(4):282-286.

742 DOBRINAS S, SOCEANU A, POPESCU V, POPOVICI IC, JITARIU D. 2021.  
743 Relationship between total phenolic content, antioxidant capacity, Fe and Cu content from  
744 tea plant samples at different brewing times. Processes. 9(1311):1-11.c

745 FERRARIS Z. 2023. *Pluchea indica* Less.iNaturalist.org.  
746 <https://www.gbif.org/occurrence/4022273660>.

747 FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional  
748 properties and visual appearance of Dampit Robusta coffee leaves tea. Advances in Food  
749 Sci. Sustain. Agric. Agro-industrial Engineering. 4(1):25-32.

750 FU L et al. 2017. Total phenolic contents and antioxidant capacities of herbal and tea  
751 infusions. Intern. J. Molecular Sci. 12:2112-2124.

Corresponding Author: paini@ukwms.ac.id

752 GAO MR et al. 2019. A theoretical and experimental study: the influence of different  
753 standards on the determination of total phenol content in the Folin–Ciocalteu assay. J.  
754 Food Measurement and Characteristic. 13:1349–1356.

755 HUANG D, OU B, PRIOR RL. 2005. The Chemistry behind antioxidant capacity assays.  
756 J. Agric. Food Chem. 53:1841-1856.

757 ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties  
758 of rosehip tea beverage: study by response surface methodology. J. Food Sci. Tech.  
759 54(11):3737–3743.

760 JAYANI NIE et al. 2022. Evaluation of stability and quality characteristics of moringa  
761 (*Moringa oleifera*) herbal tea during storage. Food Res. 6(4):399-406.

762 KIM JM et al. 2020. Effect of storage temperature on the antioxidant activity and catechins  
763 stability of Matcha (*Camellia sinensis*). Food Science and Biotechnology. 29(9):1261–  
764 1271.

765 KILIC et al. 2017. Antioxidant properties of some herbal teas (green tea, senna, corn silk,  
766 rosmery) brewed at different temperatures. Intern. J. Secondary Metabolite. 4(3):142-248  
767 Special Issue 1.

768 KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of  
769 extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves  
770 from different provenances in Thailand. Revista Brasileira de Farmacognosia. 28(2018):  
771 145–150.

772 KOWALSKA J et al. 2021. Influence of tea brewing parameters on the antioxidant  
773 potential of infusions and extracts depending on the degree of processing of the leaves  
774 of *Camellia sinensis*. Molecules.26(4773):1-17.

Corresponding Author: paini@ukwms.ac.id

775 KRUK J, ABOUL-ENAIN BH, DUCHNIK E, MARCHLEWICZ M. 2022. Antioxidative  
776 properties of phenolic compounds and their effect on oxidative stress induced by severe  
777 physical exercise. *J. Physiol. Sci.* 72(19):1-24.

778 KUMAR N, GOEL N. 2019. Phenolic acids: natural versatile molecules with promising  
779 therapeutic applications. *Biotechnology Reports.* 24(e00370):1-10.

780 LI K et al. 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the flavonoids  
781 rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity  
782 relationship of its eight flavonoids by a refined assign score method. *Chem. Central J.*  
783 12(82):1-11.

784 LIN YS. et al. 2020. Effects of storage time and temperature on antioxidants in juice from  
785 *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser. *Molecules.*  
786 25(3614): 1-12.

787 MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content  
788 and antioxidant activity test of avocado seed extract (*Persea Americana* Mill). *Jurnal MIPA*  
789 *Unsrat Online.* 1(1): 5-10. [https://media.neliti.com/media/publications/116214-ID-  
790 penentuan-kandungan-tanin-dan-uji-aktivi.pdf](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf) [In Bahasa Indonesia]

791 MARTINEZ-SOLIS J et al. 2022. Antidiabetic and toxicological effect of the tea infusion  
792 of summer collection from *Annona cherimola* Miller leaves. *Plants.* 11(3224):1-17.

793 MATHUR R, VIJAYVERGIA R. 2017. Determination of total flavonoid and phenol content  
794 in *Mimusops Elengi* Linn. *Intern. J. Pharm. Sci. Res.* 8(12): 5282-5285.

795 MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of  
796 sprouted and solid-state bioprocessed soybean. *Asia Pacific J. Clinical Nut.* 14(2): 145-  
797 152.

Corresponding Author: paini@ukwms.ac.id

798 MORAES-DE-SOUZA RA, OLDONI TLC, REGITANO-D'ARCE MAB, ALENCAR SM.  
799 2008. Antioxidant activity and phenolic composition of herbal infusions consumed in  
800 Brazil. *Cienc Tecnol. Aliment.* 6(1):41-47.

801 MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant  
802 activity: A Review. *Intern. J. Molecular Sci.* 22(3380):1-30.

803 NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus*  
804 *sabdariffa* L.): Effect of dying temperature and brewing conditions on total soluble solid,  
805 phenolic content, antioxidant capacity and sensory quality. *Beverages*.6(2):1-11 (2020).

806 NOREEN H. et al. 2017. Measurement of total phenolic content and antioxidant activity  
807 of aerial parts of medicinal plant *Coronopus didymus*. *Asian Pacific J. Tropical Med.* 10(8):  
808 792–801.

809 NURCHOLIS GW et al. 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity in-  
810 vitro and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum pictum*  
811 (L.). *Current Biochem.* 1(2): 58-65.

812 OH J et al. 2013. Antioxidant and antimicrobial activities of various leafy herbal teas. *Food*  
813 *Control.* 31(2013):403-409.

814 PROENCA C et al. 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an in vitro and in silico  
815 structure–activity relationship study. *J. Enzyme Inhibition Med. Chem.* 32(1): 1216–1228.

816 PUREWAL SS et al. 2022. Unraveling the effect of storage duration on antioxidant  
817 properties, physicochemical and sensorial parameters of ready to serve Kinnow-aml  
818 beverages. *Applied Food Res.* 2(1): 1-8.

819 RAHARJO D, HARYOTO. 2019. Antioxidant activity of mangrove *Sonneratia caseolaris*  
820 L. using the FRAP method. *ISETH.* 2019: 623-629.

Corresponding Author: paini@ukwms.ac.id



821 RAMPHINWA ML et al. 2023. Eco-physiological response of secondary metabolites of  
822 teas: review of quality attributes of herbal tea. *Frontiers in Sustain. Food Systems*.  
823 7(990334):1-14.

824 REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids.  
825 *Czech J. Food Sci.* 30(2):171-177.

826 RUAN J et al. 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea*  
827 *indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel  
828 column chromatography fractions. *Molecules*. 24(2784):1-20.

829 RUSITA YD et al. 2019. Comparison of tannin concentration on fresh and dried tea leaf  
830 (*Camelia sinensis* L.) extract using UV-visible spectrophotometric method. 1st  
831 International Conference of Health, Science & Technology (ICOHETECH) 2019 E-ISBN:  
832 978-623-92207-0-9. November 16<sup>th</sup>, 2019.LPPM Universitas Duta Bangsa Surakarta,  
833 Surakarta.

834 SANGEETHA R, VEDASREE N. 2012. In Vitro  $\alpha$ -Amylase inhibitory activity of the leaves  
835 of *Thespesia populnea*. *Intern. Scholarly Res.Network*. 2012: 1-4 (2012)

836 SHRAIM AM et al. 2021. Determination of total flavonoid content by aluminum chloride  
837 assay: A critical evaluation. *LWT Food Sci. Tech.* 150(111932):1-11.

838 SILVA-RAMIREZ KA et al. 2020. Effect of drying and steeping temperature on the  
839 phenolic content, antioxidant activity, aromatic compounds and sensory properties of  
840 *Culila polyantha* Benth. *Infusions. Processes*. 8(1378):2-20.

841 SRISOOK K et al. 2012. Antioxidant and anti-inflammatory activities of hot water extract  
842 from *Pluchea indica* Less. herbal tea. *J. Med. Plants Res.* 6(23):4077-4081.

Corresponding Author: paini@ukwms.ac.id

843 SURIYAPHAN O. 2014. Nutrition, health benefits and applications of *Pluchea indica* (L)  
844 Less leaves. Pharm. Sci. Asia. 41:1–10.

845 SU D et al. 2019. Impact of thermal processing and storage temperature on phenolic  
846 profile and antioxidant activity of different varieties of lychee juice. LWT-Food Tech.  
847 116(108578):1-9.

848 THANAJIRUSCHAYA P et al. 2010. Effect of storage ~~time~~[time period](#) and temperature on  
849 antioxidant components and properties of milled rice. Khon Kaen Univ. Res. J.15(9):843-  
850 851.

851 VONGSAK B et al. 2018. Comparison of active constituents, antioxidant capacity, and  
852 αglucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. Food  
853 Biosci. 25: 68–73.

854 WANG SC. et al. 2022. Effect of brewing conditions on polyphenols in the dark tea  
855 (*Camellia sinensis* L.) infusions: content, composition and antioxidant activities. Food  
856 Sci.Tech. 42(e36322):1-9.

857 WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica*  
858 Less as functional beverage in diabetic patients. ASSEHR. 98:164-167.

859 WIDYAWATI PS et al. 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract  
860 as antioxidant and antiwarmed over flavor (WOF) of duck meat. International Congress  
861 on Challenges of Biotechnological Research in Food and Health. November 15<sup>th</sup>  
862 2014.Agricultural Technology Faculty, Slamet Riyadi University, Surakarta.

863 WIDYAWATI PS et al. 2016. The physicochemical and antioxidant properties of *Pluchea*  
864 *indica* Less drink in tea bag packaging. IJFNS. 5: 13–120.

Corresponding Author: paini@ukwms.ac.id

865 WIDYAWATI PS et al. 2017. In vitro antioxidant capacities and antidiabetic properties of  
866 *Pluchea* leaves. IJPPS. 9(8): 203-208.

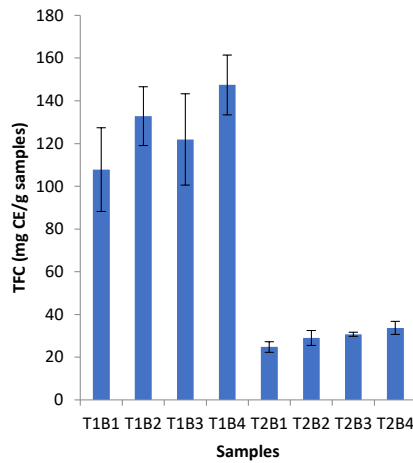
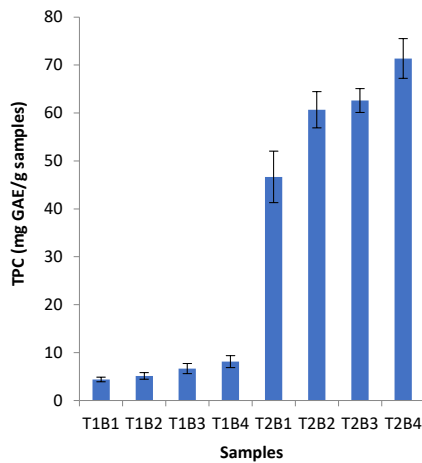
867 WIDYAWATI PS et al. 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica*  
868 Less beverage. CJFST.12(4): 125-139.

869 WIDYAWATI PS ET AL. 2022. The effect of  $\kappa$ -carrageenan proportion and hot water  
870 extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of stink  
871 lily (*Amorphophallus muelleri*) wet noodles. Molecules. 27(5062):1-16.

872 XU P, CHEN L, WANG Y. 2019. Effect of storage [time period](#) on antioxidant activity and  
873 inhibition on  $\alpha$ -amylase and  $\alpha$ -glucosidase of white tea. Food Sci. Nut. 7(2): 636-644.

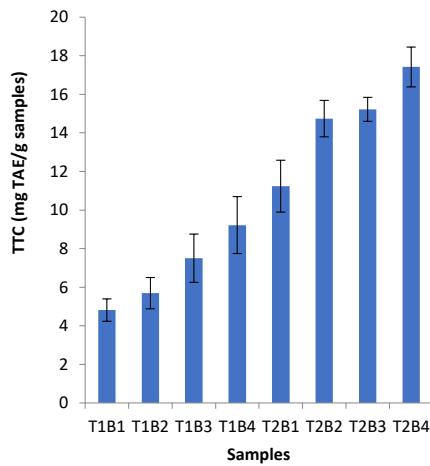
874 ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction [time period](#) on  
875 caffeine in coffee. Lantanida J. 6(2):103-202. [https://jurnal.ar-](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811)  
876 [raniry.ac.id/index.php/lantanida/article/view/3811](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811). [In Bahasa Indonesia]

877 ZHANG Y et al. 2021. Effect of storage conditions and [time period](#) on the polyphenol  
878 content of wheat flours. Processes. 9(248):1-11.



(a)

(b)



(c)

Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping temperature and storage time period (a) Total phenolic content (b) Total flavonoid content (c) Total tannin content. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-

Formatted: Left, Indent: Left: 0.5", Hanging: 0.69", Line spacing: single

888  
889  
890  
891  
892  
893  
894

stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$ .

Commented [A35]:

Table 1. Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperature and storage time period

Phenolic Compounds	Steeping Temperature (°C)	Mean±SD Un-stored	Mean±SD Stored	Mean difference ±SD	Sig (2-tailed)
Gallic Acid (µg/g samples)	60	0.2132±0.0027	0.2364±0.0015	0.0375±0.0175	0.2030
	70	0.2157±0.0013	0.2324±0.0214	0.0167±0.0227	0.4870
	80	0.2234±0.0122	0.2347±0.0078	0.0386±0.0264	0.2870
	95	0.2316±0.0104	0.2402±0.0169	0.0086±0.1990	0.8500
(+)-Catechin (µg/g samples)	60	0.3425±0.0110	0.5085±0.0111	-0.1576±0.0885	0.241
	70	0.3260±0.0265	0.5448±0.0006	-0.2188±0.0259	0.053
	80	0.3240±0.0222	0.5023±0.0773	-0.1451±0.0248	0.077
	95	0.4039±0.0320	0.5995±0.0372	-0.2049±0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756±0.1234	1.4762±0.0271	-1.2887±0.3222	0.111
	70	0.2587±0.0160	1.4245±0.2526	-1.1657±0.2695	0.103
	80	0.4175±0.0104	1.4570±0.0925	-1.0391±0.0841	0.036*
	95	0.8786±0.0434	2.6138±0.0695	-1.735±0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220±0.0268	0.6220±0.0706	-0.5999±0.9733	0.544
	70	0.1530±0.0511	1.0708±0.0289	-0.9177±0.0222	0.011*
	80	0.3666±0.0103	0.8629±0.0815	-0.1082±0.4462	0.790
	95	0.6559±0.0570	2.0230±0.0573	-1.4123±0.3203	0.101
Kaempferol (µg/g samples)	60	0.1394±0.0202	0.3675±0.0183	-0.3207±0.1122	0.154
	70	0.0514±0.0037	0.3726±0.0944	0.3213±0.0907	0.125
	80	0.3699±0.0924	0.7966±0.0366	-0.4267±0.2727	0.271
	95	0.5913±0.0239	0.9478±0.0287	-0.3565±0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103±0.0628	2.4863±0.0270	-1.8760±0.2074	0.050*
	70	0.6271±0.0099	2.3403±0.0325	-1.7131±0.3152	0.082
	80	0.7967±0.03060	2.6278±0.0211	-1.8311±0.0095	0.002*
	95	1.5386±0.0668	4.0211±0.0851	-2.4825±0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635±0.0628	0.9449±0.0501	-0.2814±0.4458	0.536
	70	0.6162±0.0099	0.9485±0.0794	-0.3323±0.0301	0.041*
	80	0.6601±0.0306	0.9099±0.0387	-0.2498±0.3127	0.461
	95	0.6642±0.0668	1.3156±0.0166	-0.6514±0.2666	0.179

Corresponding Author: paini@ukwms.ac.id

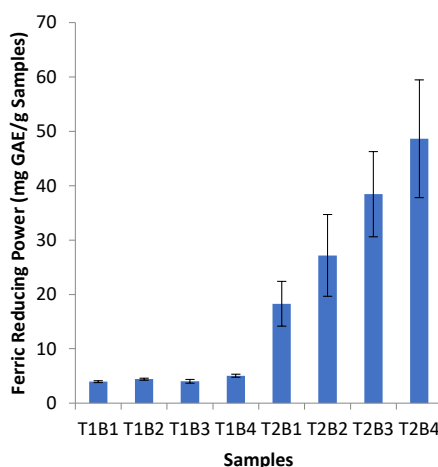
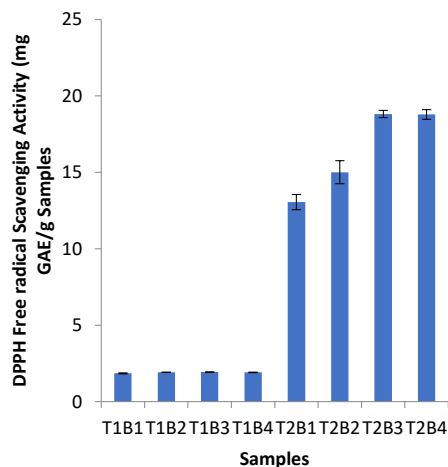
4,5-di-O-Caffeoylquinic acid ( $\mu\text{g/g}$ samples)	60	0.4906 $\pm$ 0.0060	1.1842 $\pm$ 0.0120	-0.6886 $\pm$ 0.2723	0.018*
	70	0.4807 $\pm$ 0.0034	1.0089 $\pm$ 0.0736	-0.5281 $\pm$ 0.0702	0.060
	80	0.5299 $\pm$ 0.0053	1.2382 $\pm$ 0.1435	-0.7082 $\pm$ 0.1489	0.094
	95	1.0018 $\pm$ 0.0526	1.3797 $\pm$ 0.2170	-0.3086 $\pm$ 0.3086	0.333

896 Note : Data were expressed as mean  $\pm$ standard deviation (n=2). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-  
897 steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C,  
898 stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped  
899 at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature,  
900 calculated using a paired T test at  $\alpha \leq 0.05$ . \*  $\alpha \leq 0.05$ .  
901

Formatted: Line spacing: single

Commented [A36]: Improve

902



(a)

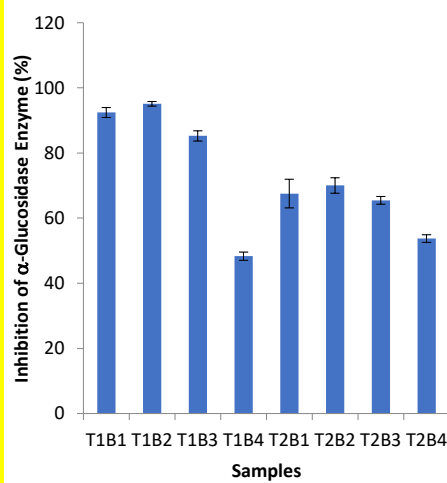
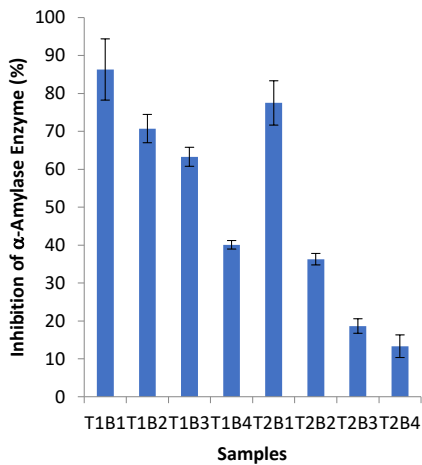
(b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage ~~time~~ time period (a) DPPH (b) FRAP. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T2B2-steeped at 70 °C, stored for 5 years; T2B3-steeped at 80 °C, stored for 5 years; T2B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$ .

Formatted: Justified, Indent: Left: 0.5", Hanging: 0.69", Line spacing: single

Commented [A37]: Improve





(a)

(b)

**Figure 3.** Antidiabetic activity of pluchea tea at different steeping temperature and storage ~~time~~ period (a)  $\alpha$ -amylase (b)  $\alpha$ -glucosidase. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$ .

Formatted: Indent: Left: 0.44", Hanging: 0.75"

Formatted: Justified, Indent: Left: 0.44", Hanging: 0.75", Line spacing: single, Tab stops: 1.19", Left

Commented [A38]: Improve

927  
928

Table 2. Pearson correlation coefficients between bioactive contents (TPC, TFC and TAC), antioxidant activity (DPPH and FRAP) and antidiabetic activity (AA and GA)\*

Formatted: Space After: 0 pt, Line spacing: single

	TPC	TFC	TTC	DPPH	FRAP	Alpha Glucosidase	Alpha Amylase
TPC	1						
TFC	-0.93589	1					
TTC	0.960028	-0.81321	1				
DPPH	0.992776	-0.93992	0.942273	1			
FRAP	0.953366	-0.82636	0.947778	0.956242	1		
Alpha Glucosidase	-0.55512	0.349873	-0.71534	-0.5272	-0.55947	1	
Alpha Amylase	-0.70842	0.429393	-0.8569	-0.69579	-0.80548	0.725161631	1

929  
930

Note: \*Correlation Ssignificant at the 0.05 level (2-tailed)



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**From Caesar Saloma/15 January 2024/ Editorial Decision/ MS 23-158R**

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Caesar Saloma <caesar.saloma@gmail.com>

Sat, Jan 20, 2024 at 5:30 PM

Dear Prof. Caesar Saloma, Ph.D

Greetings,

Attached I send the publication manuscript which I have revised according to the reviewer's suggestions and input.

Thank You

Regards

Paini SW

[Quoted text hidden]

---

 **Effect of Brewing Temperature and Storage Time on Antioxidant and Antidiabetic Properties of Pluchea Tea-Final Revision-2.docx**  
115K

1 **Effect of Steeping Temperature and Storage **Period** on the Bioactive Compounds,**  
2 **Antioxidant and Antidiabetic Activities of Infusion from Powdered Pluchea Indica**  
3 **Less**

4 Painsi Sri Widyawati<sup>1\*)</sup>, Yufita Ratnasari Wilianto<sup>2)</sup>

5 <sup>1)</sup>Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala  
6 Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

7 <sup>2)</sup>Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic  
8 University, Kalisari Street Number 1, Surabaya 60272, Indonesia

9 Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, Pluchea  
10 indica Less, storage **period**

11

12

13

14

15

16

17

18

19

20

21 ABSTRACT

22 This study was done to determine the effects of steeping temperature and storage period  
23 on the bioactive contents, antioxidant and antidiabetic activities of *Pluchea* leaf infusion.  
24 The research used a randomized block design with two factors, i.e., steeping temperature  
25 (T) and storage period (B). The *Pluchea* leaf blades were exposed to 4 steeping  
26 temperatures of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) °C with a storage period of 0 (B1)  
27 and 5 (B2) years resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1,  
28 T3B2, T4B1, T4B2). Statistical analysis using a paired t-test at  $\alpha \leq 0.05$  showed that  
29 treatments significantly affected the bioactive contents [(total phenol (TPC), total tannin  
30 (TTC), total flavonoid (TFC)], antioxidant [(DPPH scavenging activity (DPPH) and ferric  
31 reducing antioxidant power (FRAP)] potential and antidiabetic [( $\alpha$ -amylase (AA) and  $\alpha$ -  
32 glucosidase (GA) inhibition] properties of the *Pluchea* leaf infusion. TPC, TTC, DPPH,  
33 and FRAP significantly increased for the storage period and the steeping temperatures.  
34 Then, TFC decreased during the storage period but significantly increased at higher  
35 steeping temperatures. The GA and AA were significantly decreased for the storage  
36 period and the steeping temperatures. The antioxidant activities of the *Pluchea* infusion  
37 were significantly determined by TPC and TTC with correlated values (r) 0.9928 of DPPH  
38 and 0.9533 of FRAP. The antidiabetic activities of samples were not influenced by the  
39 TPC and TTC but were weakly and positively correlated with TFC,  $r=0.3499$  of GA and  
40 0.4294 of AA. The antioxidant activity of the *Pluchea* leaf infusion was inversely  
41 proportional to the antidiabetic activity. The simple phenolic compounds derived from  
42 *Pluchea* leaf infusion at different steeping temperatures and storage included gallic acid,

43 kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-caffeoylquinic acid, 3,5- di-O-  
44 caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid

45

## 46 INTRODUCTION

47 Pluchea herbal tea is a product of dried Pluchea leaf processing introduced by  
48 world people (Srisook et al., 2012; Widyawati et al., 2016) because of the efficacy of the  
49 active components in Pluchea leaves, as a herbal plant that has been widely used for  
50 traditional medicine and food (Chan et al., 2022). Pluchea leaves are composed of many  
51 nutrients and bioactive compounds useful to body health. The nutrient compositions in  
52 the Pluchea leaves include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates,  
53 calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive compounds are comprised, i.e.,  
54 chlorogenic acid, caffeic acid, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-  
55 caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-  
56 caffeoylquinic acid, quercetin, myricetin, kaempferol, total anthocyanin,  $\beta$ -carotene, and  
57 total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et  
58 al., 2022, Chan et al., 2022).

59 The steeping process of Pluchea leaves can be performed with fresh or dry leaves  
60 in infusion by hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et  
61 al., 2020; Jayani et al., 2022). In Asia, especially in Indonesia, people usually consume  
62 the Pluchea infusion by steeping 2 g of powdered Pluchea leaves in a tea bag in 100 mL  
63 of hot or boiling water. Widyawati et al. (2016) claimed that steeping 2 g of Pluchea leaf  
64 powder at 95 °C for 5 minutes exhibits total phenolic and total flavonoid contents, the  
65 ability to scavenge DPPH free radicals, and the capability to reduce ferric ions at 9.3 mg

66 gallic acid equivalent (GAE)/g sample, 22.0 mg gallic acid equivalent (GAE)/g sample,  
67 27.2 mg gallic acid equivalent (GAE)/g sample, and 10.2 mg gallic acid equivalent  
68 (GAE)/g sample, respectively. Werdani and Widyawati (2018) reported that drinking  
69 *Pluchea* leaf powder infusion in the morning and evening regularly (2 g/100 mL) can  
70 decline blood sugar levels.

71 The steeping of *Pluchea* herbal tea with hot water at 95 °C for 5 minutes certainly  
72 determines the stability and amount of extracted bioactive compounds that influence the  
73 biological activity especially antioxidant and antidiabetic activities. Silva-Ramirez et al.  
74 (2020) reported that the infusion process can influence the content and composition of  
75 the bioactive compounds and antioxidant activity of tea. Acar et al. (2022) stated that the  
76 infusion quality of herbal tea extract depends on several factors, i.e., storage and  
77 temperature. The polyphenol profile and antioxidant properties of herbal tea infusion  
78 decline with an increase in steeping/brewing and storage temperatures and longer  
79 exposure periods.

80 Several studies have mentioned the effect of steeping temperature on the  
81 bioactive compound contents and antioxidant activity, such as some white and green teas  
82 are effective with hot water at 90 °C for 7 min (Castiglioni et al., 2015), on roship tea is  
83 effective at infusion period around 6-8 min at temperatures of 84-86 °C (Ilyasoglu and  
84 Arpa, 2017), on the caffeine content extracted at the brewing temperature of coffee  
85 (Zarwinda and Sartika, 2018), and the high total phenol content and antioxidant activity  
86 of dark tea at 92 °C for 27 min (Wang et al., 2022). The study of the effect of steeping  
87 temperature on *Pluchea* infusion was carried out to afford information about the most

88 efficient preparation of powdered *Pluchea* leaves to get higher bioactive compounds,  
89 antioxidant, and antidiabetic activities.

90 Storage period tea usually for several months to years *Pluchea* herbal tea also  
91 affects the levels of the bioactive compounds and biological activity (Jayani et al., 2022).  
92 Tea or herbal tea is generally stored at ambient temperature and packed in a tea bag or  
93 aluminum foil standing pouch or a combination of both. Many researchers reported that  
94 the storage period decreases the bioactive compounds, antioxidant and antidiabetic  
95 activities, i.e., juice from *Momordica charantia* L. (Lin et al., 2020), dried *Piper bettle*  
96 extracts (Ali et al., 2018), white tea (Xu et al., 2019), kinnow-amlam beverages (Purewal et  
97 al., 2022), whole wheat flour (Zhang et al., 2021).

98 Therefore, this research studied the effect of steeping temperature and storage  
99 period on the bioactive compounds [total phenolic content (TPC), total flavonoid content  
100 (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging activity  
101 (DPPH), ferric reducing antioxidant power (FRAP)], and antidiabetic activities [( $\alpha$ -amylase  
102 (AA) and  $\alpha$ -glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea* leaves  
103 and on the phenolic compound profile.

104

## 105 MATERIALS AND METHODS

### 106 RAW MATERIALS AND PREPARATION

107 The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya,  
108 East Java, Indonesia. The *Pluchea* plants were included in the *Asteraceae* family with  
109 specifications according to the GBIF taxon ID number database:3132728 (Ferraris, 2023).  
110 *Pluchea* leaves at 1-6 levels of each branch from the shoot were collected, sorted,



111 washed, and dried to get a moisture content of around  $11.16 \pm 0.09$  % dry base  
112 (Widyawati et al., 2022). The dried *Pluchea* leaves were pulverized to a 45-mesh size  
113 powder. The *Pluchea* leaf powder was dried in an oven (Binder, Merck KGaA, Darmstadt,  
114 Germany) at 120 °C for 10 min to reduce microbial organisms. Then, 2 g of the powder  
115 was packed into a paper filter infusion bag. Packed samples were stored for 0 (un-stored)  
116 and 5 (stored) years in an aluminum standing pouch before analysis.

117 In the research, the samples of *Pluchea* herbal tea in a tea bag that was un-stored  
118 [0 (B1) year] and stored [5 (B2)] years, was steeped with 100 mL hot water at 60 (T1), 70  
119 (T2), 80 (T3), and 95 (T4) °C for 5 min, then immediately were analyzed for the bioactive  
120 compounds [(total phenol (TPC), total tannin (TTC), total flavonoid (TFC)], antioxidant  
121 potential [(DPPH scavenging activity (DPPH) and ferric reducing antioxidant power  
122 (FRAP)] potential and antidiabetic activities [( $\alpha$ -amylase (AA) and  $\alpha$ -glucosidase (GA)  
123 inhibition)]. The rest of the samples were stored at room temperature and analyzed after  
124 5 years.

125

## 126 REAGENTS

127 The reagents used in the analyses include 2,2-diphenyl-1-picrylhydrazyl (DPPH),  
128 sodium carbonate, gallic acid,  $\alpha$ -amylase,  $\alpha$ -glucosidase, pNPG (p-nitrophenyl- $\alpha$ -  
129 glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-O-caffeoylquinic  
130 acid, 4,5-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and (+)-catechin were  
131 purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, Folin–Ciocalteu’s Phenol,  
132 sodium nitric, aluminum chloride, ferric chloride, sodium dihydrogen phosphate, sodium  
133 phosphate, potassium ferricyanide, starch, acetic acid, and sodium hydroxide were

134 purchased from Merck (Kenilworth, NJ, USA). All reagents used were of analytical grade  
135 except for distilled water which was purchased from PT Aqua Industry Surabaya.

136

## 137 METHODOLOGY

### 138 ANALYSIS OF THE BIOACTIVE COMPOUNDS

#### 139 TOTAL PHENOLIC CONTENT ANALYSIS

140 Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the  
141 technique by Gao et al. (2019). About 10  $\mu$ L *Pluchea* infusion and 1 mL Folin-Ciocalteu's  
142 phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. Then  
143 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5 % was added and filled up to 10 mL volume with distilled water. The  
144 blue color intensity of the solution was measured in the spectrophotometer UV-Vis 1800  
145 (Shimadzu, Japan) at  $\lambda$  760 nm with gallic acid as the reference standard. The total  
146 phenolic content was calculated using the formula:  $y=0.00009x+0.008$  with  $R^2=0.9941$ .  
147 The results were expressed as mg gallic acid equivalent (GAE)/g samples.

148

#### 149 TOTAL FLAVONOID CONTENT ASSAY

150 The total flavonoid content (TFC) of the samples was measured based on the  
151 reaction between AlCl<sub>3</sub> and NaNO<sub>2</sub> with the aromatic ring of flavonoid compounds,  
152 especially flavonol and flavon (Shraim et al., 2021). The reaction between AlCl<sub>3</sub> and  
153 flavonoid compounds resulted in a yellow solution. About 30  $\mu$ L *Pluchea* infusion was  
154 mixed with 0.3 mL NaNO<sub>2</sub> 5 % in 10 mL volumetric flash and incubated for 5 min. The  
155 mixture was added with 0.3 mL AlCl<sub>3</sub> 10 % for 5 min. Then, 2 mL NaOH 1 M and distilled  
156 water were added to 10 mL volume. Then, the red solution was produced after NaOH

157 solution addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis  
158 1800, Shimadzu, Japan) at  $\lambda$  510 nm with (+)-catechin as the reference standard  
159 compound, and the results were expressed as mg catechin equivalents (CE)/g samples  
160 using the formula:  $y=0.00008x-0.0023$  with  $R^2= 0.9980$ .

161

## 162 TOTAL TANNIN CONTENT ANALYSIS

163 Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method  
164 (Chandran and Indira, 2016). Approximately 10  $\mu$ L *Pluchea* infusion was added 1 mL  
165 Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flask and incubated for 5 min.  
166 Then, the mixture was added with 2 mL  $\text{Na}_2\text{CO}_3$  7.5 % and filled up to 10 mL volume with  
167 distilled water. The blue dark color solution was measured UV-Vis spectrophotometer  
168 1800 (Shimadzu, Japan) at  $\lambda$  760 nm with tannic acid as the reference standard.  
169 Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/g samples using  
170 the formula:  $y=0.00009x+0.0021$  with  $R^2=0.9993$

171

## 172 ANALYSIS OF THE ANTIOXIDANT POTENTIAL

### 173 DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

174 The DPPH free radical scavenging activity (DPPH) was measured by the  
175 spectrophotometric method (Widyawati et al., 2017) to determine the ability of the  
176 phytochemicals in the *Pluchea* leaf infusion to donate hydrogen atoms to the nitrogen  
177 atom in DPPH resulting in the formation of DPPH-H compound exhibiting a yellow-colored  
178 solution. About 25  $\mu$ L *Pluchea* leaf infusion was poured into the reaction tube into which  
179 was added 3 mL DPPH solution (4 mg/100 mL). After incubation for 15 min in a dark

180 room, the absorbance was measured by a spectrophotometer (Spectrophotometer UV-  
181 Vis 1800, Shimadzu, Japan) at  $\lambda$ . 517 nm. The reference standard compound was gallic  
182 acid and the results of the analysis were expressed as mg gallic acid equivalents (GAE)/g  
183 samples that were calculated using the formula:  $y=0.146x+1.7896$  with  $R^2=0.9975$ .

184

## 185 FERRIC REDUCING POWER ANALYSIS

186 Ferric-reducing power (FRAP) was determined following the method used by  
187 Widyawati et al. (2014) method. Approximately 10  $\mu$ L of samples were added to 2.5 mL  
188 phosphate buffer pH 6.6 and 2.5 mL of 1% potassium ferricyanide in the reaction tube.  
189 Then mixture was shaken and incubated for 20 min at 50 °C. Finally, 2.5 mL chloroacetic  
190 acid 10% (w/v) was added. Into the 2.5 mL supernatant was added 2.5 mL distilled water,  
191 0.5 mL ferric chloride 0.1% w/v, and incubated for 10 min. The potency of the samples  
192 reducing iron (III) to iron (II) ion was indicated by the intensity of blue color formed that  
193 was measured using UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800,  
194 Shimadzu, Japan) at  $\lambda$  700 nm. The intensity of the blue color indicated a higher reducing  
195 capacity. The reducing power expressed as mg gallic acid equivalent (GAE)/g samples  
196 was calculated using the formula:  $y=0.0002x+0,0256$  with  $R^2=0,9906$ .

197

## 198 ANALYSIS OF THE ANTIDIABETIC PROPERTIES

### 199 $\alpha$ -AMYLASE ENZYME INHIBITION CAPACITY ASSAY

200 In vitro, inhibition of  $\alpha$ -amylase enzyme (AA) followed the procedure as described  
201 by Widyawati et al. (2020). Each 500  $\mu$ L of samples, was mixed with starch 1 % (w/v) and  
202 sodium acetate buffer pH 5 into a 250  $\mu$ L of the mixture was added an  $\alpha$ -amylase solution

203 (0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in 50 mL of 0.2 M sodium acetate  
204 pH 5. The mixture was shaken into which was and added 2 mL sodium hydroxide 1M.  
205 Before the analysis, this mixture was incubated at 37 °C for 10 min. Then, the capacity of  
206 the α-amylase enzyme to hydrolyze the starch to release glucose was measured by UV-  
207 Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ 540 nm.  
208 The inhibition percentage of α-amylase was assessed using the formula:  $(ACb - Aca) -$   
209  $(As - Ab) (ACb - Aca) \times 100 \%$ . Where ACb is the absorbance of 100 % enzyme activity  
210 (solvent with the enzyme), Aca is the absorbance of 0 % enzyme activity (solvent without  
211 the enzyme), As is the absorbance of the test sample with enzyme, Ab is the absorbance  
212 of test sample without enzyme.

213

#### 214 α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

215 The analysis of the α-glycosidase inhibitor activity (GA) was done by Widyawati et  
216 al. (2020) method with slight modification. About 150 μL samples containing 100 μL  
217 *Pluchea* infusion and 50 μL pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH 7)  
218 were reacted with 50 μL α-glycosidase 2 mM (0.0833 unit/mL), and then the mixture was  
219 incubated at 37 °C for 15 min. The reaction was stopped with the addition of 1000 μL  
220 sodium carbonate 0.2 M. The amount of these enzymes that didn't react with bioactive  
221 compounds of *Pluchea* infusion hydrolyzed p-nitrophenyl-α-D-glucopyranoside (pNPG)  
222 as a substrate to result in p-nitrophenol. The inhibition activity of the *Pluchea* infusion was  
223 measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu,  
224 Japan) at λ 405 nm. The inhibition percentage of α-glycosidase was calculated using the  
225 formula:  $(ACb - Aca) - (As - Ab) (ACb - Aca) \times 100 \%$ . Where ACb is the absorbance

226 of 100 % enzyme activity (solvent with enzyme), A<sub>Ca</sub> is the absorbance of 0 % enzyme  
227 activity (solvent without enzyme), A<sub>s</sub> is the absorbance of test sample with enzyme, A<sub>b</sub>  
228 is the absorbance of test sample without enzyme.

229

## 230 ANALYSIS OF PHENOLICS

231 The phenolic compounds of the samples were analyzed by HPLC based on **the**  
232 Kongkiatpaiboon et al. (2018) method with modifications. Each *Pluchea* infusion was  
233 sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter  
234 syringe (Whatmann, 0.2 µm, NYL). About 20 µL of **the** sample was injected in an HPLC  
235 (LC20AD series, Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC  
236 LC-20AD pump, CTO-30A column oven, CBM-20A/20 Alite system controller, and SPD-  
237 20A/20 AV UV-Vis detector. Separation of phenolic compounds in samples was carried  
238 out using a Shim-pack VP-ODS C18 column (ID 5 µm × 50 mm × 4.6 mm) with a GVP-  
239 ODS Cartridge guard column (2 pieces) (ID 10 mm × 4.6 mm). **The mobile phase used**  
240 consisted of a solution of (A) 0.5 % acetic acid in water and (B) absolute methanol.  
241 Analysis was carried out using a gradient system in the following order: initial conditions  
242 of 10 % B in A to 50 % B in A were maintained for 40 minutes; then 100 % B was  
243 maintained for 20 minutes. Next the column was re-equilibrated with 10 % B in A  
244 maintained for 10 minutes before analysis of the next sample. The sample flow rate was  
245 set at 1.0 ml/min with a controlled temperature **of 40 °C**. Detection was used at a  
246 wavelength of 280 nm. The reference standard used were gallic acid, (+)-catechin,  
247 myricetin, quercetin, kaempferol, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and

248 4,5-dicaffeoylquinic acid. All of the reference standards were dissolved in distilled water  
249 and prepared similar to the samples before being injected in HPLC.

250

## 251 EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

252 The research design used a randomized block design with two factors, i.e., the  
253 steeping temperature (T) and the storage period. *Pluchea* leaf blades were subjected to  
254 4 steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95 °C (T4), and  
255 the storage period of 0 year /fresh (B1), and 5 year/stored (B2) resulting in 8 treatment  
256 combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2).

257 The data analysis of samples was repeated for six periods. The data were  
258 analyzed by ANOVA at  $\alpha \leq 0.05$ , and continued analysis using a paired t-test at  $\alpha \leq 0.05$   
259 that was expressed as the mean  $\pm$  SD. The analysis used SPSS 17.0 software (SPSS  
260 Inc., Chicago, IL, USA).

261

## 262 RESULTS AND DISCUSSIONS

263 *Pluchea* leaf infusion is produced by young *Pluchea* leaf from 1-6 levels on each  
264 branch of the shoot (Widyawati et al., 2022), that is steeped at 95 °C for 5 min, has many  
265 biological activities, such as antioxidant activity (Widyawati et al., 2016), antidiabetic  
266 activity (Werdani and Widyawati, 2018), anti-inflammatory (Srisook et al., 2015). The  
267 chemical constituents in *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols,  
268 cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2  
269 g/100 mL steeping *Pluchea* tea has total phenolic content 9.3 mg gallic acid equivalents  
270 (GAE)/g samples, total flavonoid content 22.0 mg catechin equivalents (CE)/g samples,

271 DPPH free radical scavenging activity 27.2 mg gallic acid equivalents (GAE)/g samples,  
272 and ferric reducing power 10.2 mg gallic acid equivalents (GAE)/g samples (Widyawati et  
273 al., 2016). Previous research has **informed the composition** of phytochemical compounds  
274 in *Pluchea* leaves, such as phenolic acids such as chlorogenic acids, caffeic acids, 3-O-  
275 caffeoylquinic acids, 4-O-caffeoylquinic acids, 5-O-caffeoylquinic acids, 3,4-di-O-  
276 caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic acids; total  
277 flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin;  $\beta$ -carotene; and  
278 total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Chan et al.,  
279 2022; Widyawati et al., 2022). **The** presence of phytochemical compounds in herbal  
280 **products was** influenced by environmental factors, i.e., temperature, light exposure,  
281 oxygen level, pH, and moisture. The structure of phytochemical compounds in herbal tea  
282 is very sensitive **to** the surrounding changes. The effect arising from these changes  
283 causes the structure of the phytochemical molecule to be degraded to produce smaller  
284 size molecules or to combine to produce larger size molecules (Ali et al., 2018; Jayani et  
285 al. 2022, Ramphinwa et al., 2023).

286

## 287 **BIOACTIVE COMPOUNDS**

288

### **Phenolics Compounds**

289 The bioactive compounds are active compounds in plants that are essential to  
290 **protecting a body's** health (Nguyen and Chuyen, 2020). These compounds usually have  
291 many biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer,  
292 antimicrobial, antibacterial, anti-cholesterol, and so on (Suriyaphan, 2014; Acar et al.,  
293 2022). Phenolic compounds have potential redox properties that can scavenge free



294 radicals that can cause many chronic diseases (Noreen et al., 2017; Aryal et al., 2019;  
295 Acar et al., 2022).

296 The total phenolic content (TPC) of *Pluchea* infusion at different steeping  
297 temperatures and storage periods generally significantly increased with increasing  
298 steeping temperature and storage period based on paired t-test at  $\alpha \leq 0.05$  (Figure 1a).  
299 Steeped and stored infusion had significantly higher amounts of phenolic compounds  
300 than the samples that were steeped and un-stored. Further, the highest total phenolic  
301 content was observed in samples infused at 95 °C and stored for 5 years (at 71.38±4.14  
302 mg GAE/g sample) while the lowest was measured in the un-stored samples and infused  
303 at 60 °C (at 4.39±0.49 mg GAE/g sample). The phenolic content of stored samples that  
304 were steeped only at 60 and 95 °C showed a significant increase in their phenolic content.  
305 This implies that the steeping temperature and the storage periods significantly resulted  
306 in the high amounts of phenolic compounds in the infusions. Results also indicated that  
307 phenolic compounds were generally greater in the infusion at high steeping temperatures  
308 and long storage periods. This could have been expected that the steeping temperature  
309 and storage period could cause the process of degradation, oxidation, and  
310 leaching/release of phenolic compounds. Phenolic compounds are water soluble and thus  
311 soaking in hot water for a certain period of the period as in steeping causes the migration  
312 process of more phenolic compounds to the water because of the exposure of phenolic  
313 compounds and water Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al. (2022).  
314 Su et al. (2019) reported that temperature treatment can stimulate the release of phenolic  
315 compounds and increase antioxidant activity of lychee juice stored at different  
316 temperatures of 4 and 45 °C and different long storage (fresh and 72 hours).

317 Temperature treatment degrades (or hydrolyzes) the hydrogen bond between  
318 phenolic compounds and proteins increasing phenolic compounds when exposed to  
319 higher temperatures. The phenomena were supported by Ali et al. (2018); Jayani et al.  
320 (2022) and Ramphinwa et al. (2023). Zhang et al. (2021) reported that phenolic  
321 compounds present in plants are not completely stable, but are easily degraded during  
322 storage after harvest. Reblova (2012) claimed that antioxidant compounds can be slowly  
323 degraded with increasing temperature. Fibrianto et al. (2021) also stated that the brewing  
324 temperature affects the extracted antioxidant compounds, such as alkaloids, catechins,  
325 and tannins. Thus, there is an assumption that temperature and storage caused the  
326 degradation, oxidation, and hydrolysis of the phenolic compounds period resulting in the  
327 increased amount of the phenolic compounds at higher steeping temperature and longer  
328 storage period.

329 Simple phenolic compounds are identified in steeped and stored. *Pluchea* leaf  
330 infusion included gallic acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-  
331 O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic acids  
332 was showed in Table 1. The treatment effects using a t-test at  $\alpha \leq 0.05$  showed that gallic  
333 acid and kaempferol of *Pluchea* infusion were insignificantly different at various steeping  
334 temperatures and long storage periods. The concentration of quercetin and 3,5-di-O-  
335 caffeoylquinic acid of the un-stored and stored. *Pluchea* infusion was significantly different  
336 from the rest of the samples at 70 °C while (+)-catechin concentration of *Pluchea* infusion  
337 was only significantly different at 95 °C. The myricetin content was significantly different  
338 at 80 and 95 °C. The 3,4-di-O-caffeoylquinic acid content showed significant difference

339 at 60, 80, and 95 °C while 4,5-di-O-caffeoylquinic acid content was only significantly  
340 different at 60 °C.

341 Results further showed that gallic acids and kaempferol were relatively stable as  
342 reflected by the insignificant changes when exposed to the different steeping temperature  
343 and storage period. Myricetin, (+)-catechin, and 3,4-di-O-caffeoylquinic acid showed a  
344 drastic increase at higher steeping temperatures and longer storage periods implying that  
345 these compounds tended to be relatively labile. Quercetin, 3,5-di-O-caffeoylquinic acid,  
346 and 4,5-di-O-caffeoylquinic acid underwent moderate changes. Therefore, myricetin, (+)-  
347 catechin, and 3,4-di-O-caffeoylquinic acid were easier to dissolve or degrade to form  
348 simple phenolic acids at higher temperatures and storage periods (Su et al. (2019, Ali et  
349 al. (2018); Jayani et al. (2022); Ramphinwa et al. (2023), and Zhang et al. (2021).  
350 Degradable polyphenol compounds have a simple structure and free hydroxyl groups that  
351 can react with Folin-Ciocalteu's Phenol reagent, resulting complex blue solution that can  
352 detected as total phenolic content.

### 353 Flavonoid Content (TFC)

354 Flavonoids are the major phenolic compounds that have potential chemical and  
355 biological activities, such as radical scavenging and antimicrobial activities (Ayele et al.,  
356 2022; Chandra et al., 2014) that can protect the human body from the oxidative stress  
357 caused by many degenerative diseases, especially cancer, cardiovascular problems and  
358 aging (Mathur and Vijayvergia, 2017). The total flavonoid content of steeped *Pluchea*  
359 infusion decreased with a longer storage period. Un-stored samples exhibited higher  
360 flavonoid content than the stored samples. The statistical analysis using a paired t-test at  
361  $\alpha= 0.05$  showed that the total flavonoid content of *Pluchea* infusion was significantly

362 different between the steeped un-stored and steeped stored samples (Figure 1b). The  
363 highest total flavonoid content was significantly lower in the stored samples than those of  
364 the un-stored samples implying that the increase in the flavonoid content of the infusion  
365 was affected primarily by the steeping temperature.

#### 366 Tannin Content (TTC)

367 Tannins are bioactive compounds that provide properties, such as astringent, anti-  
368 diarrheal, antibacterial and antioxidant (Malangngi et al., 2012). Generally, results  
369 indicated that the total tannin content of *Pluchea* infusion significantly increased with  
370 increasing steeping temperature and storage period (Figure 1c). Among, the un-stored  
371 steeped samples, the tannin content was significantly lowest in samples infused at 60 °C  
372 about  $4.81 \pm 0.58$  to  $17.42 \pm 1.04$  mg TAE/g samples which were significantly different lower  
373 from that of the lowest tannin content of the stored samples. Among the stored and  
374 steeped samples, the highest tannin content was observed at samples steeped at 95 °C  
375 about  $17.42 \pm 1.04$  mg TAE/g samples, and was significantly different from that of the  
376 highest tannin content of the un-stored steeped samples at 95 °C about  $9.22 \pm 1.48$  mg  
377 TAE/g samples. Indicating that the tannin content was primarily affected by a longer  
378 storage period than high steeping temperatures. The condensation of catechins to tannins  
379 is a dominant process occurring in tea leaves that is accelerated during the maceration  
380 of raw tea leaves (Kowalska et al., 2021) and could have contributed to the observed  
381 increase in the tannin content in the treated samples.

382 However, high temperatures and long storage periods can cause the degradation  
383 of tannins to catechins. Rusita et al. (2019) emphasized that tannins are polar

384 thermostable complex compounds that are resistant to heating, indicating that even with  
385 exposure to high temperatures, the tannin remained high in the treated samples period.

386

### 387 ANTIOXIDANT ACTIVITY

388 Antioxidant activity is the capability of compounds to inhibit the oxidation of  
389 macromolecules from biological targets that are involved in oxidative chain reactions (Ali  
390 et al., 2005; Oh et al., 2013). The antioxidant activity assay was done in this research  
391 using DPPH Free Radical Scavenging Activity (DPPH) and ferric reducing power (FRAP)  
392 methods. The phenolic compounds are active antioxidants that have antioxidant  
393 capability depending on their redox properties. The structure of phenolic compounds  
394 determines the effectivity to donate hydrogen atoms which is negatively correlated with  
395 the O-H phenolic bond strength. The higher antioxidant power of phenolic compounds is  
396 caused by the weaker O-H phenolic bond (Kruk et al., 2022). The mechanism of phenolic  
397 compounds as antioxidants depends on their ability to donate hydrogen atoms and  
398 transfer electrons, and as reducing agents and singlet oxygen quenchers (Ali et al., 2005;  
399 Huang et al. 2005).

### 400 DPPH Free Radical Scavenging Activity

401 DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate  
402 antioxidant activity because this method is simple and is suitable to measure the donating  
403 hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of  
404 DPPH to change to a yellow color (Munteanu and Apetrei, 2021; Baliyan et al., 2022).  
405 Figure 2a. shows that the free radical scavenging properties of the stored and steeped  
406 samples were significantly higher than the un-stored steeped samples. It can also be

407 observed that the free radical scavenging property was significantly different among the  
408 stored and steeped samples but insignificant among the un-stored and steeped sample  
409 period. *Pluchea* infusion stored at room temperature for 5 years resulted in high free  
410 radical scavenging activity by more than 10%. Steeping at higher temperatures  
411 significantly increased the DPPH free radical scavenging activity in stored *Pluchea*  
412 infusion by around 15 to 25 %. This implies that the higher free radical scavenging  
413 property was primarily affected by the storage period than the steeping temperature.  
414 During the storage process, it is possible to form complex phenolic compounds which  
415 provide a high ability to scavenge free radicals (Thanajiruschaya et al., 2010).

416 The scavenging activity of the samples was strongly and positively correlated with  
417 total phenol and tannin contents, but inversely with total flavonoid levels. The study also  
418 demonstrated that longer storage periods and higher infusion temperatures produced  
419 many simple phenolic compounds with free hydroxyl groups capable of donating  
420 hydrogen atoms to DPPH free radicals. Many phenolic acids, such as gallic acids, (+)-  
421 catechins, myricetins, kaempferols, quercetins, 3,5-di-O-caffeoylquinic acids, 3,4-di-O-  
422 caffeoylquinic acids, 4,5-di-O-caffeoylquinic acids have established potential antioxidant  
423 activity (Kumar and Goel, 2019) (Table 1). Kruk et al (2022) stated that the capability of  
424 phenolic compounds to donor hydrogen atoms depends on chemical structure, number  
425 and position of hydroxyl groups attached to a benzene ring, a double bond between C2  
426 and C3 rings, and a carbonyl group (C=O) on the C ring at C4. The effectivity of  
427 antioxidant compound donor hydrogen atoms is determined by O-H bond dissociation  
428 energy.

429 The free radical scavenging property observed in the study was not inconsistent  
430 with the results of the study by Moraes-de-Souza et al. (2008). The research shows that  
431 the total phenolic content of herbal infusion is lowly correlated with DPPH free radical  
432 scavenging activity. However, Dobrinás et al. (2021) stated that the total phenolic content  
433 of tea infusion is positively and significantly correlated with the free radical scavenging  
434 property of tea infusion.

435

#### 436 Ferric Reducing Antioxidant Power (FRAP)

437 FRAP is an analysis of the antioxidant power of the phytochemical compounds  
438 that is based on the ability of antioxidant compounds to reduce iron ions of potassium  
439 ferricyanide ( $\text{Fe}^{3+}$ ) to potassium ferrocyanide ( $\text{Fe}^{2+}$ ). Potassium ferrocyanide reacts with  
440 ferric chloride to form a ferric-ferrous complex and results green color solution (Widyawati  
441 et al., 2017; Raharjo and Haryoto, 2019).

442 The results showed that the ferric-reducing antioxidant power (FRAP) increased  
443 at higher steeping temperatures and longer storage periods. The lowest FRAP was  
444 observed in the un-stored samples which were steeped at 60 °C at  $3.95 \pm 0.17$  mg gallic  
445 acid equivalents (GAE)/g samples, and the highest was exhibited in *Pluchea* infusion  
446 which was stored for 5 years at 95 °C at  $48.63 \pm 10.83$  mg gallic acid equivalents (GAE)/g  
447 samples (Figure 2b). FRAP increased significantly as the steeping temperature was  
448 increased. FRAP of the samples stored for 5 years was also significantly higher than the  
449 un-stored samples at  $\alpha \leq 0.05$ .

450 This is in contrast with the study on the antioxidant activity of DPPH and FRAP of  
451 matcha. The longer storage period reduces the levels of catechin content due to the

452 catechins, such as epigallocatechin gallat (EGCG), epicatechin gallat (ECG),  
453 epigallocatechin (EGC), and epicatechin (EC) which are bioactive compounds that have  
454 high antioxidant activity (Kim et al. 2020). The ferric-reducing capability of *Pluchea* could  
455 have been due to the presence of simple phenolic acids that can transfer electrons from  
456 their free hydroxyl groups. The FRAP of *Pluchea* infusion was strongly and positively  
457 significantly correlated with the DPPH, TPC, and TTC, but inversely to TFC.

458

## 459 ANTIDIABETIC ACTIVITY

### 460 $\alpha$ -Amylase enzyme inhibition activity (AA)

461 Antidiabetic activity is a measure of the potency of phenolic compounds to regulate  
462 the uptake of glucose by the cells from the blood through the mediation of  $\alpha$ -digestive  
463 enzymes i.e.,  $\alpha$ -amylase and  $\alpha$ -glucosidase, which are involved in the control of dietary  
464 carbohydrate digestion and release in the postprandial blood glucose in human body (Fu  
465 et al., 2017). The phenolic compounds can bind with the protein component of  $\alpha$ -amylase  
466 and  $\alpha$ -glucosidase enzymes (Martinez-Solis et al., 2022) resulting in the reduced activity  
467 of the enzymes. The results showed that lower steeping *Pluchea* leaf infusion was able  
468 to inhibit the action of the  $\alpha$ -amylase enzymes (Figure 3a). The *Pluchea* infusion exhibited  
469 a good  $\alpha$ -amylase enzyme inhibition activity, more than 50 % and even almost 100 % in  
470 un-stored *Pluchea* infusion steeped at 60, 70, and 80 °C with the highest at 60 °C, and in  
471 stored *Pluchea* leaf infusion which was steeped at 60 °C. The stored *Pluchea* leaf infusion  
472 steeped at 70, 80, and 95 °C for 5 minutes had lower enzyme inhibition activity of less  
473 than 50 % with the lowest at 95 °C around 13 %. Widyawati et al. (2017) found that the  
474 ability to inhibit the  $\alpha$ -amylase enzyme in un-stored *Pluchea* infusion steeped at 95 °C for



475 5 minutes was also low at 28.79 %. Increasing the steeping temperature and storage  
476 period reduced the ability of the phytochemicals in the *Pluchea* infusion to inhibit the  $\alpha$ -  
477 amylase enzyme activity period. Table 2 further shows that the AA of *Pluchea* infusion  
478 was strongly and negatively significantly correlated with TPC, TTC, DPPH, and FRAP,  
479 but it was weakly and positively significantly correlated with TFC.

480 This inhibitory activity was thought to be contributed by other bioactive compounds,  
481 besides phenolics which are sensitive to steeping temperature and storage period. Li et  
482 al. (2018) stated that there are flavonoid compounds that contribute to the ability to inhibit  
483 the  $\alpha$ -amylase enzyme. Akah et al. (2011) reported that phytochemical compounds, such  
484 as terpenoids, saponins, flavonoids, glycosides/carbohydrates, and alkaloids are good  
485 antidiabetic metabolites or  $\alpha$ -amylase enzyme activity inhibitors. Sangeetha and  
486 Vedesree (2012) explained, that the ability to inhibit the  $\alpha$ -amylase enzyme was  
487 determined by the content of the phenolic compound and protein. The  $\alpha$ -amylase inhibitor  
488 enzyme present in herbal infusion may be proteinaceous or nonproteinaceous in nature.  
489 It means that the  $\alpha$ -amylase enzyme inhibitory activity was correlated with their protein  
490 and phenolic compounds. Aleixandre et al. (2022) stated that phenolic acids have  
491 inhibition activity to  $\alpha$ -amylase enzyme depending on their structures. Besides that, the  
492 capability of phenolic acids to inhibit  $\alpha$ -amylase enzyme was determined by low half-  
493 maximum inhibitory concentration (IC<sub>50</sub>). There are C=C double bonds conjugated with a  
494 carbonyl group of phenolic structures that stabilize the binding forces to the active site of  
495 the  $\alpha$ -amylase. The hydroxyl groups can bind by non-covalent interaction, such as  
496 hydrogen bonding, cation- $\pi$  interactions, salt bridge interactions, ionic interactions, or  
497 electrostatic forces with amino acid residue at the active site in the  $\alpha$ -amylase enzyme.

498 Elevated steeping temperature and longer storage periods can easily cause the removal  
499 of the hydroxyl groups of phenolic compounds which can reduce their ability of enzyme  
500 inhibition. The phenolic acids with a greater number of hydroxyl groups exhibit a stronger  
501 capability to obstruct the  $\alpha$ -amylase enzyme.

502

### 503 $\alpha$ -Glucosidase enzyme inhibition activity (GA)

504  $\alpha$ -glucosidase is an important enzyme in carbohydrate digestion, that catalysis the  
505 hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and converts them  
506 into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et al., 2014;  
507 Proenca et al., 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -glucosidase  
508 enzyme is used to determine their antidiabetic activity. This is supported by Werdani and  
509 Widyawati (2018) stated that *Pluchea* infusion has the potential as an antidiabetic agent.  
510 Widyawati et al. (2020) found that the steeping of fresh *Pluchea* infusion at 95 °C for 5  
511 minutes has an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857 %.

512 Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -  
513 glucosidase enzyme decreased with increasing steeping temperature and storage period.  
514 Steeping at 95 °C for the un-stored *Pluchea* infusion obtained the lowest inhibitory ability,  
515 i.e.,  $48.32 \pm 1.27$  %, and the highest inhibitory activity was at 70 °C at  $95.11 \pm 0.70$ %. The  
516 results of a paired t-test showed that the GA of *Pluchea* infusion was significantly different  
517 between steeping temperature and long storage. Figure 3 further shows that the ability of  
518 *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher than the  
519 ability to inhibit the  $\alpha$ -amylase enzyme. Data analysis in Table 2. showed that the TFC of  
520 the *Pluchea* leaf infusion was influenced weakly and positively by GA and AA, but the GA

521 and AA were not affected by TPC, TTC, DPPH, and FRAP. Li et al. (2018) stated that  
522 flavonoid compounds can inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes.  
523 Dias et al. (2021) stated that flavonoid compounds, such as rutin, myricetin, kaempferol,  
524 and quercetin have antioxidant and antihyperglycemic activities. The ability to inhibit the  
525 action of enzymes from flavonoid compounds is determined by the position and number  
526 of hydroxyl groups, the number of double bonds in rings A and B, and the heterocyclic  
527 ring in ring C. Tadera et al. (2006) and Zhang et al. (2014) also explained that the  
528 flavonoid compounds of samples significantly inhibit the  $\alpha$ -glucosidase enzyme activity.

529 The ability to inhibit the  $\alpha$ -glucosidase enzyme from *Pluchea* infusion was  
530 significantly affected by the steeping temperature and long storage. Figure 3 also showed  
531 that the capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater  
532 than the  $\alpha$ -amylase enzyme because the mechanism of the two enzymes was different,  
533 according to the opinion of McCue et al. (2005). The mechanism of the  $\alpha$ -glucosidase  
534 enzyme inhibitor includes making the sugar mimic structure, binding using ionic bonds  
535 with nucleophilic, making the transition state-like structure, binding hydrogen with catalytic  
536 acid residue, interacting ionic and hydrophobic with site other than the active site, and  
537 binding covalent with enzymes through an epoxy or aziridine group (Moorthy et al., 2012).  
538 Then, the mechanism of the  $\alpha$ -amylase enzyme inhibitor includes blocking carbohydrates,  
539 limiting the digestibility and absorption of carbohydrates, and blocking the active centers  
540 of several subsites of the enzyme (Gong et al., 2020).

541 Widyawati et al. (2017) stated that phenolic and non-phenolic compounds can  
542 inhibit of the  $\alpha$ -glucosidase enzyme activity. The ability of bound phenolic compounds to  
543 inhibit  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. The presence

544 of polymerization and degradation reactions, that may be occurred in *Pluchea* infusion  
545 during storage, affects the structure and profile of phenolic and non-phenolic compounds.  
546 Asriningtyas et al. (2014) explained that the methyl-esterified quinic acid with the caffeic  
547 groups, such as 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid methyl ester,  
548 3,4,5-tri-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid, and 1,3,4,5-  
549 tetra-*O*-caffeoylquinic acid of *Pluchea* leaves inhibits the  $\alpha$ -glucosidase enzyme activity.  
550 The resulting analysis of caffeoylquinic acids (3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-  
551 caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid in stored *Pluchea* leaf infusion higher  
552 concentration than in un-stored *Pluchea* infusion, and the concentrations of the simple  
553 phenolic compounds were increased at higher steeping temperature, but the  $\alpha$ -  
554 glucosidase inhibition activity of them was reduced. It means that the methyl-esterified  
555 quinic acid with the caffeic groups had more potential to inhibit  $\alpha$ -glucosidase enzyme  
556 than free caffeoylquinic acid.

557 This study showed that the increasing steeping temperature and storage period  
558 caused degradation of polyphenol compounds to produce simple phenolic compounds,  
559 such as gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,4-di-*O*-caffeoylquinic  
560 acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid that increased the total  
561 phenolic content and total tannin content. The increase in the simple phenolic  
562 concentration of the *Pluchea* leaf infusion caused higher antioxidant activity and lower  
563 antidiabetic activity.

564

565

566

567 **CONCLUSION**

568       The Total Phenol (TPC) of *Pluchea* infusion at different steeping temperatures and  
569 storage periods generally significantly increased with increasing steeping temperature  
570 and storage periods. Steeped and stored infusion had significantly higher amounts of  
571 phenolic compounds than the samples that were steeped and un-stored. TPC was  
572 highest in the stored and steeped at 95°C and lowest in the un-stored and steeped at  
573 60°C. Un-stored steeped samples exhibited significantly higher flavonoid content than the  
574 stored steeped samples. The highest total flavonoid content was exhibited by the un-  
575 stored samples steeped at 95°C. The total tannin content of *Pluchea* leaf infusion  
576 significantly increased with increasing steeping temperature and storage period. Among  
577 the un-stored steeped samples, the tannin content was significantly lowest in the samples  
578 steeped at 60°C and highest in the samples steeped at 95°C.

579       The free radical scavenging property (DPPH) of the stored and steeped *Pluchea*  
580 leaf infusion was significantly higher than the un-stored steeped samples. The free radical  
581 scavenging property was highest in the stored samples steeped at 80 and 95°C. free  
582 radical scavenging activity of the samples was strongly and positively correlated with total  
583 phenolic and tannin contents, but inversely with total flavonoid levels. The ferric-reducing  
584 antioxidant power (FRAP) significantly increased with increasing steeping temperature  
585 and longer storage periods. The lowest FRAP was found in the un-stored samples which  
586 were steeped at 60°C and the highest was exhibited in *Pluchea* stored samples which  
587 were stored for 5 years and steeped at 95°C. The FRAP of *Pluchea* leaf infusion was  
588 significantly strong and positively correlated with the free radical scavenging property,  
589 total phenolic, and total tannin content, but inversely with total flavonoid content. The

590 inhibition of the  $\alpha$ -amylase activity was generally found to be higher at lower steeping  
591 temperatures of the un-stored *Pluchea* leaf infusion than at higher steeping temperatures  
592 of the stored sample. The  $\alpha$ -amylase enzyme inhibition capacity of the *Pluchea* leaf  
593 infusion showed a significantly strong and negative correlation with TPC, TTC, DPPH,  
594 and FRAP, but it was weakly and positively correlated significantly with TFC.

595 The ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme  
596 decreased at high steeping temperatures and long storage periods. The highest inhibitory  
597 activity was obtained in the un-stored *Pluchea* leaf infusion that was steeped at 70°C  
598 while the lowest was obtained in the un-stored sample that was steeped at 95°C. The  
599 ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher  
600 than the ability to inhibit the  $\alpha$ -amylase enzyme. The inhibition of the  $\alpha$ -glucosidase  
601 enzyme activity was significantly strong and negative TPC, TTC, DPPH, and FRAP, and  
602 it was weakly and positively correlated significantly with TFC.

603 The simple phenolic compounds identified in *Pluchea* leaf infusion may affect the  
604 presence of the bioactive compounds, antioxidant potential, and antidiabetic properties  
605 at different steeping temperatures and storage periods including gallic acids, (+)-  
606 catechins, myricetins, kaempferols, quercetins, 3,5-di-O-caffeoylquinic acids, 3,4-di-O-  
607 caffeoylquinic acids, 4,5-di-O-caffeoylquinic acids.

608

#### 609 DATA AVAILABILITY

610 Table and figure used to support this study were included in the article.

611

#### 612 CONFLICT OF INTEREST

Corresponding Author: paini@ukwms.ac.id

613 The authors declare no conflict of interest.

614

## 615 ACKNOWLEDGEMENTS

616 The authors would like to thank the Ministry of Education and Culture of the Republic of  
617 Indonesia for a fundamental research grant to higher education institutions in 2022

618

## 619 REFERENCES

620 ACAR A, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food  
621 wastes:evaluation of temperature and time effects on quality parameters. Applied Food  
622 Res. 2(100087):1-8.

623 ALEIXANDRE A et al. 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -  
624 glucosidase and influence of reaction conditions. Food Chem. 372(131231):1-9.

625 ALI et al. 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile.  
626 Food Chem. 89(2005):27-36.

627 ALI A. et al. 2018. Impact of storage conditions on the stability of predominant phenolic  
628 constituents and antioxidant activity of dried *Piper bettle* extracts. Molecules.23(24):1-13.

629 AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and  
630 methanol extract and fractions of *Gongronema latifolium* (Asclepidaceae) leaves in  
631 alloxan diabetic rats. J Applied Pharm. Sci. 01(09): 99-102.

632 ARSININGTYAS IS. et al. 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves  
633 of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product  
634 use. Natural Product Res. 28(17):1350-1353.

635 ARYAL S et al. 2019. Total phenolic content, flavonoid content and antioxidant of wild  
636 vegetables from Western Nepal. *Plants*. 8(96): 1-12.

637 AYELE DT, AKELE ML, MELESE A.T. 2022. Analysis of total phenolic contents,  
638 favonoids, antioxidant and antibacterial activities of *Croton macrostachyus* root extracts.  
639 *BMC Chem*. 16(30): 1-9.

640 BALIYAN S et al. 2022. Determination of antioxidants by DPPH radical scavenging  
641 activity and quantitative phytochemical analysis of *Ficus religiosa*. *Molecules*. 27(1326):  
642 1-19.

643 CASTIGLIONI S et al. 2015. Influence of steeping conditions (time, temperature, and  
644 particle size) on antioxidant properties and sensory attributes of some white and green  
645 teas. *Intern. J. Food Sci. Nut.* Online 1-7.

646 Chan EWC et al. 2022. *Pluchea indica*: An updated review of its botany, uses, bioactive  
647 compounds and pharmacological properties. *Pharmaceutical Sciences Asia*. 49(1): 77-  
648 85.

649 CHANDRA S et al. 2014. Assessment of total phenolic and flavonoid content, antioxidant  
650 properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit  
651 crops: A Comparative study. Hindawi Publishing Corporation Evidence-Based  
652 Complementary Alter. Med. 2014:1-9 (2014)

653 CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic, flavonoids,  
654 tannin and chlorophyll content of leaves of *Strobilanthes Kunthiana (Neelakurinji)*. *J.Med.*  
655 *Plants Studies*. 4(4):282-286.

656 DIAS MC, PINTO DCGA, SILVA AMS. 2021. Plant flavonoids: chemical characteristics  
657 and biological activity. *Molecules*. 26(5377):1-16.



658 DOBRINAS S, SOCEANU A, POPESCU V, POPOVICI IC, JITARIU D. 2021.  
659 Relationship between total phenolic content, antioxidant capacity, Fe and Cu content from  
660 tea plant samples at different brewing times. *Processes*. 9(1311):1-11.c

661 FERRARIS Z. 2023. *Pluchea indica* Less. [iNaturalist.org.](https://www.gbif.org/occurrence/4022273660)  
662 <https://www.gbif.org/occurrence/4022273660>.

663 FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional  
664 properties and visual appearance of Dampit Robusta coffee leaves tea. *Advances in Food*  
665 *Sci. Sustain. Agric. Agro-industrial Engineering*. 4(1):25-32.

666 FU L et al. 2017. Total phenolic contents and antioxidant capacities of herbal and tea  
667 infusions. *Intern. J. Molecular Sci*. 12:2112-2124.

668 GAO MR et al. 2019. A theoretical and experimental study: the influence of different  
669 standards on the determination of total phenol content in the Folin–Ciocalteu assay. *J.*  
670 *Food Measurement and Characteristic*. 13:1349–1356.

671 GONG L, FENG D, WANG T, REN Y, LIU Y, WANG J. 2020. Inhibitors of  $\alpha$ -amylase and  
672  $\alpha$ -glucosidase: Potential linkage for whole cereal foods on prevention of hyperglycemia.  
673 *Food Sci. Nut*. 2020(8):6320-6337.

674 HUANG D, OU B, PRIOR RL. 2005. The Chemistry behind antioxidant capacity assays.  
675 *J. Agric. Food Chem*. 53:1841-1856.

676 ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties  
677 of rosehip tea beverage: study by response surface methodology. *J. Food Sci. Tech*.  
678 54(11):3737–3743.

679 JAYANI NIE et al. 2022. Evaluation of stability and quality characteristics of moringa  
680 (*Moringa oleifera*) herbal tea during storage. *Food Res*. 6(4):399-406.

681 KIM JM et al. 2020. Effect of storage temperature on the antioxidant activity and catechins  
682 stability of Matcha (*Camellia sinensis*). *Food Science and Biotechnology*. 29(9):1261–  
683 1271.

684 KILIC et al. 2017. Antioxidant properties of some herbal teas (green tea, senna, corn silk,  
685 rosmery) brewed at different temperatures. *Intern. J. Secondary Metabolite*. 4(3):142-248  
686 Special Issue 1.

687 KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of  
688 extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves  
689 from different provenances in Thailand. *Revista Brasileira de Farmacognosia*. 28(2018):  
690 145–150.

691 KOWALSKA J et al. 2021. Influence of tea brewing parameters on the antioxidant  
692 potential of infusions and extracts depending on the degree of processing of the leaves  
693 of *Camellia sinensis*. *Molecules*.26(4773):1-17.

694 KRUK J, ABOUL-ENAIN BH, DUCHNIK E, MARCHLEWICZ M. 2022. Antioxidative  
695 properties of phenolic compounds and their effect on oxidative stress induced by severe  
696 physical exercise. *J. Physiol. Sci*. 72(19):1-24.

697 LI K et al. 2018. Inhibitory effects against  $\alpha$ - glucosidase and  $\alpha$ -amylase of the flavonoids  
698 rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity  
699 relationship of its eight flavonoids by a refined assign score method. *Chem. Central J*.  
700 12(82):1-11.

701 LIN YS. et al. 2020. Effects of storage time and temperature on antioxidants in juice from  
702 *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser. *Molecules*.  
703 25(3614): 1-12.

704 MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content  
705 and antioxidant activity test of avocado seed extract (*Persea Americana* Mill). Jurnal MIPA  
706 Unsrat Online. 1(1): 5-10. [https://media.neliti.com/media/publications/116214-ID-  
707 penentuan-kandungan-tanin-dan-uji-aktivi.pdf](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf) [In Bahasa Indonesia]

708 MARTINEZ-SOLIS J et al. 2022. Antidiabetic and toxicological effect of the tea infusion  
709 of summer collection from *Annona cherimola* Miller leaves. Plants. 11(3224):1-17.

710 MATHUR R, VIJAYVERGIA R. 2017. Determination of total flavonoid and phenol content  
711 in *Mimusops Elengi* Linn. Intern. J. Pharm. Sci. Res. 8(12): 5282-5285.

712 MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of  
713 sprouted and solid-state bioprocessed soybean. Asia Pacific J. Clinical Nut. 14(2): 145-  
714 152.

715 MOORTHY NS, RAMOS MJ, A FERNANDES P. 2012. Studies on  $\alpha$ -glucosidase  
716 inhibitors development: Magic molecules for the treatment of carbohydrate mediated  
717 diseases. Mini Rev Med Chem. 12:713–720.

718 MORAES-DE-SOUZA RA, OLDONI TLC, REGITANO-D'ARCE MAB, ALENCAR SM.  
719 2008. Antioxidant activity and phenolic composition of herbal infusions consumed in  
720 Brazil. Cienc Tecnol. Aliment. 6(1):41-47.

721 MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant  
722 activity: A Review. Intern. J. Molecular Sci. 22(3380):1-30.

723 NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus*  
724 *sabdariffa* L.): Effect of dying temperature and brewing conditions on total soluble solid,  
725 phenolic content, antioxidant capacity and sensory quality. Beverages.6(2):1-11 (2020).

726 NOREEN H. et al. 2017. Measurement of total phenolic content and antioxidant activity  
727 of aerial parts of medicinal plant *Coronopus didymus*. Asian Pacific J. Tropical Med. 10(8):  
728 792–801.

729 NURCHOLIS GW et al. 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity in-  
730 vitro and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum pictum*  
731 (L.). Current Biochem. 1(2): 58-65.

732 OH J et al. 2013. Antioxidant and antimicrobial activities of various leafy herbal teas. Food  
733 Control. 31(2013):403-409.

734 PROENCA C et al. 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an in vitro and in silico  
735 structure–activity relationship study. J. Enzyme Inhibition Med. Chem. 32(1): 1216–1228.

736 PUREWAL SS et al. 2022. Unraveling the effect of storage duration on antioxidant  
737 properties, physicochemical and sensorial parameters of ready to serve Kinnow-aml  
738 beverages. Applied Food Res. 2(1): 1-8.

739 RAHARJO D, HARYOTO. 2019. Antioxidant activity of mangrove *Sonneratia caseolaris*  
740 L. using the FRAP method. ISETH. 2019: 623-629.

741 RAMPHINWA ML et al. 2023. Eco-physiological response of secondary metabolites of  
742 teas: review of quality attributes of herbal tea. Frontiers in Sustain. Food Systems.  
743 7(990334):1-14.

744 REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids.  
745 Czech J. Food Sci. 30(2):171-177.

746 RUAN J et al. 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea*  
747 *indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel  
748 column chromatography fractions. Molecules. 24(2784):1-20.

749 RUSITA YD et al. 2019. Comparison of tannin concentration on fresh and dried tea leaf  
750 (*Camelia sinensis* L.) extract using UV-visible spectrophotometric method. 1st  
751 International Conference of Health, Science & Technology (ICOHETECH) 2019 E-ISBN:  
752 978-623-92207-0-9. November 16<sup>th</sup>, 2019.LPPM Universitas Duta Bangsa Surakarta,  
753 Surakarta.

754 SANGEETHA R, VEDASREE N. 2012. In Vitro  $\alpha$ -Amylase inhibitory activity of the leaves  
755 of *Thespesia populnea*. Intern. Scholarly Res.Network. 2012: 1-4 (2012)

756 SHRAIM AM et al. 2021. Determination of total flavonoid content by aluminum chloride  
757 assay: A critical evaluation. LWT Food Sci. Tech. 150(111932):1-11.

758 SILVA-RAMIREZ KA et al. 2020. Effect of drying and steeping temperature on the  
759 phenolic content, antioxidant activity, aromatic compounds and sensory properties of  
760 *Culila polyantha* Benth. Infusions. Processes. 8(1378):2-20.

761 SRISOOK K et al. 2012. Antioxidant and anti-inflammatory activities of hot water extract  
762 from *Pluchea indica* Less. herbal tea. J. Med. Plants Res. 6(23):4077-4081.

763 SURİYAPHAN O. 2014. Nutrition, health benefits and applications of *Pluchea indica* (L)  
764 Less leaves. Pharm. Sci. Asia. 41:1–10.

765 SU D et al. 2019. Impact of thermal processing and storage temperature on phenolic  
766 profile and antioxidant activity of different varieties of lychee juice. LWT-Food Tech.  
767 116(108578):1-9.

768 TADERA K, MINAMI Y, TAKAMATSU K, MATSUOKA T. 2006. Inhibition of alpha  
769 glucosidase and alpha-amylase by flavonoids. J Nutr Sci Vitaminol. 52:149–153.

770 THANAJIRUSCHAYA P et al. 2010. Effect of storage **period** and temperature on  
771 antioxidant components and properties of milled rice. Khon Kaen Univ. Res. J.15(9):843-  
772 851.

773 VONGSAK B et al. 2018. Comparison of active constituents, antioxidant capacity, and  
774 αglucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. Food  
775 Biosci. 25: 68–73.

776 WANG SC. et al. 2022. Effect of brewing conditions on polyphenols in the dark tea  
777 (*Camellia sinensis* L.) infusions: content, composition and antioxidant activities. Food  
778 Sci.Tech. 42(e36322):1-9.

779 WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica*  
780 Less as functional beverage in diabetic patients. ASSEHR. 98:164-167.

781 WIDYAWATI PS et al. 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract  
782 as antioxidant and **anti-warmed** over flavor (WOF) of duck meat. International Congress  
783 on Challenges of Biotechnological Research in Food and Health. November 15<sup>th</sup>  
784 2014.Agricultural Technology Faculty, Slamet Riyadi University, Surakarta.

785 WIDYAWATI PS et al. 2016. The physicochemical and antioxidant properties of *Pluchea*  
786 *indica* Less drink in tea bag packaging. IJFNS. 5: 13–120.

787 WIDYAWATI PS et al. 2017. In vitro antioxidant capacities and antidiabetic properties of  
788 pluchea leaves. IJPPS. 9(8): 203-208.

789 WIDYAWATI PS et al. 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica*  
790 Less beverage. CJFST.12(4): 125-139.

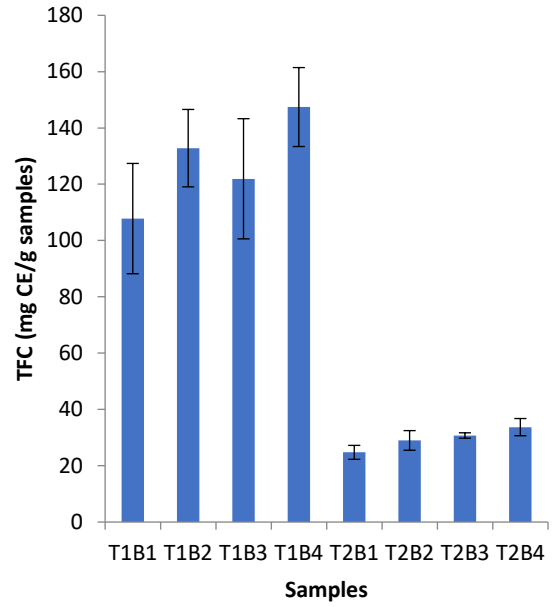
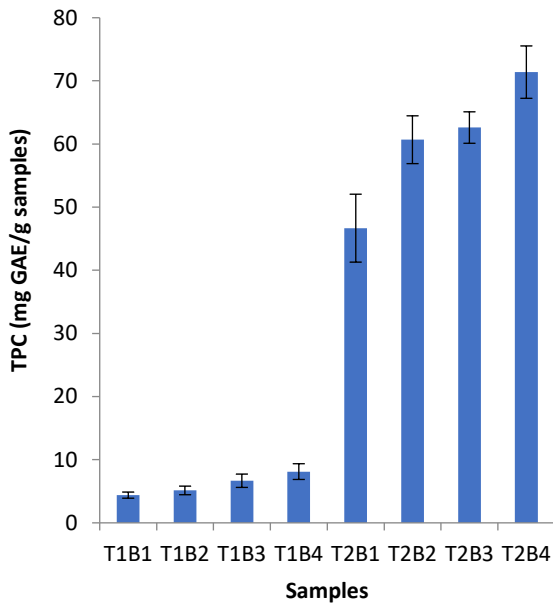
791 WIDYAWATI PS ET AL. 2022. The effect of  $\kappa$ -carrageenan proportion and hot water  
792 extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of stink  
793 lily (*Amorphophallus muelleri*) wet noodles. *Molecules*. 27(5062):1-16.

794 XU P, CHEN L, WANG Y. 2019. Effect of storage **period** on antioxidant activity and  
795 inhibition on  $\alpha$ -amylase and  $\alpha$ -glucosidase of white tea. *Food Sci. Nut.* 7(2): 636-644.

796 ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction **period** on caffeine  
797 in coffee. *Lantanida J.* 6(2):103-202. [https://jurnal.ar-](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811)  
798 [raniry.ac.id/index.php/lantanida/article/view/3811](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811). [In Bahasa Indonesia]

799 Zhang H, Wang G, Dong J. 2014. Inhibitory properties of aqueous ethanol extracts of  
800 propolis on alpha-glucosidase. *J Evid Based Complement Alternat Med.* 2015:1–8.

801 ZHANG Y et al. 2021. Effect of storage conditions and **period** on the polyphenol content  
802 of wheat flours. *Processes*. 9(248):1-11.

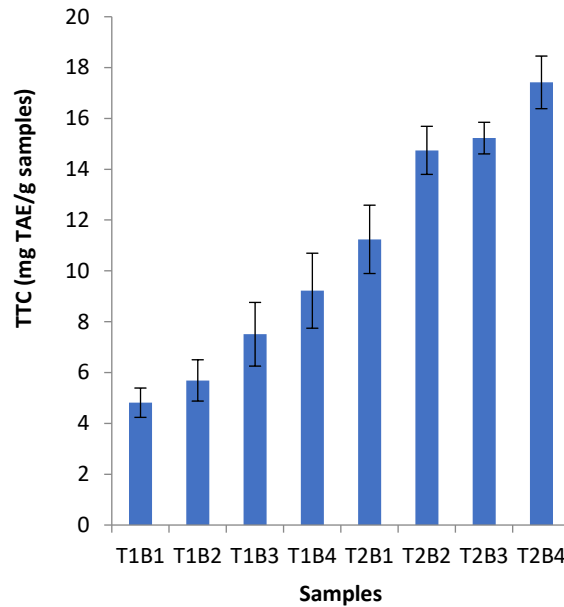


803

804

(a)

(b)



805

806

(c)

807 **Figure 1.** Bioactive compound contents of *Pluchea* infusion at different steeping  
 808 temperature and storage period (a) Total phenolic content (b) Total flavonoid  
 809 content (c) Total tannin content. Data analysis using ANOVA at  $\alpha \leq 0.05$   
 810 continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as  
 811 mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored;



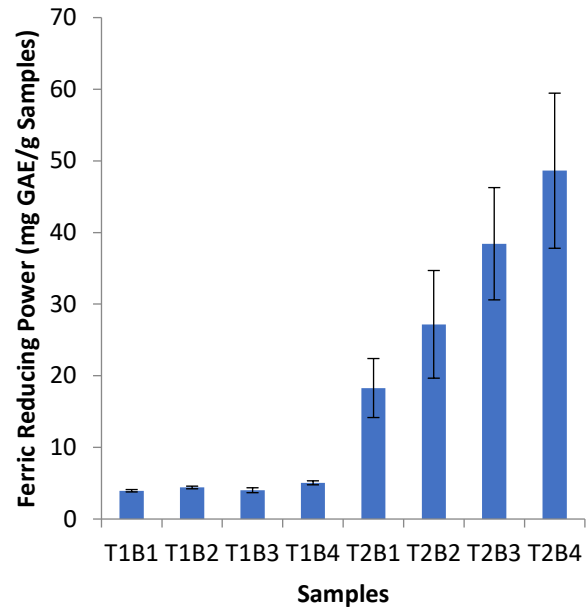
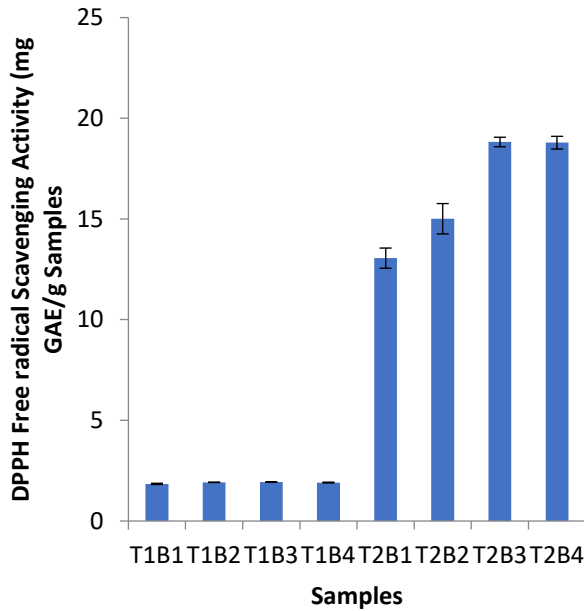
812 T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-  
813 steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-  
814 steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5  
815 years; T3B4-steeped at 95 °C, stored for 5 years.

816 Table 1. Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperature and storage period

Phenolic Compounds	Steeping Temperature (°C)	Mean±SD Un-stored	Mean±SD Stored	Mean difference ±SD	Sig (2-tailed)
Gallic Acid (µg/g samples)	60	0.2132±0.0027	0.2364±0.0015	0.0375±0.0175	0.2030
	70	0.2157±0.0013	0.2324±0.0214	0.0167±0.0227	0.4870
	80	0.2234±0.0122	0.2347±0.0078	0.0386±0.0264	0.2870
	95	0.2316±0.0104	0.2402±0.0169	0.0086±0.1990	0.8500
(+) -Catechin (µg/g samples)	60	0.3425±0.0110	0.5085±0.0111	-0.1576±0.0885	0.241
	70	0.3260±0.0265	0.5448±0.0006	-0.2188±0.0259	0.053
	80	0.3240±0.0222	0.5023±0.0773	-0.1451±0.0248	0.077
	95	0.4039±0.0320	0.5995±0.0372	-0.2049±0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756±0.1234	1.4762±0.0271	-1.2887±0.3222	0.111
	70	0.2587±0.0160	1.4245±0.2526	-1.1657±0.2695	0.103
	80	0.4175±0.0104	1.4570±0.0925	-1.0391±0.0841	0.036*
	95	0.8786±0.0434	2.6138±0.0695	-1.1735±0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220±0.0268	0.6220±0.0706	-0.5999±0.9733	0.544
	70	0.1530±0.0511	1.0708±0.0289	-0.9177±0.0222	0.011*
	80	0.3666±0.0103	0.8629±0.0815	-0.1082±0.4462	0.790
	95	0.6559±0.0570	2.0230±0.0573	-1.4123±0.3203	0.101
Kaempferol (µg/g samples)	60	0.1394±0.0202	0.3675±0.0183	-0.3207±0.1122	0.154
	70	0.0514±0.0037	0.3726±0.0944	0.3213±0.0907	0.125
	80	0.3699±0.0924	0.7966±0.0366	-0.4267±0.2727	0.271
	95	0.5913±0.0239	0.9478±0.0287	-0.3565±0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103±0.0628	2.4863±0.0270	-1.8760±0.2074	0.050*
	70	0.6271±0.0099	2.3403±0.0325	-1.7131±0.3152	0.082
	80	0.7967±0.03060	2.6278±0.0211	-1.8311±0.0095	0.002*
	95	1.5386±0.0668	4.0211±0.0851	-2.4825±0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635±0.0628	0.9449±0.0501	-0.2814±0.4458	0.536
	70	0.6162±0.0099	0.9485±0.0794	-0.3323±0.0301	0.041*
	80	0.6601±0.0306	0.9099±0.0387	-0.2498±0.3127	0.461
	95	0.6642±0.0668	1.3156±0.0166	-0.6514±0.2666	0.179

4,5-di-O-Caffeoylquinic acid ( $\mu\text{g/g}$ samples)	60	0.4906 $\pm$ 0.0060	1.1842 $\pm$ 0.0120	-0.6886 $\pm$ 0.2723	0.018*
	70	0.4807 $\pm$ 0.0034	1.0089 $\pm$ 0.0736	-0.5281 $\pm$ 0.0702	0.060
	80	0.5299 $\pm$ 0.0053	1.2382 $\pm$ 0.1435	-0.7082 $\pm$ 0.1489	0.094
	95	1.0018 $\pm$ 0.0526	1.3797 $\pm$ 0.2170	-0.3086 $\pm$ 0.3086	0.333

817 Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  
818  $\pm$ standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped  
819 at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped at 70 °C,  
820 stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped at 95 °C, stored for 5 years.  
821



822

(a)

(b)

823

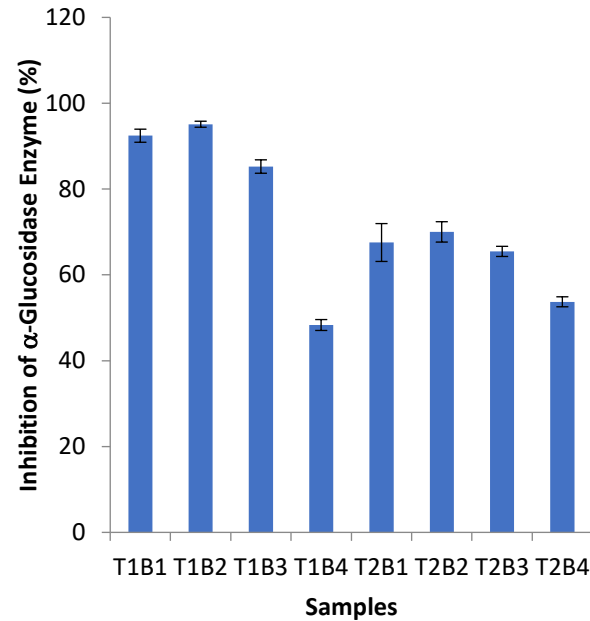
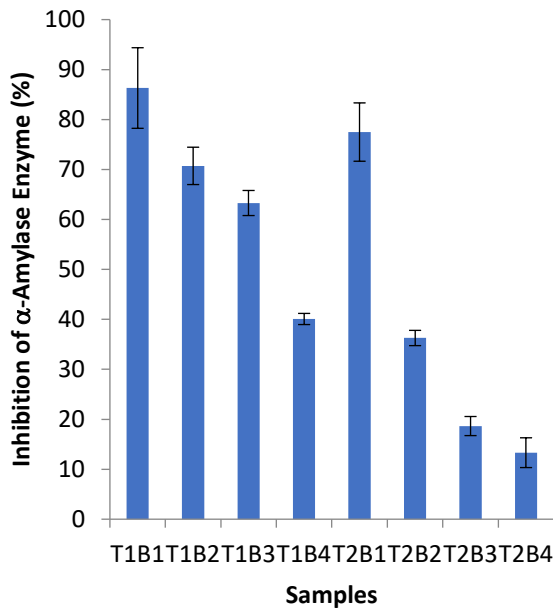
824 **Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage**  
 825 **period (a) DPPH (b) FRAP. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued**  
 826 **analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean**  
 827  **$\pm$ standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-**  
 828 **steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped**  
 829 **at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped**  
 830 **at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-**  
 831 **steeped at 95 °C, stored for 5 years.**

832

833

834

835



836

837

(a)

(b)

838

839

840

841

842

843

844

845

**Figure 3.** Antidiabetic activity of pluchea tea at different steeping temperature and storage period (a)  $\alpha$ -amylase (b)  $\alpha$ -glucosidase. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T2B2-steeped at 70 °C, stored for 5 years; T2B3-steeped at 80 °C, stored for 5 years; T2B4-steeped at 95 °C, stored for 5 years.

846 Table 2. Pearson correlation coefficients between bioactive contents (TPC, TFC and TAC), antioxidant activity (DPPH and  
 847 FRAP) and antidiabetic activity (AA and GA)

	TPC	TFC	TTC	DPPH	FRAP	Alpha Glucosidase	Alpha Amylase
TPC	1						
TFC	-0.93589	1					
TTC	0.960028	-0.81321	1				
DPPH	0.992776	-0.93992	0.942273	1			
FRAP	0.953366	-0.82636	0.947778	0.956242	1		
Alpha Glucosidase	-0.55512	0.349873	-0.71534	-0.5272	-0.55947	1	
Alpha Amylase	-0.70842	0.429393	-0.8569	-0.69579	-0.80548	0.725161631	1

848 Significant at the 0.05 level (2-tailed)

849



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**From Caesar Saloma/15 January 2024/ Editorial Decision/ MS 23-158R**

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Caesar Saloma <caesar.saloma@gmail.com>

Tue, Feb 27, 2024 at 4:57 AM

Subject: MS 23-158R

Title: Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea indica* Less Tea

Authors Paini Sri Widyawati and Yufita Ratnasari Wilianto

Dear Dr. Caesar Saloma

Greetings,

Regarding the manuscript that I have sent, please provide information about when the manuscript will be published and whether it still needs further improvements.

Thank you for your attention

Regards

Paini Sri Widyawati

On Mon, Jan 15, 2024 at 4:15 PM Caesar Saloma <caesar.saloma@gmail.com> wrote:

[Quoted text hidden]



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**From Caesar Saloma/15 January 2024/ Editorial Decision/ MS 23-158R**

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Caesar Saloma <caesar.saloma@gmail.com>

Tue, Apr 16, 2024 at 1:49 AM

Dear Prof. Dr. Caesar Saloma

Greetings,

Please give me information about the status of our manuscript with number MS 23-158R  
Title: The Effect of Brewing Temperature and Storage Time on Bioactive Compounds, Antioxidant and Antidiabetic Activity in Less Tea *Pluchea*  
Authors Paini Sri Widyawati and Yufita Ratnasari Wilianto

What month would it be published in 2024?

Thank you for your attention

Paini Sri Widyawati

On Mon, Jan 15, 2024 at 4:15 PM Caesar Saloma <caesar.saloma@gmail.com> wrote:  
[Quoted text hidden]



1 **Effect of Steeping Temperature and Storage [TimePeriod](#) on the Bioactive**  
2 **Compounds, Antioxidant and Antidiabetic Activities of Infusion from Powdered**  
3 ***Pluchea Indica Less***

4 Painsi Sri Widyawati<sup>1\*)</sup>, Yufita Ratnasari Wilianto<sup>2)</sup>

5 <sup>1)</sup>Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala  
6 Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

7 <sup>2)</sup>Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic  
8 University, Kalisari Street Number 1, Surabaya 60272, Indonesia

9 Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea*  
10 *indica Less*, storage [timeperiod](#)

11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
Corresponding Author: [painsi@ukwms.ac.id](mailto:painsi@ukwms.ac.id)

21 ABSTRACT

22 This study was done to determine the effects of steeping temperature and storage  
23 ~~timeperiod~~ on the bioactive contents, antioxidant and antidiabetic activities of *Pluchea*  
24 ~~leaf infusion~~. The research used a randomized block design with two factors, i.e., steeping  
25 temperature (T) and storage ~~timeperiod~~ (B). The ~~variety of the Pluchea leaf blades were~~  
26 ~~exposed to 4~~ steeping temperatures ~~included of~~ 60 (T1), 70 (T2), 80 (T3), and 95 (T4)  
27 (°C) with the storage ~~timeperiod-period~~ of 0 (B1) and 5 (B2) ~~(year)~~. ~~The research~~  
28 ~~resultedresulting in~~ 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2,  
29 T4B1, T4B2). Statistical analysis using a paired ~~t-T~~ test at  $\alpha \leq 0.05$  showed that  
30 treatments significantly ~~affected influenced~~ the bioactive contents (total phenol (TPC),  
31 total tannin (TTC), total flavonoid (TFC)), antioxidant [(DPPH scavenging activity (DPPH)  
32 and ferric reducing antioxidant power (FRAP)] ~~potential~~ and antidiabetic [( $\alpha$ -amylase  
33 (AA) and  $\alpha$ -glucosidase (GA) ~~inhibitorsinhibition~~)] ~~activities-properties~~ of the *Pluchea* leaf  
34 ~~infusionsamples~~. TFC decreased during storage period but significantly increased at  
35 higher steeping temperature. The AA and GA of *Pluchea* infusion increased until 70 °C  
36 ~~of the steeping temperature, but decreased until 95 °C~~. ~~The bioactive contents influenced~~  
37 ~~antioxidant and antidiabetic activities~~. TFC was decreased for storage time and significant  
38 ~~increased at higher steeping temperature~~. The AA and GA of *Pluchea* infusion increased  
39 ~~until 70 °C of the steeping temperature, but decreased until 95 °C~~. The AA and GA were  
40 strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was  
41 moderately and negatively correlated with TFC. ~~Between-T~~ the antioxidant activity of  
42 ~~DPPH and FRAP~~ and the antidiabetic activity of AA and GA of *Pluchea* infusion were  
43 strongly and positively correlated. ~~with correlation coefficient (r) values of 0.956 and~~

**Commented [A1]:** Describe treatment effects on total phenolics, tannins, antioxidant, and antidiabetic in one brief sentence each and indicate statistical significance.

**Commented [A2]:** State briefly results of the correlation analysis.

44 0.725, respectively. The treatments gave different effect of simple phenolic compounds  
45 derived from *Pluchea* leaf infusion at different steeping temperatures and storage  
46 included, such as gallic acid, kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-  
47 caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid of  
48 *Pluchea* infusion at different steeping temperature and long storage. To obtain high  
49 antioxidant activity, *Pluchea* infusion selected was stored and steeped at high  
50 temperature, however high antidiabetic activity obtained was fresh *Pluchea* infusion and  
51 steeped at low temperature.

Formatted: Font: Italic

## 52

### 53 INTRODUCTION

54 *Pluchea* herbal tea is a product of dried *Pluchea* leaf processing introduced by  
55 world people (Srisook et al., 2012; Widyawati et al., 2016) because of the efficacy of the  
56 active components in *Pluchea* leaves, as a herbal plant that has been widely used for  
57 traditional medicine and food (Chan et al., 2022). *Pluchea* leaves are composed many  
58 nutrients and bioactive compounds useful to body health. The nutrient compositions in  
59 the *Pluchea* leaves include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates,  
60 calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive compounds is comprised, i.e.,  
61 chlorogenic acid, caffeic acid, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-  
62 caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-  
63 caffeoylquinic acid, quercetin, myricetin, kaempferol, total anthocyanin,  $\beta$ -carotene, and  
64 total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et  
65 al., 2022, Chan et al., 2022).

Corresponding Author: paini@ukwms.ac.id

66 Steeping process of *Pluchea leaves* can be performed with fresh or dry leaves  
67 ~~infusion by~~in hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et  
68 al., 2020; Jayani et al., 2022). In Asia ~~area~~, especially in Indonesia, people usually  
69 consume the *Pluchea infusion* ~~with brewing of~~ steeping 2 g of powdered *Pluchea*  
70 leaves in tea bag ~~by~~in 100 mL of hot ~~water~~ or boiling water. ~~Each tea bag contained 2 g~~  
71 ~~of *Pluchea* leaf powder is steeped with 100 mL hot water or boiling water.~~ Widyawati et  
72 al. (2016) claimed that steeping of 2 g of *Pluchea leaf powder* at 95 °C for 5 minutes  
73 ~~results exhibits~~ total phenolic ~~content, and~~ total flavonoid contents, the ability to scavenge  
74 DPPH free radicals, and the capability ~~of to~~ reduce ferric ions ~~at~~ 9.3 mg gallic acid  
75 equivalent (GAE)/g samples, 22.0 mg gallic acid equivalent (GAE)/g samples, 27.2 mg  
76 gallic acid equivalent (GAE)/g samples, and 10.2 mg gallic acid equivalent (GAE)/g  
77 samples, respectively. Werdani and Widyawati (2018) reported that drinking of *Pluchea*  
78 *leaf powder infusion* in the morning and evening regularly (2 g/100 mL) can decline blood  
79 sugar levels.

80 The steeping of *Pluchea herbal tea* with hot water at 95 °C for 5 minutes certainly  
81 determines the stability and amount of extracted bioactive compounds, that influences  
82 the biological activity, especially antioxidant and antidiabetic activities. Silva-Ramirez et  
83 al. (2020) reported that the infusion process can influence the ~~if~~ content and composition  
84 of the bioactive compounds and antioxidant activity of tea. Acar et al. (2022) informed  
85 that infusion quality of *herbal* tea extract depends on several factors, i.e., ~~time~~ ~~storage~~  
86 and temperature. Polyphenol profile and antioxidant properties of *herbal* tea infusion  
87 decline with an increase in steeping/brewing and storage temperatures, and longer  
88 exposure ~~time~~ ~~periods~~.

89 Several studies have mentioned the effect of steeping temperature ~~to on the~~  
90 bioactive compound contents and antioxidant activity, such as some white and green teas  
91 are effective with hot water at 90 °C for 7 min (Castiglioni et al., 2015), ~~on~~ roseship tea is  
92 effectively ~~at infusion~~ ~~timeperiod~~ around 6-8 min at temperatures of 84-86 °C (Ilyasoglu  
93 and Arpa, 2017), ~~on the caffeine content extracted the coffee at the~~ brewing temperature  
94 ~~of coffee influences the caffeine content extracted~~ (Zarwinda and Sartika, 2018), ~~and the~~  
95 ~~steeping the high total phenol content and antioxidant activity~~ of dark tea at 92 °C for 27  
96 min ~~results the highest total phenol content and antioxidant activity~~ (Wang et al., 2022).  
97 The study of the effect of steeping temperature to *Pluchea* infusion was carried out to  
98 afford information about ~~the most efficient~~ preparation of *powdered Pluchea leaves* ~~most~~  
99 ~~efficiently~~ to get higher ~~the~~ bioactive compounds, antioxidant and antidiabetic activities.

100 ~~On the other hand, storage~~ ~~Storage~~ ~~timeperiod~~ *tea* usually for several months until  
101 ~~years~~ of *Pluchea* herbal tea also affects the levels of the bioactive compounds and  
102 biological activity ~~because this~~ ~~herbal tea~~ usually is stored for a several months until years  
103 (Jayani et al., 2022). Tea or herbal tea is generally stored in ambient temperature and  
104 packed in tea bag or ~~Alu~~ foil standing proud or a combination of both. Many researchers  
105 ~~informed~~ ~~reported~~ that storage ~~timeperiod~~ decreases the bioactive compounds,  
106 antioxidant and antidiabetic activities, i.e., juice from *Momordica charantia* L. (Lin et al.,  
107 2020), dried *Piper bettle* extracts (Ali et al., 2018), white tea (Xu et al., 2019), kinnow-  
108 amla beverages (Purewal et al., 2022), whole wheat flour (Zhang et al., 2021).

109 Therefore, *this research studied the effect of steeping temperature and storage*  
110 *timeperiod on the bioactive compounds [(total phenolic content (TPC), total flavonoid*  
111 *content (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging*

Commented [A3]: IS THIS ALUMINUM? If so, replace Alu with aluminum

Commented [A4]: Do you mean standing pouch?

112 activity (DPPH), ferric reducing antioxidant power (FRAP)] and antidiabetic activities [( $\alpha$ -  
113 amylase (AA) and  $\alpha$ -glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea*  
114 leaves. The study was done to determine total phenolic content (TPC), total flavonoid  
115 content (TFC), total tannin content (TTC), DPPH free radical scavenging activity (DPPH),  
116 ferric reducing antioxidant power (FRAP),  $\alpha$ -amylase (AA) and  $\alpha$ -glycosidase (GA)  
117 inhibition activities, and on the phenolic compound profile.

## 119 MATERIALS AND METHODS

### 120 RAW MATERIALS AND PREPARATION

121 The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya,  
122 East Java, Indonesia. The *Pluchea* plants were included in Asteraceae family with  
123 specification according to the GBIF taxon ID number database:3132728 (Ferraris, 2023).  
124 *Pluchea* leaves at 1-6 level of each branch ~~offrom~~ the shoot were collected, sorted,  
125 washed and dried to ~~get a~~ moisture content ~~of~~ around  $11.16 \pm 0.09$  % dry basise  
126 (Widyawati et al., 2022). The ~~powdoring of~~ dried *Pluchea* leaves was ~~done~~ pulverized to  
127 ~~get a~~ 45-mesh size powder. ~~And then, the heating of T~~the *Pluchea* leaf powder was ~~done~~  
128 ~~using a drying~~dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 °C for  
129 10 min to reduce microbial organisms. ~~and Then, 2 g of the powder were~~ packed using  
130 ~~into a paper filter~~ infusion bag, ~~that made from paper filter around 2 g/bag. And then all~~  
131 ~~of samples called~~Packed samples were *Pluchea* herbal tea was stored for 0 (un-stored)  
132 and 5 (stored)years in standing pouch before analysis.

133 In the research, the one tea bag of *Pluchea* herbal tea that stored 0 (B1) and 5  
134 (B2) year, was steeped with 100 mL hot water at various temperatures, including 60 (T1),

135 70 (T2), 80 (T3), and 95 (T4) °C for 5 min with infusion method ~~that obtained~~obtaining 8  
136 treatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2.  
137 After the temperature of *Pluchea* infusion similar to ambient temperature was analyzed  
138 further.

## 140 REAGENTS

141 The ~~compounds~~reagents used ~~to analyze~~in the analyses including include 2,2-  
142 diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid, α-amylase, α-  
143 glucosidase, pNPG (p-nitrophenyl-α-glucopyranoside), (+)-catechin, kaempferol,  
144 myricetin, quercetin, 3,4-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, 3,5-di-O-  
145 caffeoylquinic acid, and (+)-catechin were purchased from Sigma-Aldrich (St. Louis, MO,  
146 USA). Methanol, Folin-Ciocalteu's Phenol, sodium nitric, aluminum chloride, ferric  
147 chloride, sodium dihydrogen phosphate, sodium phosphate, potassium ferricyanide,  
148 starch, acetic acid, and sodium hydroxide were purchased from Merck (Kenilworth, NJ,  
149 USA). All reagents used were of analytical grade except for distilled water which was  
150 purchased from PT Aqua Industry Surabaya.

## 152 METHODOLOGY

### 153 ANALYSIS OF THE BIOACTIVE COMPOUNDS

#### 154 TOTAL PHENOLIC CONTENT ANALYSIS

155 Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the  
156 technique by Gao et al. (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's  
157 phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. And

**Commented [A5]:** Confusing, needs to be re-written eg The unstored samples were steeped in 100 mL distilled water at 60, 70, 80, and 95 °C for 5 min, then immediately were analyzed for the bioactive compounds [(total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC)], antioxidant potential [(DPPH free radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP)] and antidiabetic activities [(α-amylase (AA) and α-glycosidase (GA) inhibition)]. The rest of the samples were stored at (describe storage conditions) and analyze after 5 years..

**Commented [A6]:** Confusing

**Formatted:** Centered

158 then 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5 % was ~~entered added and filled up to 10 mL volume with distilled~~  
159 ~~water and distilled water was added until 10 mL volume~~. The color intensity of solution  
160 was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at  $\lambda$  760 nm  
161 with gallic acid as the reference standard. The total phenolic content was calculated using  
162 the formula:  $y=0.00009x+0.008$  with  $R^2=0.9941$ . The results were expressed as mg gallic  
163 acid equivalent (GAE)/g samples.

Commented [A7]: Specify what color

164

#### 165 TOTAL FLAVONOID CONTENT ASSAY

166 Total flavonoid content (TFC) of the samples was measured based on the reaction  
167 between AlCl<sub>3</sub> and NaNO<sub>2</sub> with ~~an the~~ aromatic ring of flavonoid compounds, especially  
168 flavonol and flavon (Shraim et al., 2021). The reaction between AlCl<sub>3</sub> and flavonoid  
169 compounds resulted ~~in~~ a yellow solution. About 30  $\mu$ L *Pluchea* infusion was mixed with  
170 0.3 mL NaNO<sub>2</sub> 5 % in 10 mL volumetric flask and incubated for 5 min. The mixture was  
171 added with 0.3 mL AlCl<sub>3</sub> 10 % for 5 min. And then, 2 mL NaOH 1 M and distilled water  
172 were added until 10 mL volume. Then, the red solution was produced after NaOH solution  
173 addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800,  
174 Shimadzu, Japan) at  $\lambda$  510 nm with (+)-catechin as the reference standard compound,  
175 and the results were expressed as mg catechin equivalents (CE)/g samples using the  
176 formula:  $y=0.00008x-0.0023$  with  $R^2= 0.9980$ .

177

#### 178 TOTAL TANNIN CONTENT ANALYSIS

179 Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method  
180 (Chandran and Indira, 2016). Approximately 10  $\mu$ L *Pluchea* infusion was added ~~with~~ 1 mL



181 Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flask and incubated for 5 min.  
182 Then, the mixture was added with 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5 % and filled up to 10 mL volume with  
183 distilled water. ~~was added until 10 mL volume.~~ The blue dark color solution ~~that was~~  
184 measured in UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at λ 760 nm with tannic  
185 acid as the reference standard. Calculation of TTC was expressed as mg tannic acid  
186 equivalents (TAE)/g samples used the formula:  $y=0.00009x+0.0021$  with  $R^2=0.9993$

## 188 ANALYSIS OF THE ANTIOXIDANT POTENTIAL

Formatted: Centered

### 189 DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

190 The DPPH free radical scavenging activity (DPPH) was measured by the  
191 spectrophotometric method (Widyawati et al., 2017) to determine the ability of the  
192 phytochemicals antioxidant activity of the *Pluchea* leaf infusion to ~~doner donate~~  
193 hydrogen atom to the nitrogen atom in DPPH resulting in the formation of -DPPH-H  
194 compound with exhibiting a yellow-colored solution. About 25 μL *Pluchea* leaf infusion  
195 was ~~entered poured~~ into reaction tube ~~and into which was added~~ added 3 mL DPPH  
196 solution (4 mg/100 mL). ~~And then the solution was~~ After incubation for 15 min in a dark  
197 room, ~~the and~~ absorbance was measured by a spectrophotometer (Spectrophotometer  
198 UV-Vis 1800, Shimadzu, Japan) at λ. 517 nm. The reference standard compound was  
199 gallic acid and the results of analysis were expressed as mg gallic acid equivalents  
200 (GAE)/g samples that calculated using formula:  $y=0.146x+1.7896$  with  $R^2=0.9975$ .

### 202 FERRIC REDUCING POWER ANALYSIS

203 Ferric reducing power (FRAP) was determined following the method used by  
204 Widyawati et al. (2014) method. Approximately 10  $\mu$ L of samples were added 2.5 mL  
205 phosphate buffer pH 6.6 and 2.5 mL and 1% potassium ferricyanide 1% in the reaction  
206 tube. And then mixture was shaken and ~~incubation~~ incubated for 20 min at 50 °C. Finally,  
207 2.5 mL chloroacetic acid 10% (w/v) was added. Into the 2.5 mL supernatant was added  
208 2.5 mL distilled water, 0.5 mL ferric chloride 0.1% (w/v) and incubated for 10 min.  
209 Potency of the samples reducing iron (III) to iron (II) ion was ~~signed~~ indicated by the  
210 intensity of blue color formed that was measured using UV-Vis spectrophotometer  
211 (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  700 nm. Intensity of the blue  
212 color indicated higher reducing capacity. The reducing power expressed as mg gallic acid  
213 equivalent (GAE)/g samples was calculated using the formula:  $y=0.0002x+0,0256$  with  
214  $R^2=0,9906$ .

215

#### 216 $\alpha$ -AMYLASE ENZYME INHIBITION CAPACITY ASSAY

217 In vitro inhibition of  $\alpha$ -amylase enzyme (AA) followed the procedure as described  
218 by Widyawati et al. (2020). Each 500  $\mu$ L of samples, was mixed with starch 1 % (w/v) and  
219 sodium acetate buffer pH 5, ~~were mixed. Then, into each~~ 250  $\mu$ L of the mixture and was  
220 added an  $\alpha$ -amylase solution (0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in  
221 50 mL of 0.2 M sodium acetate pH 5). Mixture was shaken and into which was added 2  
222 mL sodium hydroxide 1M. Before the analysis, this mixture was incubated at 37 °C for 10  
223 min. Then, the capacity of the  $\alpha$ -amylase enzyme to hydrolyzed the starch to release  
224 glucose was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800,  
225 Shimadzu, Japan) that could be analyzed based on absorbance at  $\lambda$  540 nm. The

226 inhibition percentage of  $\alpha$ -amylase was assessed using the formula:  $(ACb - ACa) - (As$   
227  $- Ab) (ACb - ACa) \times 100 \%$ . Where, ACb is the absorbance of 100 % enzyme activity  
228 (solvent with the enzyme), ACa is the absorbance of 0 % enzyme activity (solvent without  
229 the enzyme), As is the absorbance of test sample with enzyme, Ab is absorbance of test  
230 sample without enzyme.

231

#### 232 $\alpha$ -GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

233 The analysis of the  $\alpha$ -glycosidase inhibitor activity (GA) was done by Widyawati et  
234 al. (2020) method with slight modification. About 150  $\mu$ L samples ~~contained~~ containing  
235 100  $\mu$ L *Pluchea* infusion and 50  $\mu$ L pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M  
236 at pH 7) were reacted with 50  $\mu$ L  $\alpha$ -glycosidase 2 mM (0.0833 unit/mL), and then the  
237 mixture was incubated at 37 °C for 15 min. ~~Finally, the~~The reaction was stopped with the  
238 addition of 1000  $\mu$ L sodium carbonate 0.2 M. The residue of this enzyme hydrolyzed p-  
239 nitrophenyl- $\alpha$ -D-glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The

Commented [A8]: Confusing. Rewrite

240 ~~inhibition~~ activity of ~~steeping the~~ *Pluchea* ~~tea-infusion to enzyme~~ was measured by UV-  
241 vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at  $\lambda$  405 nm.

242 The inhibition percentage of  $\alpha$ -glycosidase was calculated using formula:  $(ACb - ACa) -$   
243  $(As - Ab) (ACb - ACa) \times 100 \%$ . Where, ACb is the absorbance of 100 % enzyme activity  
244 (solvent with enzyme), ACa is the absorbance of 0 % enzyme activity (solvent without  
245 enzyme), As is the absorbance of test sample with enzyme, Ab is the absorbance of test  
246 sample without enzyme.

247

#### 248 HPLC ANALYSIS OF PHENOLICS

Corresponding Author: paini@ukwms.ac.id

249 The phenolic compounds of the samples were analyzed by HPLC based on  
250 Kongkiatpaiboon et al. (2018) method with modifications. Each *Pluchea* infusion was  
251 sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter  
252 syringe (Whatmann, 0.2 µm, NYL). About 20 µL of sample was injected in an HPLC  
253 (LC20AD series, Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC  
254 LC-20AD pump, CTO-30A column oven, CBM-20A/20 Alite system controller, and SPD-  
255 20A/20 AV UV-Vis detector. Separation of phenolic compounds in samples was carried  
256 out using a Shim-pack VP-ODS C18 column (ID 5 µm × 50 mm × 4.6 mm) with a GVP-  
257 ODS Cartridge guard column (2 pieces) (ID 10 mm × 4.6 mm). ~~Analytical conditions:~~ The  
258 mobile phase used consisted of a solution of (A) 0.5 % acetic acid in water and (B)  
259 absolute methanol. Analysis was carried out using a gradient system in the following  
260 order: initial conditions of 10 % B in A to 50 % B in A were maintained for 40 minutes;  
261 then 100 % B was maintained for 20 minutes. Next the column was re-equilibrated with  
262 10 % B in A maintained for 10 minutes before analysis of the next sample. The sample  
263 flow rate was set at 1.0 ml/min with a controlled temperature at 40 °C. Detection was used  
264 at a wavelength of 280 nm. The reference standard used were gallic acid, (+)-catechin,  
265 myricetin, quercetin, kaempferol, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and  
266 4,5-dicaffeoylquinic acid. All of the reference standard was dissolved in distilled water  
267 and prepared similar to the samples before injected in HPLC.

268

## 269 EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

270 The research design used a randomized block design with two factors, i.e., the  
271 steeping temperature (T) and the storage ~~time~~ timeperiod (B). *Pluchea* leaf blades were

272 subjected to 4 steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95  
273 °C (T4), and the storage ~~timeperiod~~ of 0 year /~~fresh-un-stored~~ (B1), and 5 year/stored  
274 (B2). ~~The research resultedresulting in~~ 8 treatment combinations (T1B1, T1B2, T2B1,  
275 T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic was repeated two  
276 ~~timeperiods~~. The data of samples were analyzed by ANOVA at  $\alpha \leq 0.05$ , and continued  
277 analysis using a paired T test at  $\alpha \leq 0.05$ . treatment means of specific phenolic  
278 compounds that were identified were expressed as the mean  $\pm$  SD. The analysis used  
279 SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

280

## 281 RESULTS AND DISCUSSIONS

282 *Pluchea* leaf infusion is produced by young *Pluchea* leaf from 1-6 level on each  
283 branch the shoot (Widyawati et al., 2022), that is steeped at 95 °C for 5 min, has many  
284 biological activities, such as antioxidant activity (Widyawati et al., 2016), antidiabetic  
285 activity (Werdani and Widyawati, 2018), anti-inflammatory (Srisook et al., 2015). The  
286 chemical constituents in *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols,  
287 cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2  
288 g/100 mL steeping *Pluchea* tea has total phenolic content 9.3 mg gallic acid equivalents  
289 (GAE)/g samples, total flavonoid content 22.0 mg catechin equivalents (CE)/g samples,  
290 DPPH free radical scavenging activity 27.2 mg gallic acid equivalents (GAE)/g samples,  
291 and ferric reducing power 10.2 mg gallic acid equivalents (GAE)/g samples (Widyawati et  
292 al., 2016). Previous research has informed related to the composition of phytochemical  
293 compounds in *Pluchea* leaves, such as phenolic acids such as chlorogenic acids, caffeic  
294 acids, 3-O-caffeoylquinic acids, 4-O-caffeoylquinic acids, 5-O-caffeoylquinic acids, 3,4-

**Commented [A9]:** Were all analyses replicated 2 periods only? What do you mean by 'continued analysis using a paired t-test at  $\alpha \leq 0.05$ '? Rewrite this part of the paragraph.

295 di-O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic  
296 acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin;  $\beta$ -  
297 carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al.,  
298 2019; Chan et al., 2022; Widyawati et al., 2022). Presence of phytochemical compounds  
299 in herbal product were influenced by environmental factors, i.e., temperature, light  
300 exposure, oxygen level, pH and moisture. The structure of phytochemical compounds in  
301 herbal tea is very sensitive of the surrounding changes. The effect arising from these  
302 changes causes the structure of the phytochemical molecule to be degraded to produce  
303 smaller size molecules or to combine to produce larger size molecules (Ali et al., 2018;  
304 Jayani et al. 2022, Ramphinwa et al., 2023). Therefore, this study was conducted to  
305 determine the effect of steeping temperature and storage ~~time~~period of *Pluchea* tea on  
306 levels of the bioactive compounds, antioxidant and antidiabetic properties and phenolic  
307 compound profile.

**Commented [A10]:** Delete this part. Information in here are already found in the Introduction section.

## 309 BIOACTIVE COMPOUNDS

### 310 Phenolics Compounds

**Formatted:** Centered

311 The bioactive compounds are active compounds in plants that are essential to  
312 protect a body health (Nguyen and Chuyen, 2020). These compounds usually have many  
313 biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer,  
314 antimicrobial, antibacterial, anti-cholesterol and so on (Suriyaphan, 2014; Acar et al.,  
315 2022). Phenolic compounds have potential redox properties that can scavenge free  
316 radicals that can cause a number of chronic diseases (Noreen et al., 2017; Aryal et al.,  
317 2019; Acar et al., 2022).

318 The total phenolic content (TPC) of *Pluchea* infusion at different steeping  
319 temperature and storage period generally significantly increased with increasing steeping  
320 temperature and storage period based on paired ~~T-t~~ test at  $\alpha \leq 0.05$  (Figure 1a). Steeped  
321 and stored infusion had significantly higher amounts of phenolic compounds than the  
322 samples that were steeped and un-stored. Further, the highest total phenolic content was  
323 observed in samples infused at 95 °C and stored for 5 years (at 71.38±4.14 mg GAE/g  
324 samples) while the lowest was measured in the un-stored samples and infused at 60 °C  
325 (at mg GAE/g sample). Phenolic content of stored samples that were infused at  
326 different temperatures that then stored were steeped only at 60 and 95 °C also showed a  
327 significant increase in their phenolic content. This implies that the steeping temperature  
328 and the storage periods significantly resulted in the high amounts of the phenolic  
329 compounds of the infusions. Results also indicated that phenolic compounds were  
330 generally greater in the infusion at high steeping temperatures and long storage period  
331 (Figure 1a). This could have been due to that fact that during steeping fresh *Pluchea* tea  
332 had a lower total phenolic content than stored *Pluchea* tea for 5 years, besides that the  
333 higher the steeping temperature also caused the greater the extracted total phenolic  
334 content. The temperature of infusion influenced total phenolic content, it could relate to  
335 This could have been due to the fact that the steeping temperature and storage period  
336 can cause the process of degradation, oxidation, and leaching/release of phenolic  
337 compounds. Phenolic compounds are water soluble and thus soaking in hot water for a  
338 certain period of period as in steeping causes the migration process of more phenolic  
339 compounds to the water because of longer increasing contact exposure between of  
340 phenolic compounds to water (Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al.

341 (2022). Su et al. (2019) reported that temperature treatment can stimulate the release  
342 of phenolic compounds of lychee juice stored at different temperatures of 4 and 45 °C  
343 and different long storage (fresh and 72 hours).  
344 this compounds and water. The same phenomena also occurred in Castiglioni  
345 et al. (2015); Kilic et al. (2017), and Acar et al. (2022).  
346 This occurrence showed that steeping temperature and storage period caused the  
347 process of degradation and oxidation of phenolic compounds. Su et al. (2019) reported  
348 that temperature treatment can stimulate the release of phenolic compounds and  
349 increase antioxidant activity of lychee juice stored at different temperatures of 4 and 45  
350 °C and different long storage (fresh and 72 hours). Hydrogen bonding is affected by  
351 Temperature treatment because the degrades (or hydrolyzes) the hydrogen bond  
352 between phenolic compounds and proteins can be degraded that the measured levels  
353 resulting in an increase of phenolic compounds when exposed to are higher  
354 temperatures. The phenomena were supported by (Ali et al. (2018); Jayani et al. (2022),  
355 and Ramphinwa et al. (2023). Zhang et al. (2021) reported that phenolic compounds  
356 present in plants are not completely stable, but are easily degraded during storage after  
357 harvest. Reblova (2012) claimed that antioxidant compounds can be slowly degraded  
358 with increasing temperature. Besides that, Fibrianto et al. (2021) also stated that the  
359 brewing temperature has an effect on the extracted antioxidant compounds, such as  
360 alkaloids, catechins and tannins. Thus, there is an assumption that temperature and  
361 storage caused the degradation, oxidation and hydrolysis of the phenolic compounds that  
362 the phenolic compounds in *Pluchea* infusion are degraded due to oxidation and hydrolysis  
363 because of temperature and storage time period and can be easily extracted during



364 steeping, thus resulting in the increased amount of the phenolic content  
365 compounds as the at higher steeping temperature and longer storage increase period.

366 Based on using of a reference standard could be informed that Simple phenolic  
367 compounds identified in steeped and stored in *Pluchea leaf* infusion, including gallic  
368 acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-O-caffeoylquinic acids,  
369 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic acids was showed in Table 1.

370 The treatment effects results of statistical analysis using a paired T test at  $\alpha \leq 0.05$  showed  
371 that gallic acid and kaempferol contents of *Pluchea* infusion were insignificantly different  
372 at various steeping temperature and long storage periods. Nevertheless, the The  
373 concentration of quercetin and 3,5-di-O-caffeoylquinic acid of the un-stored and stored  
374 *Pluchea* infusion was significantly different from the rest of the samples between of two  
375 treatments except at 70 °C. The while (+)-catechin concentration of *Pluchea* infusion was  
376 only significantly different at 95 °C, but the myricetin content was significantly different  
377 different concentration at 80 and 95 °C. The 3,4-di-O-caffeoylquinic acid content showed  
378 significace difference at 60, 80 and 95 °C and while 4,5-O-dicaffeoylquinic acid  
379 compounds content from *Pluchea* infusion were was only significantly different at 60 °C,  
380 however the concentration of 3,4 dicaffeoylquinic acid was also significantly different at  
381 80 and 95 °C.

382 Based on the analysis of concentration of Results further showed simple phenolic  
383 compounds showed that gallic acids and kaempferol were relatively stable phenolic acid  
384 because of as reflected by the insignificant changes when exposed no changes at to the  
385 different steeping temperature and storage time period, with concentration about 0.24 ±  
386 0.00 to 0.24 ± 0.02 µg/g samples and 0.14 ± 0.02 to 0.95 ± 0.03 µg/g samples, respectively.

387 ~~However, myricetin~~Myricetin, (+)-catechin and 3,4-di-O-caffeoylquinic acid showed a  
388 drastic ~~increasing~~ increase at higher steeping temperature and longer storage period  
389 ~~implying -It's meant~~ that these compounds tended to be relatively labile. Quercetin, 3,5-  
390 di-O-caffeoylquinic acid and 4,5-di-O-caffeoylquinic acid underwent moderate changes  
391 ~~compared to the other two groups of phenolic acids.~~ Therefore, myricetin, (+)-catechin  
392 and 3,4-di-O-caffeoylquinic acid were easier to dissolve or degraded to form simple  
393 phenolic compounds at higher steeping temperature and storage ~~time~~period. ~~can cause~~  
394 ~~macromolecules of three phenolic acids in herbal tea convenient degradable to form~~  
395 ~~simple phenolic compounds for storage, as explained by~~ (Su et al. (2019), Ali et al. (2018);  
396 Jayani et al. (2022); Ramphinwa et al. (2023), and Zhang et al. (2021). Degradable  
397 polyphenol compounds have a simple structure and free hydroxyl groups that can react  
398 with Folin-Ciocalteu's Phenol reagent, resulting complex blue solution that can detected  
399 as total phenolic content.

Commented [A11]: Does the negative sign mean an increase or decrease

#### 400 Flavonoid Content (TFC)

401 Flavonoids are the major phenolic compounds that have potential chemical and  
402 biological activities, such as radical scavenging and antimicrobial activities (Ayele et al.,  
403 2022; Chandra et al., 2014) that can protect the human body from the oxidative stress  
404 caused many degenerative diseases, especially cancer, cardiovascular problems and  
405 ageing (Mathur and Vijayvergia, 2017). The total flavonoid content of steeped *Pluchea*  
406 infusion decreased with longer storage period. Un-stored samples exhibited higher  
407 flavonoid content than the stored samples. The statistical analysis using a paired T test  
408 at  $\alpha= 0.05$  showed that total flavonoid content of *Pluchea* infusion was significantly  
409 different between ~~two treatments~~the steeped un-stored and steeped stored samples

Commented [A12]: What does the negative (-) sign implies? What is your basis of classifying the simple phenolic compounds as relatively labile, moderate?

Formatted: Centered

410 (Figure 1b). The highest total flavonoid content was exhibited by ~~fresh the un-stored~~  
411 ~~samples steeped at 95 °C at~~ about 147.42±14.03 mg CE/g samples. Total flavonoid  
412 content was significantly lower in the stored ~~samples regardless of steeping temperature~~  
413 ~~than those of the un-stored around 24.75±2.47 to 33.71±3.06 mg CE/g samples~~ implying  
414 that the increase in the flavonoid content of the infusion was affected primarily by the  
415 steeping temperature.

Commented [A13]: cite similar studies to support your findings

#### 416 Tannin Content (TTC)

Formatted: Centered

417 Tannins are bioactive compounds that provide properties, such as astringent, anti-  
418 diarrheal, antibacterial and antioxidant (Malangngi et al., 2012). Generally, results  
419 indicated that the total tannin content of *Pluchea* infusion significantly increased with  
420 increasing steeping temperature and storage period (Figure 1c). Among, the un-stored  
421 steeped samples, the tannin content was significantly lowest in ~~the~~ samples infused at 60  
422 °C ~~at~~ about 4.81±0.58 to 17.42±1.04 mg TAE/g samples, ~~which is was~~ significantly  
423 different ~~lower~~ from ~~that of~~ the lowest tannin content of the stored samples. Among the  
424 stored and steeped samples, the highest tannin content was observed at samples  
425 steeped at 95 °C about 17.42 ± 1.04 mg TAE/g samples and was significantly different  
426 from that of the highest tannin content of the un-stored steeped samples at 95 °C about  
427 9.22 ± 1.48 mg TAE/g samples. ~~Indicating that the tannin content was~~ primarily affected  
428 ~~by both high steeping temperature and long storage period~~ ~~than high steeping~~  
429 ~~temperature and that the presence of high tannin content was primarily brought about by~~  
430 ~~long storage period.~~ Kowalska et al. (2021) informed that ~~the~~ condensation of catechins  
431 to tannins ~~of polyphenolic compounds~~ is a dominant process ~~occurred~~ ~~occurring~~ in tea  
432 leaves that is accelerated during maceration of raw ~~material~~ tea leaves (Kowalska et al.

433 (2021) could have had contributed to the observed increase in the tannin content in the  
434 treated samples. However, the high temperature can degrade polyphenolic compounds  
435 to form simple phenolic compounds that is essential to body health. The results showed,  
436 that the higher the brewing temperature and the longer the storage time caused the tannin  
437 compound to degrade to result catechin compounds. This phenomenon is in line with the  
438 increase in total phenol levels and the concentration of (+) catechin compounds. Ali et al.  
439 (2018) said that pH, storage temperature, chemical structure and concentration, light,  
440 oxygen, enzymes and metal ions affect the presence of bioactive compounds in the  
441 material. Nevertheless,

442 Although, high temperature and long storage period can cause the degradation of  
443 tannins to catechins, Rusita et al. (2019) emphasized that tannins are a polar  
444 thermostable complex compounds, that is are resistant to heating, indicating that even  
445 with the exposure to high temperature, the tannins still remained high in the treated  
446 samples as a result the tannin content in *Pluchea* tea increases with increasing steeping  
447 temperature and storage time period, this is caused tannins are thermostable complex  
448 compounds.

#### 450 ANTIOXIDANT ACTIVITY

451 Antioxidant activity is capability of compounds to inhibit the oxidation of  
452 macromolecules from biological target that involve in oxidative chain reactions (Ali et al.,  
453 2005; Oh et al., 2013). The antioxidant activity assay was done in this research using  
454 DPPH Free Radical Scavenging Activity (DPPH) and ferric reducing power (FRAP)  
455 methods. The phenolic compounds are an active antioxidant that have antioxidant

456 capability ~~that~~ depends on their redox properties. The structure of phenolic compounds  
457 determine ~~the~~ effectivity to ~~doner donate~~ hydrogen atom which is negatively correlated  
458 with the O-H phenolic bond strength. The higher antioxidant power of phenolic  
459 compounds is caused ~~by~~ the weaker O-H phenolic bond (Kruk et al., 2022). The  
460 mechanism of phenolic compounds ~~is involved~~ as antioxidants ~~through depends on their~~  
461 the ability to donate hydrogen atom ~~ands~~, transfer electrons, ~~and as~~ reducing agents and  
462 singlet oxygen quenchers (Ali et al., 2005; Huang et al. 2005).

Commented [A14]: what do you mean? rewrite

#### 464 DPPH Free Radical Scavenging Activity

Formatted: Centered

465 DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate  
466 antioxidant activity because this method ~~is simple~~ that is suitable to measure the donating  
467 hydrogen atoms capability of ~~herbal infusion~~. This reaction can cause the purple color of  
468 ~~DPPH to change to yellow color~~ (Munteanu and Apetrei, 2021; Baliyan et al., 2022).  
469 ~~Figure 2a shows that the free radical scavenging property of the stored and steeped~~  
470 ~~samples were significantly higher than the un-stored steeped samples. The result of~~  
471 ~~DPPH assay~~ It can also be observed ~~indicates~~ that the ~~free radical scavenging property~~  
472 ~~DPPH values accrued~~ was significantly different among the stored and steeped samples  
473 ~~but insignificant among the un-stored and steeped samples at higher steeping~~  
474 ~~temperature and longer storage timeperiod. Statistical analysis by ANOVA using a paired~~  
475 ~~T test at  $\alpha \leq 0.05$  proven that the higher the steeping temperature of fresh *Pluchea*~~  
476 ~~infusion (T1B1, T2B1, T3B1, and T4B1) was consistent the ability to DPPH free radicals~~  
477 ~~scavenging activity, whereas the stored *Pluchea* infusion resulted in the higher activity~~  
478 ~~and the values went up as rising of the infusion temperature. *Pluchea* infusion stored at~~

479 room temperature for 5 years resulted in the high DPPH-free radical scavenging activity  
480 by more than 100 %. Steeping at higher temperatures significantly increased the DPPH  
481 free radical scavenging activity in stored *Pluchea* infusion by around 15 to 25 %. Steeping  
482 at 80-95 °C in stored *Pluchea* infusion insignificantly affected the free radical scavenging  
483 property of the bioactive compounds (Figure 2a). This implies that that the higher free  
484 radical scavenging property was primarily affected by the storage period than steeping  
485 temperature. During the storage process it is possible to form complex phenolic  
486 compounds which provide a high ability to scavenge DPPH-free radicals  
487 (Thanajiruschaya et al., 2010)

488 Scavenging The scavenging activity of DPPH free radicals of the the samples was  
489 strongly and positively correlated with total phenolic and tannin contents levels,  
490 but inversely to with total flavonoid levels. Based on Pearson correlation at Table 2, the  
491 correlated coefficient values (*r*) between DPPH and TPC, TTC and TFC were 0.993,  
492 0.942, and 0.940, respectively. During the storage process it is possible to form complex  
493 phenolic compounds which provide a high ability to scavenge DPPH free radicals  
494 (Thanajiruschaya et al., 2010). This research study also demonstrated that longer storage  
495 time period and higher infusion temperature produced many simple phenolic compounds  
496 with free hydroxyl groups capable to donor hydrogen atom to DPPH free radical. Many  
497 phenolic acids, such as gallic acids, (+)-catechins, myricetins, kaempferols, quercetins,  
498 3,5-di-O-caffeoylquinic acids, 3,4-di-O-caffeoylquinic acids, 4,5-di-O-caffeoylquinic acids  
499 have established potential antioxidant activity (Kumar and Goel, 2019) (Table 1). Kruk  
500 et al (2022) informed that the capability of phenolic compounds to donor hydrogen atom  
501 depends on chemical structure, number and position of hydroxyl groups attached to a

Commented [A15]:

Commented [A16R15]: Clarify on how you were able to come up with free radical scavenging activity by more than 100 %. Steeping temperatures significantly increased the free radical scavenging activity in stored *Pluchea* infusion by around 15 to 25 %

Commented [A17]: Explain/interpret this observation based on the data that you were able to obtain.

502 benzene ring, a double bond between C2 and C3 rings and a carbonyl group (C=O) on  
503 the C ring at C4. The effectivity of antioxidant compounds donor hydrogen atom is  
504 determined by O-H bond dissociation energy.

505 The DPPH-free radical scavenging property observed in the study was not in  
506 consistent with the results of the study by Moraes-de-Souza et al. (2008). The research  
507 shows that total phenolic content of herbal infusion is low correlated with DPPH-free  
508 radical scavenging activity. However, Dobrinas et al. (2021) informed that total phenolic  
509 content of tea infusion is positively and significantly correlated with the free radical  
510 scavenging property/inhibitor activity of DPPH of tea infusion.

511

#### Ferric Reducing Antioxidant Power (FRAP)

Formatted: Centered

512 FRAP is an analysis of antioxidant power of the phytochemical compounds based  
513 on the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic  
514 acid, and ferric chloride to produce a color complex, that can be measured at  $\lambda$  700 nm  
515 (Fu et al., 2011; Al-Temimi and Choudhary, 2013). The principle of the assay measures  
516 that is based of the ability of antioxidant compounds to reduce iron ions of potassium  
517 ferrocyanide ( $\text{Fe}^{3+}$ ) to be potassium ferrocyanide ( $\text{Fe}^{2+}$ ). Potassium ferrocyanide reacts  
518 with ferric chloride to form a ferric-ferrous complex and results green color solution  
519 (Widyawati et al., 2017; Raharjo and Haryoto, 2019).

520 The results showed that the ferric reducing antioxidant power (FRAP) increased  
521 with at higher steeping temperature and longer storage time period. The lowest FRAP was  
522 observed in the un-stored samples which was steeped at 60 °C at  $3.95 \pm 0.17$  mg gallic  
523 acid equivalents (GAE)/g samples, and the highest was owned exhibited by in *Pluchea*

525 infusion which was stored for 5 years at 95 °C at 48.63 ±10.83 mg gallic acid equivalents  
526 (GAE)/g samples (Figure 2b). FRAP increased significantly as steeping temperature was  
527 increased. FRAP of the samples stored for 5 years was also significantly higher than the  
528 un-stored samples at  $\alpha \leq 0.05$ . Based on Pearson correlation, the FRAP of *Pluchea*  
529 infusion was strongly and positively significant correlated with the DPPH, TPC and TTC,  
530 but inversely to TFC. The correlated coefficient values (r) between FRAP and DPPH,  
531 TPC, TTC and TFC were 0.956, 0.953, 0.948 and -0.826, respectively.

532 This case was is in contrast to with the study on the antioxidant activity of DPPH  
533 and FRAP on of matcha, because The the longer storage time period reduces the levels  
534 of catechin content due to the catechins, such as epigallocatechin gallat (EGCG),  
535 epicatechin gallat (ECG), epigallocatechin (EGC), and epicatechin (EC) which are  
536 bioactive compounds that have high antioxidant activity (Kim et al. 2020), and also the  
537 case of the effect of temperature and storage time in betel (*Piper bettle* L.) extract. Light  
538 and temperature influence degradation of phenolic compounds of betel that determine  
539 antioxidant activity. Different structure of phenolic compounds determines their stability  
540 to degrade accelerating of light and temperature. Hydroxychavicol is the best stability of  
541 phenolic compounds of betel compared with eugenol, isoeugenol and allyl pyrocatechol  
542 (Ali et al., 2018). Thanajiruschaya et al. (2010) revealed that the antioxidant activity of  
543 rice stored at high temperatures is greater than that stored at low temperatures. The ferric  
544 reducing capability of *Pluchea* could have due infusion corresponded to the presence to  
545 of simple phenolic acid values that have the ability to transfer electron from their free  
546 hydroxyl groups of, presence of them in samples could accrue antioxidant activity  
547 because of ability of the electron transfer from free hydroxyl groups of phenolic acids.

Commented [A18]: Relate these with Figure 2b. Rewrite



548 [The FRAP of \*Pluchea\* infusion was strongly and positively significant correlated with the](#)  
549 [DPPH, TPC and TTC, but inversely to TFC.](#)

550 ANTIDIABETIC ACTIVITY

551 [α-Amylase enzyme inhibition activity](#) (AA)

Formatted: Centered

552 [Antidiabetic activity is a measure of the potency of phenolic compounds to regulate](#)  
553 [the uptake of glucose by the cells from the blood through the mediation of 2-degestive](#)  
554 [enzymes i.e., α-amylase and α-glucosidase, which are involved the control of dietary](#)  
555 [carbohydrate digestion and release in the postprandial blood glucose in human body \(Fu](#)  
556 [et al., 2017\). The phenolic compounds have the capability to bind with the protein](#)  
557 [component of α-amylase and α-glucosidase enzymes \(Martinez-Solis et al., 2022\)](#)  
558 [resulting in the reduced activity of the enzymes. The results showed, that the lower](#)  
559 [steeping \*Pluchea\* leaf infusion was able to inhibit the action of the α-amylase enzymes](#)  
560 [\(Figure 3a\). The \*Pluchea\* infusion had very good activity, exhibited a good α-amylase](#)  
561 [enzyme inhibition activity of more than 50 % and even almost 100 % for fresh in the un-](#)  
562 [stored \*Pluchea\* infusion which steeped was brewed at 60, 70 and 80 °C with highest at](#)  
563 [60 °C, and in stored \*Pluchea\* leaf infusion which was steeped at 60 °C. Whereas The](#)  
564 [stored fresh \*Pluchea\* leaf infusion steeped at 70, 80 and 95 °C for 5 minutes had lower](#)  
565 [enzyme inhibition activity an activity of of less than 50 % with lowest at 95 °C inhibiting the](#)  
566 [α-amylase enzyme of less than 50 %, which was equal to 40.08±1.12 %. Widyawati et al.](#)  
567 [\(2017\) detected found that the ability to inhibit the α-amylase enzyme from in fresh un-](#)  
568 [stored \*Pluchea\* infusion steeped at 95 °C for 5 minutes by was also low at 28.79 %.](#)  
569 [Increasing the steeping temperature and storage time period reduced the ability to of the](#)  
570 [phytochemicals in the \*Pluchea\* infusions to inhibit the α-amylase enzyme activity. The](#)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Commented [A19]: Why?/Explain

571 results of the analysis based on a paired T test at  $\alpha \leq 0.05$  showed, that the steeping  
572 temperature and storage timeperiod had a significant effect on the ability to inhibit the  $\alpha$ -  
573 amylase enzyme. Based on Pearson correlation, the Table 2 further shows that the AA of  
574 *Pluchea* infusion was strongly and negatively significant correlated with TPC, TTC, DPPH  
575 and FRAP, but it was moderately and negatively significant correlated with TFC. The  
576 correlated coefficient values ( $r$ ) between AA and TPC, TTC, DPPH, FRAP and TFC were  
577 0.708, -0.857, -0.696, -0.806 and 0.429, respectively.

578 This inhibitory activity was thought to be contributed by other bioactive compounds,  
579 besides phenolics which are sensitive to steeping temperature and storage timeperiod. Li  
580 et al. (2018) stated that there are flavonoid compounds that contribute to the ability to  
581 inhibit the  $\alpha$ -amylase enzyme. Flavonoid compounds with a hydroxyl structure at C-4' in  
582 ring B are more effective than C-6 in ring A. Akah et al. (2011) informed reported that the  
583 phytochemical compounds, such as terpenoids, saponins, flavonoids, glycosides and  
584 carbohydrate, and alkaloids are good antidiabetic metabolites or  $\alpha$ -amylase enzyme  
585 activity inhibitor. Sangeetha and Vedaşree (2012) explained, that the ability to inhibit the  
586  $\alpha$ -amylase enzyme was determined by the content of the phenolic compound and protein.  
587 The  $\alpha$ -amylase inhibitor enzyme present in *Pluchea* infusion may be proteinaceous in  
588 nature. Alexandre et al. (2022) informed that phenolic acids have inhibition activity to  $\alpha$ -  
589 amylase enzyme depending their structures. Besides that, capability of phenolic acids to  
590 inhibit  $\alpha$ -amylase enzyme was determined by low half-maximum inhibitory concentration  
591 ( $IC_{50}$ ). There are C=C double bond conjugated with a carbonyl group of phenolic  
592 structures that stabilizes the binding forces to the active site of the  $\alpha$ -amylase. The  
593 hydroxyl groups of them are able to bind by non-covalent interaction, such as hydrogen

Commented [A20]: Implications? Explain

Commented [A21]: What content or what is in the content the influenced the ability to inhibit the enzyme?

Commented [A22]: How will this affect the ability to inhibit the enzyme?

594 binding, cation- $\pi$  interactions, salt bridge interactions, ionic interactions or electrostatic  
595 forces with amino acid residue at the active site in  $\alpha$ -amylase enzyme. ~~Elevated steeping~~  
596 ~~temperature and longer storage period~~ ~~The steeping temperature and storage time can~~  
597 ~~easily cause the~~ removal of the ~~e~~ hydroxyl groups of phenolic compounds that can reduce  
598 ~~their~~ ability of enzyme inhibition. The phenolic acids with a greater number of hydroxyl  
599 groups ~~are exhibits~~ stronger capability to obstruct the  $\alpha$ -amylase enzyme.

**Commented [A23]:** Lines 585 to 595, Either delete or rewrite for better readability and understanding referring to enzyme activity inhibition

#### 600 $\alpha$ -Glucosidase enzyme inhibition activity (GA)

**Commented [A24]:** This part is disorganized. Avoid duplicating statements, observation facts etc

**Formatted:** Centered

601 ~~Alpha~~ $\alpha$ -glucosidase is an important enzyme in carbohydrates digestion, that  
602 catalysis the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and  
603 converts them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et  
604 al., 2014; Proenca et al., 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -  
605 glucosidase enzyme is used to determine ~~their~~ antidiabetics activity. ~~This is supported~~  
606 ~~by~~ Werdani and Widyawati (2018) ~~stated~~, that **Pluchea infusion** has the potential as an  
607 antidiabetic agent. Widyawati et al. (2020) found that brewing fresh **Pluchea infusion** at  
608 95 °C for 5 minutes has an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857 %.

609 ~~The results showed~~, ~~Figure 3b shows~~ that the ability of the **Pluchea leaf infusion**  
610 to inhibit the  $\alpha$ -glucosidase enzyme decreased with increasing steeping temperature and  
611 storage ~~time~~period. Steeping at 95 °C ~~for fresh~~ of the un-stored **Pluchea leaf** infusion (~~un-~~  
612 ~~stored~~) obtained the lowest inhibitory ability, i.e.,  $48.32 \pm 1.27$  %, and the highest inhibitory  
613 activity was found at 70 °C ~~steeping temperature for fresh~~ **Pluchea** infusion, which was at  
614  $95.11 \pm 0.70$ % ~~(Figure 3b)~~. The results of a paired T test showed that GA of **Pluchea**  
615 **infusion** was significantly different ~~at both~~ between steeping temperature and long storage.  
616 ~~The antidiabetic activity of~~ **Pluchea infusion** ~~Figure 3 further showed~~ shows that the ability

**Formatted:** Font: Not Italic

**Commented [A25]:** Explain

617 of *Pulchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher than the  
618 ability to inhibit the  $\alpha$ -amylase enzyme. Li et al. (2018) informed that flavonoid compounds  
619 have the ability to inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. This is  
620 due to the total flavonoids in steeped *Pluchea* infusion which tended to have the same  
621 pattern as the ability to inhibit the activity of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes.  
622 The statistical analysis using Pearson correlation showed that GA of *Pluchea* infusion  
623 was strongly and negatively correlated with TPC, TTC, DPPH and FRAP  
624 ~~with r was -0.555, -0.715, -0.527 and -0.560, respectively.~~ However, GA was  
625 moderately and positively correlated to TFC, ~~with r was 0.350 and strongly and positively~~  
626 ~~correlated to AA, with r was 0.725.~~ Flavonoid compounds, such as rutin, myricetin,  
627 kaempferol, and quercetin ~~which~~ have antioxidant and antihyperglycemic activities. The  
628 ability to inhibit the action of enzymes from flavonoid compounds is determined by the  
629 position and number of hydroxyl groups and the number of double bonds in rings A and  
630 B and the heterocyclic ring in ring C. The ability to inhibit the  $\alpha$ -glucosidase enzyme from  
631 *Pluchea* infusion was significantly affected by the steeping temperature and long storage.  
632 The capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater than  
633 the  $\alpha$ -amylase enzyme because the mechanism of two enzymes was different, according  
634 to the opinion of McCue et al. (2005). Widyawati et al. (2017) informed that phenolic and  
635 non-phenolic compounds determine the inhibitory activity of the  $\alpha$ -glucosidase enzyme.  
636 The ability of bound phenolic compounds to inhibit  $\alpha$ -glucosidase enzymes was higher  
637 than free phenolic compounds. The presence of polymerization and degradation  
638 reactions, that may be occurred in *Pluchea* infusion during storage, affects the structure  
639 and profile of phenolic and non-phenolic compounds. Asriningtyas et al. (2014) claimed

Formatted: Font: Italic

Commented [A26]: This does not explain explain lines 597-599 with the manner it is written. Include statements that connect the explanation with the observation. Having 'same pattern' is not observed in the figure/graph

Commented [A27]: Interpret/Implications

Commented [A28]: Delete literature citations that are unnecessary to explain the findings

640 that *Pluchea* leaves contain 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid  
641 methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid,  
642 and 1,3,4,5-tetra-*O*-caffeoylquinic acid. Quinic acid is methyl esterified with the number  
643 of caffeic groups in the molecule that determines the activity of inhibiting the  $\alpha$ -  
644 glucosidase enzyme. Analysis of caffeoylquinic acids in *Pluchea* infusion was obtained  
645 that the higher steeping temperature and long storage caused increased concentration  
646 of them, but the  $\alpha$ -glucosidase inhibition activity of them was reduced. Aleixandre et al.  
647 (2022) reported that the simple phenolic acids forming a dipole-dipole interaction of active  
648 site from  $\alpha$ -glucosidase enzyme are effectively inhibiting the enzyme.

649 This study ~~was obtained information~~ showed that the increasing of steeping  
650 temperature and storage ~~time period~~ caused a degradation reaction of polyphenol  
651 compounds to produce simple phenolic compounds, such as gallic acid, (+)-catechin,  
652 myricetin, quercetin, kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic  
653 acid, and 4,5-di-*O*-caffeoylquinic acid, supported the results of total phenolic content and  
654 total tannin content assays. Increased concentration of simple phenolic compounds  
655 determined the ability of these compounds as antioxidant agents, but reduced their  
656 capability as antidiabetic agents.

## 658 CONCLUSION

659 The steeping temperature and storage ~~time period~~ of *Pluchea* infusion significantly  
660 influenced bioactive contents, antioxidant and antidiabetic activities. TPC, TTC, and TFC  
661 were significantly different at various steeping temperature and storage period based on  
662 statistical analysis using a paired ~~T-t~~ test at  $\alpha \leq 0.05$ . ~~There was the difference of t~~The

Corresponding Author: paini@ukwms.ac.id

**Commented [A29]:** Unnecessary because this is not included as one of the derived simple phenolic acids

**Commented [A30]:** Not clear, re-write

**Commented [A31]:** Organize the discussion to explain the observation one at a period. ex:

1) 'Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme decreased with increasing steeping temperature and storage period.' (This must be followed by an explanation or support.)

2) However, GA was moderately and positively correlated to TFC and positively correlated to AA..(This must be followed by implications/support/explanation.)

The ability to inhibit the  $\alpha$ -glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping temperature and long storage.(This can be integrated in 1)

4) The capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater than the  $\alpha$ -amylase enzyme because the mechanism of two enzymes was different, according to the opinion of McCue et al. (2005). (This can be integrated in 1)

The mechanism must be explained -the mechanism of two enzymes was different,

5) Flavonoid compounds, such as rutin, myricetin, kaempferol, and quercetin which have antioxidant and antihyperglycemic activities

6) . Li et al. (2018) informed that flavonoid compounds have the ability to inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. ( May also be integrated in 1)

7) Widyawati et al. (2017) informed that phenolic and non-phenolic compounds determine the inhibitory activity of the  $\alpha$ -glucosidase enzyme. The ability of bound phenolic compounds to inhibit  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. (May also be incorporated in 1).

8) Lines 618 to 629 into 1)

**Commented [A32]:** Reconcile with your discussion

**Commented [A33]:** Suggested conclusion

## CONCLUSION

The total phenolic content (TPC) of *Pluchea* infusion at different steeping temperature and storage period generally significantly increased with increasing steeping temperature and storage period. Steeped and stored infusion had significantly higher amounts of phenolic compounds than the samples that were steeped and un-stored. TPC was highest in the store

663 phenolic compound profile in ~~fresh~~ ~~the~~ ~~unstored~~ and stored ~~of~~ *Pluchea* infusion ~~and~~ ~~at~~  
664 various steeping temperature. ~~The~~ ~~included~~ simple phenolic compounds ~~were~~ ~~detected~~  
665 ~~in~~ *Pluchea* infusion ~~including~~ ~~such~~ ~~as~~ gallic acid, (+)-catechin, quercetin, myricetin,  
666 kaempferol, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-  
667 caffeoylquinic acid. The results of statistical analysis using a paired ~~T~~ ~~t~~ test at  $\alpha \leq 0.05$   
668 showed that gallic acid and kaempferol of *Pluchea* infusion were insignificantly different  
669 at various steeping temperature and long storage. ~~Nevertheless,~~ ~~T~~ the concentration of  
670 quercetin and 3,5-dicaffeoylquinic acid of *Pluchea* infusion was significantly different of  
671 two treatments except at 70 °C. The (+)-catechin concentration of *Pluchea* infusion was  
672 significantly different at 95 °C, but the myricetin was different concentration at 80 and 95  
673 °C. The 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid compounds from *Pluchea*  
674 infusion were significantly different at 60 °C, however the concentration of 3,4-  
675 dicaffeoylquinic acid was also significantly different at 80 and 95 °C. TPC, TTC and TFC  
676 of *Pluchea* infusion were significantly different at various steeping temperature and  
677 storage period. TPC and TTC significantly increased with increasing steeping  
678 temperature and long storage, but TFC significantly increased at various steeping  
679 temperature and significantly decreased at long storage. The bioactive compounds of  
680 *Pluchea* infusion influenced antioxidant activities (DPPH and FRAP) and antidiabetic  
681 activity (AA and GA). The DPPH was strongly and positively correlated with TPC and  
682 TTC, but it was strongly and negatively correlated with TFC, with coefficient  $r$  0.993,  
683 0.942, and -0.940, respectively. The correlated pattern between FRAP and bioactive  
684 contents of *Pluchea* infusion was similar to it between DPPH and bioactive contents. The  
685 correlated coefficient values ( $r$ ) between FRAP and TPC, TTC and TFC were 0.953, 0.948

686 and -0.826, respectively. The AA and GA were strongly and negatively correlated with  
687 TPC, TTC, DPPH and FRAP, but it was moderately and negatively significant correlated  
688 with TFC. Between the antioxidant activity of DPPH and FRAP and the antidiabetic  
689 activity of AA and GA of Pluchea infusion were strongly and positively correlated with  
690 correlation coefficient (r) values of 0.956 and 0.725, respectively.

691

#### 692 DATA AVAILABILITY

693 Table and figure used to support of this study were included in the article.

694

#### 695 CONFLICT OF INTEREST

696 The authors declare no conflict of interest.

697

#### 698 ACKNOWLEDGEMENTS

699 The authors would like to thank the he Ministry of Education and Culture of the Republic  
700 of Indonesia for fundamental research grant to higher education institutions in 2022

701

#### 702 REFERENCES

- 703 ACAR A, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food  
704 wastes:evaluation of temperature and time effects on quality parameters. Applied Food  
705 Res. 2(100087):1-8.
- 706 ALEIXANDRE A et al. 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -  
707 glucosidase and influence of reaction conditions. Food Chem. 372(131231):1-9.

**Commented [A34]:** Check for authors that may had been deleted in the paper due to revisions done.

708 ALI et al. 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile.  
709 Food Chem. 89(2005):27-36.

710 ALI A. et al. 2018. Impact of storage conditions on the stability of predominant phenolic  
711 constituents and antioxidant activity of dried *piper bettle* extracts. Molecules.23(24):1-13.

712 AL-TEMIMI A, CHOUDHARY R. 2013. Determination of antioxidant activity in different  
713 kinds of plants *in vivo* and *in vitro* by using diverse technical methods. J Nut. Food Sci.  
714 3(1):1-6.

715 AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and  
716 methanol extract and fractions of *Gongronema latifolium* (Asclepidaceae) leaves in  
717 alloxan diabetic rats. J Applied Pharm. Sci. 01(09): 99-102.

718 ARSININGTYAS IS. et al. 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves  
719 of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product  
720 use. Natural Product Res. 28(17):1350-1353.

721 ARYAL S et al. 2019. Total phenolic content, flavonoid content and antioxidant of wild  
722 vegetables from Western Nepal. Plants. 8(96): 1-12.

723 AYELE DT, AKELE ML, MELESE A.T. 2022. Analysis of total phenolic contents,  
724 favonoids, antioxidant and antibacterial activities of *Croton macrostachyus* root extracts.  
725 BMC Chem. 16(30): 1-9.

726 BALIYAN S et al. 2022. Determination of antioxidants by DPPH radical scavenging  
727 activity and quantitative phytochemical analysis of *Ficus religiosa*. Molecules. 27(1326):  
728 1-19.



729 CASTIGLIONI S et al. 2015. Influence of steeping conditions (time, temperature, and  
730 particle size) on antioxidant properties and sensory attributes of some white and green  
731 teas. Intern. J. Food Sci. Nut. Online 1-7.

732 Chan EWC et al. 2022. *Pluchea indica*: An updated review of its botany, uses, bioactive  
733 compounds and pharmacological properties. Pharmaceutical Sciences Asia. 49(1): 77-  
734 85.

735 CHANDRA S et al. 2014. Assessment of total phenolic and flavonoid content, antioxidant  
736 properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit  
737 crops: A Comparative study. Hindawi Publishing Corporation Evidence-Based  
738 Complementary Alter. Med. 2014:1-9 (2014)

739 CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic, flavonoids,  
740 tannin and chlorophyll content of leaves of *Strobilanthes Kunthiana (Neelakurinji)*. J.Med.  
741 Plants Studies. 4(4):282-286.

742 DOBRINAS S, SOCEANU A, POPESCU V, POPOVICI IC, JITARIU D. 2021.  
743 Relationship between total phenolic content, antioxidant capacity, Fe and Cu content from  
744 tea plant samples at different brewing times. Processes. 9(1311):1-11.c

745 FERRARIS Z. 2023. *Pluchea indica* Less.iNaturalist.org.  
746 <https://www.gbif.org/occurrence/4022273660>.

747 FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional  
748 properties and visual appearance of Dampit Robusta coffee leaves tea. Advances in Food  
749 Sci. Sustain. Agric. Agro-industrial Engineering. 4(1):25-32.

750 FU L et al. 2017. Total phenolic contents and antioxidant capacities of herbal and tea  
751 infusions. Intern. J. Molecular Sci. 12:2112-2124.

Corresponding Author: paini@ukwms.ac.id

752 GAO MR et al. 2019. A theoretical and experimental study: the influence of different  
753 standards on the determination of total phenol content in the Folin–Ciocalteu assay. J.  
754 Food Measurement and Characteristic. 13:1349–1356.

755 HUANG D, OU B, PRIOR RL. 2005. The Chemistry behind antioxidant capacity assays.  
756 J. Agric. Food Chem. 53:1841-1856.

757 ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties  
758 of rosehip tea beverage: study by response surface methodology. J. Food Sci. Tech.  
759 54(11):3737–3743.

760 JAYANI NIE et al. 2022. Evaluation of stability and quality characteristics of moringa  
761 (*Moringa oleifera*) herbal tea during storage. Food Res. 6(4):399-406.

762 KIM JM et al. 2020. Effect of storage temperature on the antioxidant activity and catechins  
763 stability of Matcha (*Camellia sinensis*). Food Science and Biotechnology. 29(9):1261–  
764 1271.

765 KILIC et al. 2017. Antioxidant properties of some herbal teas (green tea, senna, corn silk,  
766 rosmery) brewed at different temperatures. Intern. J. Secondary Metabolite. 4(3):142-248  
767 Special Issue 1.

768 KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of  
769 extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves  
770 from different provenances in Thailand. Revista Brasileira de Farmacognosia. 28(2018):  
771 145–150.

772 KOWALSKA J et al. 2021. Influence of tea brewing parameters on the antioxidant  
773 potential of infusions and extracts depending on the degree of processing of the leaves  
774 of *Camellia sinensis*. Molecules.26(4773):1-17.

Corresponding Author: paini@ukwms.ac.id

775 KRUK J, ABOUL-ENAIN BH, DUCHNIK E, MARCHLEWICZ M. 2022. Antioxidative  
776 properties of phenolic compounds and their effect on oxidative stress induced by severe  
777 physical exercise. *J. Physiol. Sci.* 72(19):1-24.

778 KUMAR N, GOEL N. 2019. Phenolic acids: natural versatile molecules with promising  
779 therapeutic applications. *Biotechnology Reports.* 24(e00370):1-10.

780 LI K et al. 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the flavonoids  
781 rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity  
782 relationship of its eight flavonoids by a refined assign score method. *Chem. Central J.*  
783 12(82):1-11.

784 LIN YS. et al. 2020. Effects of storage time and temperature on antioxidants in juice from  
785 *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser. *Molecules.*  
786 25(3614): 1-12.

787 MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content  
788 and antioxidant activity test of avocado seed extract (*Persea Americana* Mill). *Jurnal MIPA*  
789 *Unsrat Online.* 1(1): 5-10. [https://media.neliti.com/media/publications/116214-ID-  
790 penentuan-kandungan-tanin-dan-uji-aktivi.pdf](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf) [In Bahasa Indonesia]

791 MARTINEZ-SOLIS J et al. 2022. Antidiabetic and toxicological effect of the tea infusion  
792 of summer collection from *Annona cherimola* Miller leaves. *Plants.* 11(3224):1-17.

793 MATHUR R, VIJAYVERGIA R. 2017. Determination of total flavonoid and phenol content  
794 in *Mimusops Elengi* Linn. *Intern. J. Pharm. Sci. Res.* 8(12): 5282-5285.

795 MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of  
796 sprouted and solid-state bioprocessed soybean. *Asia Pacific J. Clinical Nut.* 14(2): 145-  
797 152.

Corresponding Author: paini@ukwms.ac.id

798 MORAES-DE-SOUZA RA, OLDONI TLC, REGITANO-D'ARCE MAB, ALENCAR SM.  
799 2008. Antioxidant activity and phenolic composition of herbal infusions consumed in  
800 Brazil. *Cienc Tecnol. Aliment.* 6(1):41-47.

801 MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant  
802 activity: A Review. *Intern. J. Molecular Sci.* 22(3380):1-30.

803 NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus*  
804 *sabdariffa* L.): Effect of dying temperature and brewing conditions on total soluble solid,  
805 phenolic content, antioxidant capacity and sensory quality. *Beverages*.6(2):1-11 (2020).

806 NOREEN H. et al. 2017. Measurement of total phenolic content and antioxidant activity  
807 of aerial parts of medicinal plant *Coronopus didymus*. *Asian Pacific J. Tropical Med.* 10(8):  
808 792–801.

809 NURCHOLIS GW et al. 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity in-  
810 vitro and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum pictum*  
811 (L.). *Current Biochem.* 1(2): 58-65.

812 OH J et al. 2013. Antioxidant and antimicrobial activities of various leafy herbal teas. *Food*  
813 *Control.* 31(2013):403-409.

814 PROENCA C et al. 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an in vitro and in silico  
815 structure–activity relationship study. *J. Enzyme Inhibition Med. Chem.* 32(1): 1216–1228.

816 PUREWAL SS et al. 2022. Unraveling the effect of storage duration on antioxidant  
817 properties, physicochemical and sensorial parameters of ready to serve Kinnow-aml  
818 beverages. *Applied Food Res.* 2(1): 1-8.

819 RAHARJO D, HARYOTO. 2019. Antioxidant activity of mangrove *Sonneratia caseolaris*  
820 L. using the FRAP method. *ISETH.* 2019: 623-629.

Corresponding Author: paini@ukwms.ac.id

821 RAMPHINWA ML et al. 2023. Eco-physiological response of secondary metabolites of  
822 teas: review of quality attributes of herbal tea. *Frontiers in Sustain. Food Systems*.  
823 7(990334):1-14.

824 REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids.  
825 *Czech J. Food Sci.* 30(2):171-177.

826 RUAN J et al. 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea*  
827 *indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel  
828 column chromatography fractions. *Molecules*. 24(2784):1-20.

829 RUSITA YD et al. 2019. Comparison of tannin concentration on fresh and dried tea leaf  
830 (*Camelia sinensis* L.) extract using UV-visible spectrophotometric method. 1st  
831 International Conference of Health, Science & Technology (ICOHETECH) 2019 E-ISBN:  
832 978-623-92207-0-9. November 16<sup>th</sup>, 2019.LPPM Universitas Duta Bangsa Surakarta,  
833 Surakarta.

834 SANGEETHA R, VEDASREE N. 2012. In Vitro  $\alpha$ -Amylase inhibitory activity of the leaves  
835 of *Thespesia populnea*. *Intern. Scholarly Res.Network*. 2012: 1-4 (2012)

836 SHRAIM AM et al. 2021. Determination of total flavonoid content by aluminum chloride  
837 assay: A critical evaluation. *LWT Food Sci. Tech.* 150(111932):1-11.

838 SILVA-RAMIREZ KA et al. 2020. Effect of drying and steeping temperature on the  
839 phenolic content, antioxidant activity, aromatic compounds and sensory properties of  
840 *Culila polyantha* Benth. *Infusions. Processes*. 8(1378):2-20.

841 SRISOOK K et al. 2012. Antioxidant and anti-inflammatory activities of hot water extract  
842 from *Pluchea indica* Less. herbal tea. *J. Med. Plants Res.* 6(23):4077-4081.

Corresponding Author: paini@ukwms.ac.id

843 SURIYAPHAN O. 2014. Nutrition, health benefits and applications of *Pluchea indica* (L)  
844 Less leaves. Pharm. Sci. Asia. 41:1–10.

845 SU D et al. 2019. Impact of thermal processing and storage temperature on phenolic  
846 profile and antioxidant activity of different varieties of lychee juice. LWT-Food Tech.  
847 116(108578):1-9.

848 THANAJIRUSCHAYA P et al. 2010. Effect of storage ~~time~~[time period](#) and temperature on  
849 antioxidant components and properties of milled rice. Khon Kaen Univ. Res. J.15(9):843-  
850 851.

851 VONGSAK B et al. 2018. Comparison of active constituents, antioxidant capacity, and  
852 αglucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. Food  
853 Biosci. 25: 68–73.

854 WANG SC. et al. 2022. Effect of brewing conditions on polyphenols in the dark tea  
855 (*Camellia sinensis* L.) infusions: content, composition and antioxidant activities. Food  
856 Sci.Tech. 42(e36322):1-9.

857 WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica*  
858 Less as functional beverage in diabetic patients. ASSEHR. 98:164-167.

859 WIDYAWATI PS et al. 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract  
860 as antioxidant and antiwarmed over flavor (WOF) of duck meat. International Congress  
861 on Challenges of Biotechnological Research in Food and Health. November 15<sup>th</sup>  
862 2014.Agricultural Technology Faculty, Slamet Riyadi University, Surakarta.

863 WIDYAWATI PS et al. 2016. The physicochemical and antioxidant properties of *Pluchea*  
864 *indica* Less drink in tea bag packaging. IJFNS. 5: 13–120.

Corresponding Author: paini@ukwms.ac.id

865 WIDYAWATI PS et al. 2017. In vitro antioxidant capacities and antidiabetic properties of  
866 *Pluchea* leaves. IJPPS. 9(8): 203-208.

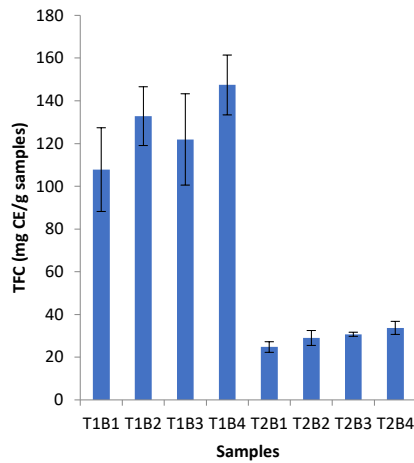
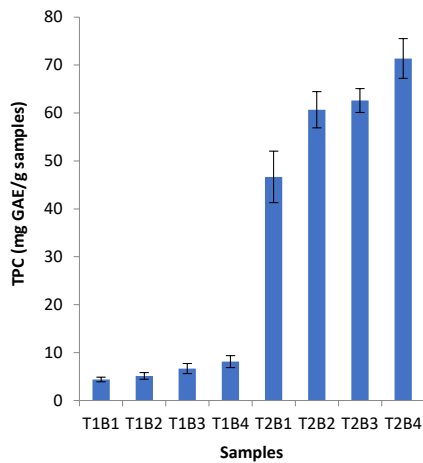
867 WIDYAWATI PS et al. 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica*  
868 Less beverage. CJFST.12(4): 125-139.

869 WIDYAWATI PS ET AL. 2022. The effect of  $\kappa$ -carrageenan proportion and hot water  
870 extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of stink  
871 lily (*Amorphophallus muelleri*) wet noodles. Molecules. 27(5062):1-16.

872 XU P, CHEN L, WANG Y. 2019. Effect of storage [time period](#) on antioxidant activity and  
873 inhibition on  $\alpha$ -amylase and  $\alpha$ -glucosidase of white tea. Food Sci. Nut. 7(2): 636-644.

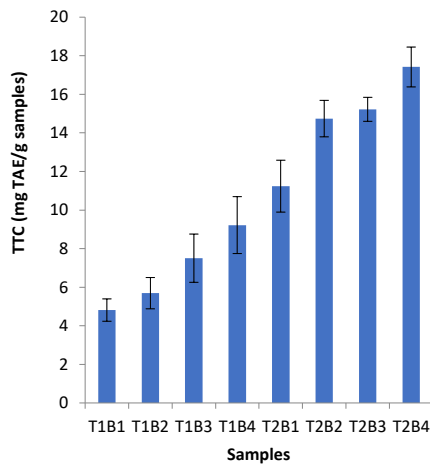
874 ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction [time period](#) on  
875 caffeine in coffee. Lantanida J. 6(2):103-202. [https://jurnal.ar-](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811)  
876 [raniry.ac.id/index.php/lantanida/article/view/3811](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811). [In Bahasa Indonesia]

877 ZHANG Y et al. 2021. Effect of storage conditions and [time period](#) on the polyphenol  
878 content of wheat flours. Processes. 9(248):1-11.



(a)

(b)



(c)

Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping temperature and storage time period (a) Total phenolic content (b) Total flavonoid content (c) Total tannin content. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-

Formatted: Left, Indent: Left: 0.5", Hanging: 0.69", Line spacing: single



888  
889  
890  
891  
892  
893  
894

stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$ .

Commented [A35]:

Table 1. Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperature and storage time period

Phenolic Compounds	Steeping Temperature (°C)	Mean±SD Un-stored	Mean±SD Stored	Mean difference ±SD	Sig (2-tailed)
Gallic Acid (µg/g samples)	60	0.2132±0.0027	0.2364±0.0015	0.0375±0.0175	0.2030
	70	0.2157±0.0013	0.2324±0.0214	0.0167±0.0227	0.4870
	80	0.2234±0.0122	0.2347±0.0078	0.0386±0.0264	0.2870
	95	0.2316±0.0104	0.2402±0.0169	0.0086±0.1990	0.8500
(+)-Catechin (µg/g samples)	60	0.3425±0.0110	0.5085±0.0111	-0.1576±0.0885	0.241
	70	0.3260±0.0265	0.5448±0.0006	-0.2188±0.0259	0.053
	80	0.3240±0.0222	0.5023±0.0773	-0.1451±0.0248	0.077
	95	0.4039±0.0320	0.5995±0.0372	-0.2049±0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756±0.1234	1.4762±0.0271	-1.2887±0.3222	0.111
	70	0.2587±0.0160	1.4245±0.2526	-1.1657±0.2695	0.103
	80	0.4175±0.0104	1.4570±0.0925	-1.0391±0.0841	0.036*
	95	0.8786±0.0434	2.6138±0.0695	-1.735±0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220±0.0268	0.6220±0.0706	-0.5999±0.9733	0.544
	70	0.1530±0.0511	1.0708±0.0289	-0.9177±0.0222	0.011*
	80	0.3666±0.0103	0.8629±0.0815	-0.1082±0.4462	0.790
	95	0.6559±0.0570	2.0230±0.0573	-1.4123±0.3203	0.101
Kaempferol (µg/g samples)	60	0.1394±0.0202	0.3675±0.0183	-0.3207±0.1122	0.154
	70	0.0514±0.0037	0.3726±0.0944	0.3213±0.0907	0.125
	80	0.3699±0.0924	0.7966±0.0366	-0.4267±0.2727	0.271
	95	0.5913±0.0239	0.9478±0.0287	-0.3565±0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103±0.0628	2.4863±0.0270	-1.8760±0.2074	0.050*
	70	0.6271±0.0099	2.3403±0.0325	-1.7131±0.3152	0.082
	80	0.7967±0.03060	2.6278±0.0211	-1.8311±0.0095	0.002*
	95	1.5386±0.0668	4.0211±0.0851	-2.4825±0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635±0.0628	0.9449±0.0501	-0.2814±0.4458	0.536
	70	0.6162±0.0099	0.9485±0.0794	-0.3323±0.0301	0.041*
	80	0.6601±0.0306	0.9099±0.0387	-0.2498±0.3127	0.461
	95	0.6642±0.0668	1.3156±0.0166	-0.6514±0.2666	0.179

Corresponding Author: paini@ukwms.ac.id

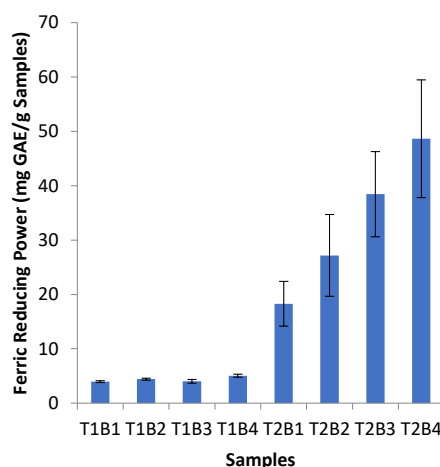
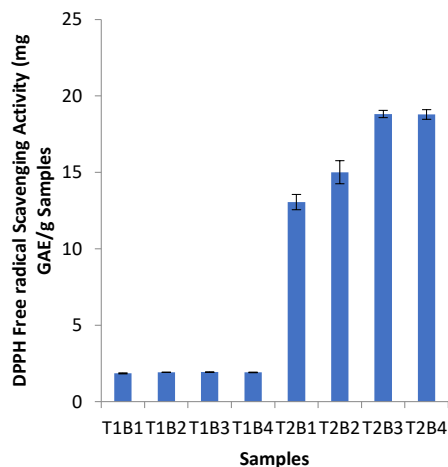
4,5-di-O-Caffeoylquinic acid ( $\mu\text{g/g}$ samples)	60	0.4906 $\pm$ 0.0060	1.1842 $\pm$ 0.0120	-0.6886 $\pm$ 0.2723	0.018*
	70	0.4807 $\pm$ 0.0034	1.0089 $\pm$ 0.0736	-0.5281 $\pm$ 0.0702	0.060
	80	0.5299 $\pm$ 0.0053	1.2382 $\pm$ 0.1435	-0.7082 $\pm$ 0.1489	0.094
	95	1.0018 $\pm$ 0.0526	1.3797 $\pm$ 0.2170	-0.3086 $\pm$ 0.3086	0.333

896 Note : Data were expressed as mean  $\pm$ standard deviation (n=2). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-  
897 steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C,  
898 stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped  
899 at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature,  
900 calculated using a paired T test at  $\alpha \leq 0.05$ . \*  $\alpha \leq 0.05$ .  
901

Formatted: Line spacing: single

Commented [A36]: Improve

902



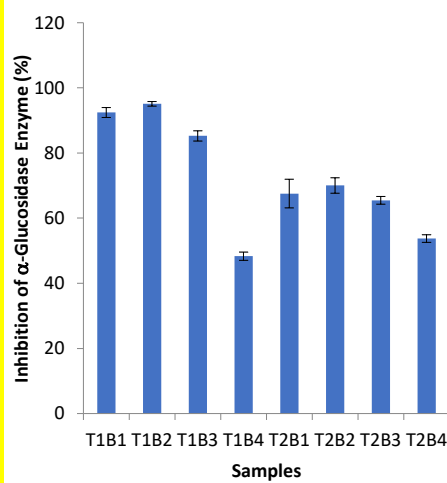
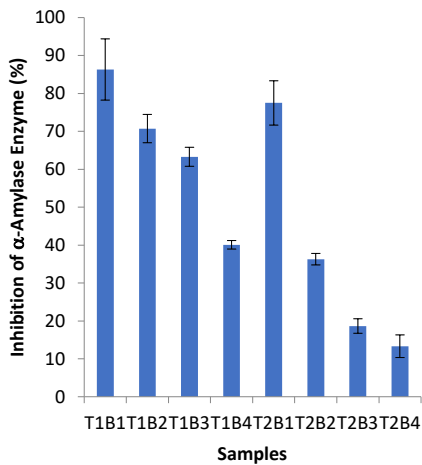
(a)

(b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage ~~time~~ time period (a) DPPH (b) FRAP. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T2B2-steeped at 70 °C, stored for 5 years; T2B3-steeped at 80 °C, stored for 5 years; T2B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$ .

Formatted: Justified, Indent: Left: 0.5", Hanging: 0.69", Line spacing: single

Commented [A37]: Improve



(a)

(b)

**Figure 3.** Antidiabetic activity of pluchea tea at different steeping temperature and storage ~~time~~ period (a)  $\alpha$ -amylase (b)  $\alpha$ -glucosidase. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$ .

Formatted: Indent: Left: 0.44", Hanging: 0.75"

Formatted: Justified, Indent: Left: 0.44", Hanging: 0.75", Line spacing: single, Tab stops: 1.19", Left

Commented [A38]: Improve

927  
928

Table 2. Pearson correlation coefficients between bioactive contents (TPC, TFC and TAC), antioxidant activity (DPPH and FRAP) and antidiabetic activity (AA and GA)\*

Formatted: Space After: 0 pt, Line spacing: single

	TPC	TFC	TTC	DPPH	FRAP	Alpha Glucosidase	Alpha Amylase
TPC	1						
TFC	-0.93589	1					
TTC	0.960028	-0.81321	1				
DPPH	0.992776	-0.93992	0.942273	1			
FRAP	0.953366	-0.82636	0.947778	0.956242	1		
Alpha Glucosidase	-0.55512	0.349873	-0.71534	-0.5272	-0.55947	1	
Alpha Amylase	-0.70842	0.429393	-0.8569	-0.69579	-0.80548	0.725161631	1

929  
930

Note: \*Correlation Ssignificant at the 0.05 level (2-tailed)



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**From Caesar Saloma/15 January 2024/ Editorial Decision/ MS 23-158R**

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Wed, Apr 17, 2024 at 9:09 AM

[Quoted text hidden]



**Philippine Journal of Science**  
**Science and Technology Information Institute**  
**Department of Science and Technology**  
**DOST Complex, Gen. Santos Ave., Bicutan 1631**  
**Taguig City, Metro Manila, Philippines**  
Telephone no. : 837 - 2191  
Email: [pjs@stii.dost.gov.ph](mailto:pjs@stii.dost.gov.ph); [philjournsci@gmail.com](mailto:philjournsci@gmail.com)  
Website: <https://philjournalsci.dost.gov.ph>  
Scopus: <https://www.scopus.com/sourceid/19700175735>



---

**R2 Ms 23-158 Reviewer 2 Comments on Revised Manuscript.docx**

140K

1 **Effect of Steeping Temperature and Storage [TimePeriod](#) on the Bioactive**  
2 **Compounds, Antioxidant and Antidiabetic Activities of Infusion from Powdered**  
3 ***Pluchea Indica Less***

4 Painsi Sri Widyawati<sup>1)</sup>, Yufita Ratnasari Wilianto<sup>2)</sup>

5 <sup>1)</sup>Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala  
6 Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

7 <sup>2)</sup>Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic  
8 University, Kalisari Street Number 1, Surabaya 60272, Indonesia

9 Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea*  
10 *indica Less*, storage [timeperiod](#)

11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
Corresponding Author: [paini@ukwms.ac.id](mailto:paini@ukwms.ac.id)



21 ABSTRACT

22 This study was done to determine the effects of steeping temperature and storage  
23 ~~timeperiod~~ on the bioactive contents, antioxidant and antidiabetic activities of *Pluchea*  
24 ~~leaf infusion~~. The research used a randomized block design with two factors, i.e., steeping  
25 temperature (T) and storage ~~timeperiod~~ (B). The ~~variety of the Pluchea leaf blades were~~  
26 ~~exposed to 4~~ steeping temperatures ~~included of~~ 60 (T1), 70 (T2), 80 (T3), and 95 (T4)  
27 ( $^{\circ}\text{C}$ ) with the storage ~~timeperiod-period~~ of 0 (B1) and 5 (B2) ~~(year)~~. ~~The research~~  
28 ~~resultedresulting in~~ 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2,  
29 T4B1, T4B2). Statistical analysis using a paired ~~t-T~~ test at  $\alpha \leq 0.05$  showed that  
30 treatments significantly ~~affected influenced~~ the bioactive contents (total phenol (TPC),  
31 total tannin (TTC), total flavonoid (TFC)), antioxidant [(DPPH scavenging activity (DPPH)  
32 and ferric reducing antioxidant power (FRAP)] ~~potential~~ and antidiabetic [( $\alpha$ -amylase  
33 (AA) and  $\alpha$ -glucosidase (GA) ~~inhibitorsinhibition~~] ~~activities-properties of the Pluchea leaf~~  
34 ~~infusionsamples~~. TFC decreased during storage period but significantly increased at  
35 higher steeping temperature. The AA and GA of *Pluchea* infusion increased until 70  $^{\circ}\text{C}$   
36 ~~of the steeping temperature, but decreased until 95  $^{\circ}\text{C}$~~ . ~~The bioactive contents influenced~~  
37 ~~antioxidant and antidiabetic activities~~. TFC was decreased for storage time and significant  
38 ~~increased at higher steeping temperature~~. The AA and GA of *Pluchea* infusion increased  
39 ~~until 70  $^{\circ}\text{C}$  of the steeping temperature, but decreased until 95  $^{\circ}\text{C}$~~ . The AA and GA were  
40 strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was  
41 moderately and negatively correlated with TFC. ~~Between-T~~ the antioxidant activity of  
42 ~~DPPH and FRAP~~ and the antidiabetic activity of AA and GA of *Pluchea* infusion were  
43 strongly and positively correlated. ~~with correlation coefficient (r) values of 0.956 and~~

**Commented [A1]:** Describe treatment effects on total phenolics, tannins, antioxidant, and antidiabetic in one brief sentence each and indicate statistical significance.

**Commented [A2]:** State briefly results of the correlation analysis.

44 0.725, respectively. The treatments gave different effect of simple phenolic compounds  
45 derived from *Pluchea* leaf infusion at different steeping temperatures and storage  
46 included, such as gallic acid, kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-  
47 caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid of  
48 *Pluchea* infusion at different steeping temperature and long storage. To obtain high  
49 antioxidant activity, *Pluchea* infusion selected was stored and steeped at high  
50 temperature, however high antidiabetic activity obtained was fresh *Pluchea* infusion and  
51 steeped at low temperature.

Formatted: Font: Italic

## 52

### 53 INTRODUCTION

54 *Pluchea* herbal tea is a product of dried *Pluchea* leaf processing introduced by  
55 world people (Srisook et al., 2012; Widyawati et al., 2016) because of the efficacy of the  
56 active components in *Pluchea* leaves, as an herbal plant that has been widely used for  
57 traditional medicine and food (Chan et al., 2022). *Pluchea* leaves are composed many  
58 nutrients and bioactive compounds useful to body health. The nutrient compositions in  
59 the *Pluchea* leaves include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates,  
60 calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive compounds is comprised, i.e.,  
61 chlorogenic acid, caffeic acid, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-  
62 caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-  
63 caffeoylquinic acid, quercetin, myricetin, kaempferol, total anthocyanin,  $\beta$ -carotene, and  
64 total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et  
65 al., 2022, Chan et al., 2022).

Corresponding Author: paini@ukwms.ac.id

66 Steeping process of *Pluchea leaves* can be performed with fresh or dry leaves  
67 ~~infusion by~~ in hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et  
68 al., 2020; Jayani et al., 2022). In Asia ~~area~~, especially in Indonesia, people usually  
69 consume the *Pluchea infusion* ~~with brewing of~~ steeping 2 g of powdered *Pluchea*  
70 leaves in tea bag ~~by~~ in 100 mL of hot ~~water~~ or boiling water. ~~Each tea bag contained 2 g~~  
71 ~~of *Pluchea* leaf powder is steeped with 100 mL hot water or boiling water.~~ Widyawati et  
72 al. (2016) claimed that steeping of 2 g of *Pluchea leaf powder* at 95 °C for 5 minutes  
73 ~~results exhibits~~ total phenolic ~~content, and~~ total flavonoid contents, the ability to scavenge  
74 DPPH free radicals, and the capability ~~of to~~ reduce ferric ions ~~at~~ 9.3 mg gallic acid  
75 equivalent (GAE)/g samples, 22.0 mg gallic acid equivalent (GAE)/g samples, 27.2 mg  
76 gallic acid equivalent (GAE)/g samples, and 10.2 mg gallic acid equivalent (GAE)/g  
77 samples, respectively. Werdani and Widyawati (2018) reported that drinking of *Pluchea*  
78 *leaf powder infusion* in the morning and evening regularly (2 g/100 mL) can decline blood  
79 sugar levels.

80 The steeping of *Pluchea herbal tea* with hot water at 95 °C for 5 minutes certainly  
81 determines the stability and amount of extracted bioactive compounds, that influences  
82 the biological activity, especially antioxidant and antidiabetic activities. Silva-Ramirez et  
83 al. (2020) reported that the infusion process can influence the ~~if~~ content and composition  
84 of the bioactive compounds and antioxidant activity of tea. Acar et al. (2022) informed  
85 that infusion quality of *herbal* tea extract depends on several factors, i.e., ~~time~~ ~~storage~~  
86 and temperature. Polyphenol profile and antioxidant properties of *herbal* tea infusion  
87 decline with an increase in steeping/brewing and storage temperatures, and longer  
88 exposure ~~time~~ ~~periods~~.

89 Several studies have mentioned the effect of steeping temperature ~~to on the~~  
90 bioactive compound contents and antioxidant activity, such as some white and green teas  
91 are effective with hot water at 90 °C for 7 min (Castiglioni et al., 2015), ~~on~~ roseship tea is  
92 effectively ~~at infusion~~ ~~timeperiod~~ around 6-8 min at temperatures of 84-86 °C (Ilyasoglu  
93 and Arpa, 2017), ~~on the caffeine content extracted the coffee at the~~ brewing temperature  
94 ~~of coffee influences the caffeine content extracted~~ (Zarwinda and Sartika, 2018), ~~and the~~  
95 ~~steeping the high total phenol content and antioxidant activity~~ of dark tea at 92 °C for 27  
96 min ~~results the highest total phenol content and antioxidant activity~~ (Wang et al., 2022).  
97 The study of the effect of steeping temperature to *Pluchea* infusion was carried out to  
98 afford information about ~~the most efficient~~ preparation of *powdered Pluchea leaves* ~~most~~  
99 ~~efficiently~~ to get higher ~~the~~ bioactive compounds, antioxidant and antidiabetic activities.

100 ~~On the other hand, storage~~ ~~Storage~~ ~~timeperiod~~ *tea* usually for several months until  
101 ~~years~~ of *Pluchea* herbal tea also affects the levels of the bioactive compounds and  
102 biological activity ~~because this~~ ~~herbal tea~~ usually is stored for a several months until years  
103 (Jayani et al., 2022). Tea or herbal tea is generally stored in ambient temperature and  
104 packed in tea bag or ~~Alu~~ foil standing proud or a combination of both. Many researchers  
105 ~~informed~~ ~~reported~~ that storage ~~timeperiod~~ decreases the bioactive compounds,  
106 antioxidant and antidiabetic activities, i.e., juice from *Momordica charantia* L. (Lin et al.,  
107 2020), dried *Piper bettle* extracts (Ali et al., 2018), white tea (Xu et al., 2019), kinnow-  
108 amla beverages (Purewal et al., 2022), whole wheat flour (Zhang et al., 2021).

109 Therefore, *this research studied the effect of steeping temperature and storage*  
110 *timeperiod on the bioactive compounds [(total phenolic content (TPC), total flavonoid*  
111 *content (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging*

Commented [A3]: IS THIS ALUMINUM? If so, replace Alu with aluminum

Commented [A4]: Do you mean standing pouch?

112 activity (DPPH), ferric reducing antioxidant power (FRAP)] and antidiabetic activities [( $\alpha$ -  
113 amylase (AA) and  $\alpha$ -glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea*  
114 leaves. The study was done to determine total phenolic content (TPC), total flavonoid  
115 content (TFC), total tannin content (TTC), DPPH free radical scavenging activity (DPPH),  
116 ferric reducing antioxidant power (FRAP),  $\alpha$ -amylase (AA) and  $\alpha$ -glycosidase (GA)  
117 inhibition activities, and on the phenolic compound profile.

## 119 MATERIALS AND METHODS

### 120 RAW MATERIALS AND PREPARATION

121 The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya,  
122 East Java, Indonesia. The *Pluchea* plants were included in Asteraceae family with  
123 specification according to the GBIF taxon ID number database:3132728 (Ferraris, 2023).  
124 *Pluchea* leaves at 1-6 level of each branch ~~offrom~~ the shoot were collected, sorted,  
125 washed and dried to ~~get a~~ moisture content ~~of~~ around  $11.16 \pm 0.09$  % dry basise  
126 (Widyawati et al., 2022). The ~~powdoring of~~ dried *Pluchea* leaves was ~~done~~ pulverized to  
127 ~~get a~~ 45-mesh size powder. ~~And then, the heating of T~~the *Pluchea* leaf powder was ~~done~~  
128 ~~using a drying~~dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 °C for  
129 10 min to reduce microbial organisms. ~~and Then, 2 g of the powder were~~ packed using  
130 ~~into a paper filter~~ infusion bag, ~~that made from paper filter around 2 g/bag. And then all~~  
131 ~~of samples called~~Packed samples were *Pluchea* herbal tea was stored for 0 (un-stored)  
132 and 5 (stored)years in standing pouch before analysis.

133 In the research, the one tea bag of *Pluchea* herbal tea that stored 0 (B1) and 5  
134 (B2) year, was steeped with 100 mL hot water at various temperatures, including 60 (T1),

135 70 (T2), 80 (T3), and 95 (T4) °C for 5 min with infusion method ~~that obtained~~obtaining 8  
136 treatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2.  
137 After the temperature of *Pluchea* infusion similar to ambient temperature was analyzed  
138 further.

## 140 REAGENTS

141 The ~~compounds~~reagents used ~~to analyze~~in the analyses including include 2,2-  
142 diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid, α-amylase, α-  
143 glucosidase, pNPG (p-nitrophenyl-α-glucopyranoside), (+)-catechin, kaempferol,  
144 myricetin, quercetin, 3,4-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, 3,5-di-O-  
145 caffeoylquinic acid, and (+)-catechin were purchased from Sigma-Aldrich (St. Louis, MO,  
146 USA). Methanol, Folin–Ciocalteu’s Phenol, sodium nitric, aluminum chloride, ferric  
147 chloride, sodium dihydrogen phosphate, sodium phosphate, potassium ferricyanide,  
148 starch, acetic acid, and sodium hydroxide were purchased from Merck (Kenilworth, NJ,  
149 USA). All reagents used were of analytical grade except for distilled water which was  
150 purchased from PT Aqua Industry Surabaya.

## 152 METHODOLOGY

### 153 ANALYSIS OF THE BIOACTIVE COMPOUNDS

#### 154 TOTAL PHENOLIC CONTENT ANALYSIS

155 Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the  
156 technique by Gao et al. (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu’s  
157 phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. And

**Commented [A5]:** Confusing, needs to be re-written eg The unstored samples were steeped in 100 mL distilled water at 60, 70, 80, and 95 °C for 5 min, then immediately were analyzed for the bioactive compounds [(total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC)], antioxidant potential [(DPPH free radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP)] and antidiabetic activities [(α-amylase (AA) and α-glycosidase (GA) inhibition)]. The rest of the samples were stored at (describe storage conditions) and analyze after 5 years..

**Commented [A6]:** Confusing

**Formatted:** Centered

158 then 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5 % was ~~entered added and filled up to 10 mL volume with distilled~~  
159 ~~water and distilled water was added until 10 mL volume~~. The color intensity of solution  
160 was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at  $\lambda$  760 nm  
161 with gallic acid as the reference standard. The total phenolic content was calculated using  
162 the formula:  $y=0.00009x+0.008$  with  $R^2=0.9941$ . The results were expressed as mg gallic  
163 acid equivalent (GAE)/g samples.

Commented [A7]: Specify what color

164

#### 165 TOTAL FLAVONOID CONTENT ASSAY

166 Total flavonoid content (TFC) of the samples was measured based on the reaction  
167 between AlCl<sub>3</sub> and NaNO<sub>2</sub> with ~~an the~~ aromatic ring of flavonoid compounds, especially  
168 flavonol and flavon (Shraim et al., 2021). The reaction between AlCl<sub>3</sub> and flavonoid  
169 compounds resulted ~~in~~ a yellow solution. About 30  $\mu$ L *Pluchea* infusion was mixed with  
170 0.3 mL NaNO<sub>2</sub> 5 % in 10 mL volumetric flask and incubated for 5 min. The mixture was  
171 added with 0.3 mL AlCl<sub>3</sub> 10 % for 5 min. And then, 2 mL NaOH 1 M and distilled water  
172 were added until 10 mL volume. Then, the red solution was produced after NaOH solution  
173 addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800,  
174 Shimadzu, Japan) at  $\lambda$  510 nm with (+)-catechin as the reference standard compound,  
175 and the results were expressed as mg catechin equivalents (CE)/g samples using the  
176 formula:  $y=0.00008x-0.0023$  with  $R^2= 0.9980$ .

177

#### 178 TOTAL TANNIN CONTENT ANALYSIS

179 Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method  
180 (Chandran and Indira, 2016). Approximately 10  $\mu$ L *Pluchea* infusion was added ~~with~~ 1 mL

181 Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flash and incubated for 5 min.  
182 Then, the mixture was added with 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5 % and filled up to 10 mL volume with  
183 distilled water. ~~was added until 10 mL volume.~~ The blue dark color solution ~~that was~~  
184 measured in UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at λ 760 nm with tannic  
185 acid as the reference standard. Calculation of TTC was expressed as mg tannic acid  
186 equivalents (TAE)/g samples used the formula:  $y=0.00009x+0.0021$  with  $R^2=0.9993$

## 188 ANALYSIS OF THE ANTIOXIDANT POTENTIAL

Formatted: Centered

### 189 DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

190 The DPPH free radical scavenging activity (DPPH) was measured by the  
191 spectrophotometric method (Widyawati et al., 2017) to determine the ability of the  
192 phytochemicals antioxidant activity of the *Pluchea* leaf infusion to ~~doner donate~~  
193 hydrogen atom to the nitrogen atom in DPPH resulting in the formation of -DPPH-H  
194 compound with exhibiting a yellow-colored solution. About 25 μL *Pluchea* leaf infusion  
195 was ~~entered poured~~ into reaction tube ~~and into which was added~~ added 3 mL DPPH  
196 solution (4 mg/100 mL). ~~And then the solution was~~After incubationed for 15 min in a dark  
197 room, ~~the and~~ absorbance was measured by a spectrophotometer (Spectrophotometer  
198 UV-Vis 1800, Shimadzu, Japan) at λ. 517 nm. The reference standard compound was  
199 gallic acid and the results of analysis were expressed as mg gallic acid equivalents  
200 (GAE)/g samples that calculated using formula:  $y=0.146x+1.7896$  with  $R^2=0.9975$ .

### 202 FERRIC REDUCING POWER ANALYSIS



203 Ferric reducing power (FRAP) was determined following the method used by  
204 Widyawati et al. (2014) method. Approximately 10  $\mu$ L of samples were added 2.5 mL  
205 phosphate buffer pH 6.6 and 2.5 mL and 1% potassium ferricyanide 1% in the reaction  
206 tube. And then mixture was shaken and ~~incubation~~ incubated for 20 min at 50 °C. Finally,  
207 2.5 mL chloroacetic acid 10% (w/v) was added. Into the 2.5 mL supernatant was added  
208 2.5 mL distilled water, 0.5 mL ferric chloride 0.1% (w/v) and incubated for 10 min.  
209 Potency of the samples reducing iron (III) to iron (II) ion was ~~signed~~ indicated by the  
210 intensity of blue color formed that was measured using UV-Vis spectrophotometer  
211 (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  700 nm. Intensity of the blue  
212 color indicated higher reducing capacity. The reducing power expressed as mg gallic acid  
213 equivalent (GAE)/g samples was calculated using the formula:  $y=0.0002x+0,0256$  with  
214  $R^2=0,9906$ .

215

#### 216 $\alpha$ -AMYLASE ENZYME INHIBITION CAPACITY ASSAY

217 In vitro inhibition of  $\alpha$ -amylase enzyme (AA) followed the procedure as described  
218 by Widyawati et al. (2020). Each 500  $\mu$ L of samples, was mixed with starch 1 % (w/v) and  
219 sodium acetate buffer pH 5, ~~were mixed. Then, into each~~ 250  $\mu$ L of the mixture and was  
220 added an  $\alpha$ -amylase solution (0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in  
221 50 mL of 0.2 M sodium acetate pH 5). Mixture was shaken and into which was added 2  
222 mL sodium hydroxide 1M. Before the analysis, this mixture was incubated at 37 °C for 10  
223 min. Then, the capacity of the  $\alpha$ -amylase enzyme to hydrolyzed the starch to release  
224 glucose was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800,  
225 Shimadzu, Japan) that could be analyzed based on absorbance at  $\lambda$  540 nm. The

226 inhibition percentage of  $\alpha$ -amylase was assessed using the formula:  $(ACb - ACa) - (As$   
227  $- Ab) (ACb - ACa) \times 100 \%$ . Where, ACb is the absorbance of 100 % enzyme activity  
228 (solvent with the enzyme), ACa is the absorbance of 0 % enzyme activity (solvent without  
229 the enzyme), As is the absorbance of test sample with enzyme, Ab is absorbance of test  
230 sample without enzyme.

231

#### 232 $\alpha$ -GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

233 The analysis of the  $\alpha$ -glycosidase inhibitor activity (GA) was done by Widyawati et  
234 al. (2020) method with slight modification. About 150  $\mu$ L samples ~~contained~~ containing  
235 100  $\mu$ L *Pluchea* infusion and 50  $\mu$ L pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M  
236 at pH 7) were reacted with 50  $\mu$ L  $\alpha$ -glycosidase 2 mM (0.0833 unit/mL), and then the  
237 mixture was incubated at 37 °C for 15 min. ~~Finally, the~~The reaction was stopped with the  
238 addition of 1000  $\mu$ L sodium carbonate 0.2 M. The residue of this enzyme hydrolyzed p-  
239 nitrophenyl- $\alpha$ -D-glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The

Commented [A8]: Confusing. Rewrite

240 ~~inhibition~~ activity of ~~steeping the~~ *Pluchea* ~~tea-infusion to enzyme~~ was measured by UV-  
241 vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at  $\lambda$  405 nm.

242 The inhibition percentage of  $\alpha$ -glycosidase was calculated using formula:  $(ACb - ACa) -$   
243  $(As - Ab) (ACb - ACa) \times 100 \%$ . Where, ACb is the absorbance of 100 % enzyme activity  
244 (solvent with enzyme), ACa is the absorbance of 0 % enzyme activity (solvent without  
245 enzyme), As is the absorbance of test sample with enzyme, Ab is the absorbance of test  
246 sample without enzyme.

247

#### 248 HPLC ANALYSIS OF PHENOLICS

Corresponding Author: paini@ukwms.ac.id

249 The phenolic compounds of the samples were analyzed by HPLC based on  
250 Kongkiatpaiboon et al. (2018) method with modifications. Each *Pluchea* infusion was  
251 sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter  
252 syringe (Whatmann, 0.2 µm, NYL). About 20 µL of sample was injected in an HPLC  
253 (LC20AD series, Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC  
254 LC-20AD pump, CTO-30A column oven, CBM-20A/20 Alite system controller, and SPD-  
255 20A/20 AV UV-Vis detector. Separation of phenolic compounds in samples was carried  
256 out using a Shim-pack VP-ODS C18 column (ID 5 µm × 50 mm × 4.6 mm) with a GVP-  
257 ODS Cartridge guard column (2 pieces) (ID 10 mm × 4.6 mm). ~~Analytical conditions:~~ The  
258 mobile phase used consisted of a solution of (A) 0.5 % acetic acid in water and (B)  
259 absolute methanol. Analysis was carried out using a gradient system in the following  
260 order: initial conditions of 10 % B in A to 50 % B in A were maintained for 40 minutes;  
261 then 100 % B was maintained for 20 minutes. Next the column was re-equilibrated with  
262 10 % B in A maintained for 10 minutes before analysis of the next sample. The sample  
263 flow rate was set at 1.0 ml/min with a controlled temperature at 40 °C. Detection was used  
264 at a wavelength of 280 nm. The reference standard used were gallic acid, (+)-catechin,  
265 myricetin, quercetin, kaempferol, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and  
266 4,5-dicaffeoylquinic acid. All of the reference standard was dissolved in distilled water  
267 and prepared similar to the samples before injected in HPLC.

268

## 269 EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

270 The research design used a randomized block design with two factors, i.e., the  
271 steeping temperature (T) and the storage ~~time~~ timeperiod (B). *Pluchea* leaf blades were

272 subjected to 4 steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95  
273 °C (T4), and the storage ~~timeperiod~~ of 0 year /~~fresh-un-stored~~ (B1), and 5 year/stored  
274 (B2). ~~The research resultedresulting in~~ 8 treatment combinations (T1B1, T1B2, T2B1,  
275 T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic was repeated two  
276 ~~timeperiods~~. The data of samples were analyzed by ANOVA at  $\alpha \leq 0.05$ , and continued  
277 analysis using a paired T test at  $\alpha \leq 0.05$ . treatment means of specific phenolic  
278 compounds that were identified were expressed as the mean  $\pm$  SD. The analysis used  
279 SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

**Commented [A9]:** Were all analyses replicated 2 periods only? What do you mean by 'continued analysis using a paired t-test at  $\alpha \leq 0.05$ '? Rewrite this part of the paragraph.

## 281 RESULTS AND DISCUSSIONS

282 *Pluchea* leaf infusion is produced by young *Pluchea* leaf from 1-6 level on each  
283 branch the shoot (Widyawati et al., 2022), that is steeped at 95 °C for 5 min, has many  
284 biological activities, such as antioxidant activity (Widyawati et al., 2016), antidiabetic  
285 activity (Werdani and Widyawati, 2018), anti-inflammatory (Srisook et al., 2015). The  
286 chemical constituents in *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols,  
287 cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2  
288 g/100 mL steeping *Pluchea* tea has total phenolic content 9.3 mg gallic acid equivalents  
289 (GAE)/g samples, total flavonoid content 22.0 mg catechin equivalents (CE)/g samples,  
290 DPPH free radical scavenging activity 27.2 mg gallic acid equivalents (GAE)/g samples,  
291 and ferric reducing power 10.2 mg gallic acid equivalents (GAE)/g samples (Widyawati et  
292 al., 2016). Previous research has informed related to the composition of phytochemical  
293 compounds in *Pluchea* leaves, such as phenolic acids such as chlorogenic acids, caffeic  
294 acids, 3-O-caffeoylquinic acids, 4-O-caffeoylquinic acids, 5-O-caffeoylquinic acids, 3,4-

295 di-O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic  
296 acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin;  $\beta$ -  
297 carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al.,  
298 2019; Chan et al., 2022; Widyawati et al., 2022). Presence of phytochemical compounds  
299 in herbal product were influenced by environmental factors, i.e., temperature, light  
300 exposure, oxygen level, pH and moisture. The structure of phytochemical compounds in  
301 herbal tea is very sensitive of the surrounding changes. The effect arising from these  
302 changes causes the structure of the phytochemical molecule to be degraded to produce  
303 smaller size molecules or to combine to produce larger size molecules (Ali et al., 2018;  
304 Jayani et al. 2022, Ramphinwa et al., 2023). Therefore, this study was conducted to  
305 determine the effect of steeping temperature and storage ~~time~~period of *Pluchea* tea on  
306 levels of the bioactive compounds, antioxidant and antidiabetic properties and phenolic  
307 compound profile.

**Commented [A10]:** Delete this part. Information in here are already found in the Introduction section.

## 309 BIOACTIVE COMPOUNDS

### 310 Phenolics Compounds

**Formatted:** Centered

311 The bioactive compounds are active compounds in plants that are essential to  
312 protect a body health (Nguyen and Chuyen, 2020). These compounds usually have many  
313 biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer,  
314 antimicrobial, antibacterial, anti-cholesterol and so on (Suriyaphan, 2014; Acar et al.,  
315 2022). Phenolic compounds have potential redox properties that can scavenge free  
316 radicals that can cause a number of chronic diseases (Noreen et al., 2017; Aryal et al.,  
317 2019; Acar et al., 2022).

318 The total phenolic content (TPC) of *Pluchea* infusion at different steeping  
319 temperature and storage period generally significantly increased with increasing steeping  
320 temperature and storage period based on paired ~~T-t~~ test at  $\alpha \leq 0.05$  (Figure 1a). Steeped  
321 and stored infusion had significantly higher amounts of phenolic compounds than the  
322 samples that were steeped and un-stored. Further, the highest total phenolic content was  
323 observed in samples infused at 95 °C and stored for 5 years (at 71.38±4.14 mg GAE/g  
324 samples) while the lowest was measured in the un-stored samples and infused at 60 °C  
325 (at mg GAE/g sample). Phenolic content of stored samples that were infused at  
326 different temperatures that then stored were steeped only at 60 and 95 °C also showed a  
327 significant increase in their phenolic content. This implies that the steeping temperature  
328 and the storage periods significantly resulted in the high amounts of the phenolic  
329 compounds of the infusions. Results also indicated that phenolic compounds were  
330 generally greater in the infusion at high steeping temperatures and long storage period  
331 (Figure 1a). This could have been due to that fact that during steeping fresh *Pluchea* tea  
332 had a lower total phenolic content than stored *Pluchea* tea for 5 years, besides that the  
333 higher the steeping temperature also caused the greater the extracted total phenolic  
334 content. The temperature of infusion influenced total phenolic content, it could relate to  
335 This could have been due to the fact that the steeping temperature and storage period  
336 can cause the process of degradation, oxidation, and leaching/release of phenolic  
337 compounds. Phenolic compounds are water soluble and thus soaking in hot water for a  
338 certain period of period as in steeping causes the migration process of more phenolic  
339 compounds to the water because of longer increasing contact exposure between of  
340 phenolic compounds to water (Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al.

341 (2022). Su et al. (2019) reported that temperature treatment can stimulate the release  
342 of phenolic compounds of lychee juice stored at different temperatures of 4 and 45 °C  
343 and different long storage (fresh and 72 hours).  
344 this compounds and water. The same phenomena also occurred in Castiglioni  
345 et al. (2015); Kilic et al. (2017), and Acar et al. (2022).  
346 This occurrence showed that steeping temperature and storage period caused the  
347 process of degradation and oxidation of phenolic compounds. Su et al. (2019) reported  
348 that temperature treatment can stimulate the release of phenolic compounds and  
349 increase antioxidant activity of lychee juice stored at different temperatures of 4 and 45  
350 °C and different long storage (fresh and 72 hours). Hydrogen bonding is affected by  
351 Temperature treatment because the degrades (or hydrolyzes) the hydrogen bond  
352 between phenolic compounds and proteins can be degraded that the measured levels  
353 resulting in an increase of phenolic compounds when exposed to are higher  
354 temperatures. The phenomena were supported by (Ali et al. (2018); Jayani et al. (2022),  
355 and Ramphinwa et al. (2023). Zhang et al. (2021) reported that phenolic compounds  
356 present in plants are not completely stable, but are easily degraded during storage after  
357 harvest. Reblova (2012) claimed that antioxidant compounds can be slowly degraded  
358 with increasing temperature. Besides that, Fibrianto et al. (2021) also stated that the  
359 brewing temperature has an effect on the extracted antioxidant compounds, such as  
360 alkaloids, catechins and tannins. Thus, there is an assumption that temperature and  
361 storage caused the degradation, oxidation and hydrolysis of the phenolic compounds that  
362 the phenolic compounds in *Pluchea* infusion are degraded due to oxidation and hydrolysis  
363 because of temperature and storage time period and can be easily extracted during

364 steeping, thus resulting in the increased amount of the phenolic content  
365 compounds as the at higher steeping temperature and longer storage increase period.

366 Based on using of a reference standard could be informed that Simple phenolic  
367 compounds identified in steeped and stored in *Pluchea leaf* infusion, including gallic  
368 acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-O-caffeoylquinic acids,  
369 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic acids was showed in Table 1.

370 The treatment effects results of statistical analysis using a paired T test at  $\alpha \leq 0.05$  showed  
371 that gallic acid and kaempferol contents of *Pluchea* infusion were insignificantly different  
372 at various steeping temperature and long storage periods. Nevertheless, the The  
373 concentration of quercetin and 3,5-di-O-caffeoylquinic acid of the un-stored and stored  
374 *Pluchea* infusion was significantly different from the rest of the samples between of two  
375 treatments except at 70 °C. The while (+)-catechin concentration of *Pluchea* infusion was  
376 only significantly different at 95 °C, but the myricetin content was significantly different  
377 different concentration at 80 and 95 °C. The 3,4-di-O-caffeoylquinic acid content showed  
378 significace difference at 60, 80 and 95 °C and while 4,5-O-dicaffeoylquinic acid  
379 compounds content from *Pluchea* infusion were was only significantly different at 60 °C,  
380 however the concentration of 3,4 dicaffeoylquinic acid was also significantly different at  
381 80 and 95 °C.

382 Based on the analysis of concentration of Results further showed simple phenolic  
383 compounds showed that gallic acids and kaempferol were relatively stable phenolic acid  
384 because of as reflected by the insignificant changes when exposed no changes at to the  
385 different steeping temperature and storage time period, with concentration about 0.24 ±  
386 0.00 to 0.24 ± 0.02 µg/g samples and 0.14 ± 0.02 to 0.95 ± 0.03 µg/g samples, respectively.



387 ~~However, myricetin~~Myricetin, (+)-catechin and 3,4-di-O-caffeoylquinic acid showed a  
388 drastic ~~increasing~~ increase at higher steeping temperature and longer storage period  
389 ~~implying -It's meant~~ that these compounds tended to be relatively labile. Quercetin, 3,5-  
390 di-O-caffeoylquinic acid and 4,5-di-O-caffeoylquinic acid underwent moderate changes  
391 ~~compared to the other two groups of phenolic acids.~~ Therefore, myricetin, (+)-catechin  
392 and 3,4-di-O-caffeoylquinic acid were easier to dissolve or degraded to form simple  
393 phenolic compounds at higher steeping temperature and storage ~~time~~period. ~~can cause~~  
394 ~~macromolecules of three phenolic acids in herbal tea convenient degradable to form~~  
395 ~~simple phenolic compounds for storage, as explained by~~ (Su et al. (2019), Ali et al. (2018);  
396 Jayani et al. (2022); Ramphinwa et al. (2023), and Zhang et al. (2021). Degradable  
397 polyphenol compounds have a simple structure and free hydroxyl groups that can react  
398 with Folin-Ciocalteu's Phenol reagent, resulting complex blue solution that can detected  
399 as total phenolic content.

Commented [A11]: Does the negative sign mean an increase or decrease

#### 400 **Flavonoid Content (TFC)**

401 Flavonoids are the major phenolic compounds that have potential chemical and  
402 biological activities, such as radical scavenging and antimicrobial activities (Ayele et al.,  
403 2022; Chandra et al., 2014) that can protect the human body from the oxidative stress  
404 caused many degenerative diseases, especially cancer, cardiovascular problems and  
405 ageing (Mathur and Vijayvergia, 2017). The total flavonoid content of steeped *Pluchea*  
406 infusion decreased with longer storage period. Un-stored samples exhibited higher  
407 flavonoid content than the stored samples. The statistical analysis using a paired T test  
408 at  $\alpha= 0.05$  showed that total flavonoid content of *Pluchea* infusion was significantly  
409 different between ~~two treatments~~the steeped un-stored and steeped stored samples

Commented [A12]: What does the negative (-) sign implies? What is your basis of classifying the simple phenolic compounds as relatively labile, moderate?

Formatted: Centered

410 (Figure 1b). The highest total flavonoid content was exhibited by ~~fresh the un-stored~~  
411 ~~samples steeped at 95 °C at~~ about 147.42±14.03 mg CE/g samples. Total flavonoid  
412 content was significantly lower in the stored ~~samples regardless of steeping temperature~~  
413 ~~than those of the un-stored around 24.75±2.47 to 33.71±3.06 mg CE/g samples~~ implying  
414 that the increase in the flavonoid content of the infusion was affected primarily by the  
415 steeping temperature.

Commented [A13]: cite similar studies to support your findings

#### 416 Tannin Content (TTC)

Formatted: Centered

417 Tannins are bioactive compounds that provide properties, such as astringent, anti-  
418 diarrheal, antibacterial and antioxidant (Malangngi et al., 2012). Generally, results  
419 indicated that the total tannin content of *Pluchea* infusion significantly increased with  
420 increasing steeping temperature and storage period (Figure 1c). Among, the un-stored  
421 steeped samples, the tannin content was significantly lowest in ~~the~~ samples infused at 60  
422 °C ~~at~~ about 4.81±0.58 to 17.42±1.04 mg TAE/g samples, ~~which is was~~ significantly  
423 different ~~lower~~ from ~~that of~~ the lowest tannin content of the stored samples. Among the  
424 stored and steeped samples, the highest tannin content was observed at samples  
425 steeped at 95 °C about 17.42 ± 1.04 mg TAE/g samples and was significantly different  
426 from that of the highest tannin content of the un-stored steeped samples at 95 °C about  
427 9.22 ± 1.48 mg TAE/g samples. ~~Indicating that the tannin content was primarily~~ affected  
428 ~~by both high steeping temperature and long storage period than high steeping~~  
429 ~~temperature and that the presence of high tannin content was primarily brought about by~~  
430 ~~long storage period. Kowalska et al. (2021) informed that~~ the condensation of catechins  
431 to tannins ~~of polyphenolic compounds~~ is a dominant process ~~occurred occurring~~ in tea  
432 leaves that is accelerated during maceration of raw ~~material~~ tea leaves (Kowalska et al.

433 (2021) could have had contributed to the observed increase in the tannin content in the  
434 treated samples. However, the high temperature can degrade polyphenolic compounds  
435 to form simple phenolic compounds that is essential to body health. The results showed,  
436 that the higher the brewing temperature and the longer the storage time caused the tannin  
437 compound to degrade to result catechin compounds. This phenomenon is in line with the  
438 increase in total phenol levels and the concentration of (+) catechin compounds. Ali et al.  
439 (2018) said that pH, storage temperature, chemical structure and concentration, light,  
440 oxygen, enzymes and metal ions affect the presence of bioactive compounds in the  
441 material. Nevertheless,

442 Although, high temperature and long storage period can cause the degradation of  
443 tannins to catechins, Rusita et al. (2019) emphasized that tannins are a polar  
444 thermostable complex compounds, that is are resistant to heating, indicating that even  
445 with the exposure to high temperature, the tannins still remained high in the treated  
446 samples as a result the tannin content in *Pluchea* tea increases with increasing steeping  
447 temperature and storage time period, this is caused tannins are thermostable complex  
448 compounds.

449

#### 450 ANTIOXIDANT ACTIVITY

451 Antioxidant activity is capability of compounds to inhibit the oxidation of  
452 macromolecules from biological target that involve in oxidative chain reactions (Ali et al.,  
453 2005; Oh et al., 2013). The antioxidant activity assay was done in this research using  
454 DPPH Free Radical Scavenging Activity (DPPH) and ferric reducing power (FRAP)  
455 methods. The phenolic compounds are an active antioxidant that have antioxidant

Formatted: Centered

456 capability ~~that depends~~ on their redox properties. The structure of phenolic compounds  
457 determine ~~the~~ effectivity to ~~doner donate~~ hydrogen atom which is negatively correlated  
458 with the O-H phenolic bond strength. The higher antioxidant power of phenolic  
459 compounds is caused ~~by~~ the weaker O-H phenolic bond (Kruk et al., 2022). The  
460 mechanism of phenolic compounds ~~is involved~~ as antioxidants ~~through depends on their~~  
461 the ability to donate hydrogen atom ~~ands~~, transfer electrons, ~~and as~~ reducing agents and  
462 singlet oxygen quenchers (Ali et al., 2005; Huang et al. 2005).

Commented [A14]: what do you mean? rewrite

#### 464 DPPH Free Radical Scavenging Activity

Formatted: Centered

465 DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate  
466 antioxidant activity because this method ~~is simple~~ that is suitable to measure the donating  
467 hydrogen atoms capability of ~~herbal infusion~~. This reaction can cause the purple color of  
468 ~~DPPH to change to yellow color~~ (Munteanu and Apetrei, 2021; Baliyan et al., 2022).  
469 ~~Figure 2a shows that the free radical scavenging property of the stored and steeped~~  
470 ~~samples were significantly higher than the un-stored steeped samples. The result of~~  
471 ~~DPPH assay~~ It can also be observed ~~indicates~~ that the ~~free radical scavenging property~~  
472 ~~DPPH values accrued~~ was significantly different among the stored and steeped samples  
473 ~~but insignificant among the un-stored and steeped samples at higher steeping~~  
474 ~~temperature and longer storage timeperiod. Statistical analysis by ANOVA using a paired~~  
475 ~~T test at  $\alpha \leq 0.05$  proven that the higher the steeping temperature of fresh *Pluchea*~~  
476 ~~infusion (T1B1, T2B1, T3B1, and T4B1) was consistent the ability to DPPH free radicals~~  
477 ~~scavenging activity, whereas the stored *Pluchea* infusion resulted in the higher activity~~  
478 ~~and the values went up as rising of the infusion temperature. *Pluchea* infusion stored at~~

479 room temperature for 5 years resulted in the high DPPH-free radical scavenging activity  
480 by more than 100 %. Steeping at higher temperatures significantly increased the DPPH  
481 free radical scavenging activity in stored *Pluchea* infusion by around 15 to 25 %. Steeping  
482 at 80-95 °C in stored *Pluchea* infusion insignificantly affected the free radical scavenging  
483 property of the bioactive compounds (Figure 2a). This implies that that the higher free  
484 radical scavenging property was primarily affected by the storage period than steeping  
485 temperature. During the storage process it is possible to form complex phenolic  
486 compounds which provide a high ability to scavenge DPPH-free radicals  
487 (Thanajiruschaya et al., 2010)

488 Scavenging The scavenging activity of DPPH free radicals of the the samples was  
489 strongly and positively correlated with total with total phenolic and tannin contents levels,  
490 but inversely to with total flavonoid levels. Based on Pearson correlation at Table 2, the  
491 correlated coefficient values (r) between DPPH and TPC, TTC and TFC were 0.993,  
492 0.942, and 0.940, respectively. During the storage process it is possible to form complex  
493 phenolic compounds which provide a high ability to scavenge DPPH free radicals  
494 (Thanajiruschaya et al., 2010). This research study also demonstrated that longer storage  
495 time period and higher infusion temperature produced many simple phenolic compounds  
496 with free hydroxyl groups capable to donor hydrogen atom to DPPH free radical. Many  
497 phenolic acids, such as gallic acids, (+)-catechins, myricetins, kaempferols, quercetins,  
498 3,5-di-O-caffeoylquinic acids, 3,4-di-O-caffeoylquinic acids, 4,5-di-O-caffeoylquinic acids  
499 have established potential antioxidant activity (Kumar and Goel, 2019) (Table 1). Kruk  
500 et al (2022) informed that the capability of phenolic compounds to donor hydrogen atom  
501 depends on chemical structure, number and position of hydroxyl groups attached to a

Commented [A15]:

Commented [A16R15]: Clarify on how you were able to come up with free radical scavenging activity by more than 100 %. Steeping temperatures significantly increased the free radical scavenging activity in stored *Pluchea* infusion by around 15 to 25 %

Commented [A17]: Explain/interpret this observation based on the data that you were able to obtain.

502 benzene ring, a double bond between C2 and C3 rings and a carbonyl group (C=O) on  
503 the C ring at C4. The effectivity of antioxidant compounds donor hydrogen atom is  
504 determined by O-H bond dissociation energy.

505 The DPPH-free radical scavenging property observed in the study was not in  
506 consistent with the results of the study by Moraes-de-Souza et al. (2008). The research  
507 shows that total phenolic content of herbal infusion is low correlated with DPPH-free  
508 radical scavenging activity. However, Dobrinas et al. (2021) informed that total phenolic  
509 content of tea infusion is positively and significantly correlated with the free radical  
510 scavenging property/inhibitor activity of DPPH of tea infusion.

511

#### Ferric Reducing Antioxidant Power (FRAP)

Formatted: Centered

512 FRAP is an analysis of antioxidant power of the phytochemical compounds based  
513 on the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic  
514 acid, and ferric chloride to produce a color complex, that can be measured at  $\lambda$  700 nm  
515 (Fu et al., 2011; Al-Temimi and Choudhary, 2013). The principle of the assay measures  
516 that is based of the ability of antioxidant compounds to reduce iron ions of potassium  
517 ferrocyanide ( $\text{Fe}^{3+}$ ) to be potassium ferrocyanide ( $\text{Fe}^{2+}$ ). Potassium ferrocyanide reacts  
518 with ferric chloride to form a ferric-ferrous complex and results green color solution  
519 (Widyawati et al., 2017; Raharjo and Haryoto, 2019).

520 The results showed that the ferric reducing antioxidant power (FRAP) increased  
521 with at higher steeping temperature and longer storage time period. The lowest FRAP was  
522 observed in the un-stored samples which was steeped at 60 °C at  $3.95 \pm 0.17$  mg gallic  
523 acid equivalents (GAE)/g samples, and the highest was owned exhibited by in *Pluchea*

525 infusion which was stored for 5 years at 95 °C at 48.63 ±10.83 mg gallic acid equivalents  
526 (GAE)/g samples (Figure 2b). FRAP increased significantly as steeping temperature was  
527 increased. FRAP of the samples stored for 5 years was also significantly higher than the  
528 un-stored samples at  $\alpha \leq 0.05$ . Based on Pearson correlation, the FRAP of *Pluchea*  
529 infusion was strongly and positively significant correlated with the DPPH, TPC and TTC,  
530 but inversely to TFC. The correlated coefficient values (r) between FRAP and DPPH,  
531 TPC, TTC and TFC were 0.956, 0.953, 0.948 and -0.826, respectively.

532 This case was is in contrast to with the study on the antioxidant activity of DPPH  
533 and FRAP on of matcha, because The the longer storage time period reduces the levels  
534 of catechin content due to the catechins, such as epigallocatechin gallat (EGCG),  
535 epicatechin gallat (ECG), epigallocatechin (EGC), and epicatechin (EC) which are  
536 bioactive compounds that have high antioxidant activity (Kim et al. 2020), and also the  
537 case of the effect of temperature and storage time in betel (*Piper bettle* L.) extract. Light  
538 and temperature influence degradation of phenolic compounds of betel that determine  
539 antioxidant activity. Different structure of phenolic compounds determines their stability  
540 to degrade accelerating of light and temperature. Hydroxychavicol is the best stability of  
541 phenolic compounds of betel compared with eugenol, isoeugenol and allyl pyrocatechol  
542 (Ali et al., 2018). Thanajiruschaya et al. (2010) revealed that the antioxidant activity of  
543 rice stored at high temperatures is greater than that stored at low temperatures. The ferric  
544 reducing capability of *Pluchea* could have due infusion corresponded to the presence to  
545 of simple phenolic acid values that have the ability to transfer electron from their free  
546 hydroxyl groups of, presence of them in samples could accrue antioxidant activity  
547 because of ability of the electron transfer from free hydroxyl groups of phenolic acids.

Commented [A18]: Relate these with Figure 2b. Rewrite

548 [The FRAP of \*Pluchea\* infusion was strongly and positively significant correlated with the](#)  
549 [DPPH, TPC and TTC, but inversely to TFC.](#)

550 ANTIDIABETIC ACTIVITY

551  **$\alpha$ -Amylase enzyme inhibition activity (AA)**

Formatted: Centered

552 Antidiabetic activity is a measure of the potency of phenolic compounds to regulate  
553 the uptake of glucose by the cells from the blood through the mediation of 2-degestive  
554 enzymes i.e.,  $\alpha$ -amylase and  $\alpha$ -glucosidase, which are involved the control of dietary  
555 carbohydrate digestion and release in the postprandial blood glucose in human body (Fu  
556 et al., 2017). The phenolic compounds have the capability to bind with the protein  
557 component of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Martinez-Solis et al., 2022)  
558 resulting in the reduced activity of the enzymes. The results showed, that ~~the lower~~  
559 ~~steeping *Pluchea* leaf infusion~~ was able to inhibit the action of the  $\alpha$ -amylase enzymes  
560 (Figure 3a). The *Pluchea* infusion ~~had very good activity, exhibited a good  $\alpha$ -mylase~~  
561 ~~enzyme inhibition activity~~ of more than 50 % and even almost 100 % ~~for fresh in the un-~~  
562 ~~stored *Pluchea* infusion which steeped was brewed~~ at 60, 70 and 80 °C with highest at  
563 60 °C, and in stored *Pluchea* leaf infusion which was steeped at 60 °C. ~~Whereas The~~  
564 ~~stored fresh *Pluchea* leaf~~ infusion steeped at 70, 80 and 95 °C for 5 minutes had lower  
565 enzyme inhibition activity ~~an activity of of less than 50 % with lowest at 95 °C, inhibiting the~~  
566  ~~$\alpha$ -amylase enzyme of less than 50 %, which was equal to 40.08±1.12 %.~~ Widyawati et al.  
567 (2017) ~~detected found that~~ the ability to inhibit the  $\alpha$ -amylase enzyme ~~from in fresh un-~~  
568 ~~stored *Pluchea* infusion steeped at 95 °C for 5 minutes~~ ~~by~~ was also low at 28.79 %.  
569 Increasing the steeping temperature and storage ~~timeperiod~~ reduced the ability ~~to of the~~  
570 ~~phytochemicals in the *Pluchea* infusions to~~ inhibit the  $\alpha$ -amylase enzyme activity. ~~The~~

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Commented [A19]: Why?/Explain



571 results of the analysis based on a paired T test at  $\alpha \leq 0.05$  showed, that the steeping  
572 temperature and storage timeperiod had a significant effect on the ability to inhibit the  $\alpha$ -  
573 amylase enzyme. Based on Pearson correlation, the Table 2 further shows that the AA of  
574 *Pluchea* infusion was strongly and negatively significant correlated with TPC, TTC, DPPH  
575 and FRAP, but it was moderately and negatively significant correlated with TFC. The  
576 correlated coefficient values ( $r$ ) between AA and TPC, TTC, DPPH, FRAP and TFC were  
577 0.708, -0.857, -0.696, -0.806 and 0.429, respectively.

578 This inhibitory activity was thought to be contributed by other bioactive compounds,  
579 besides phenolics which are sensitive to steeping temperature and storage timeperiod. Li  
580 et al. (2018) stated that there are flavonoid compounds that contribute to the ability to  
581 inhibit the  $\alpha$ -amylase enzyme. Flavonoid compounds with a hydroxyl structure at C-4' in  
582 ring B are more effective than C-6 in ring A. Akah et al. (2011) informed reported that the  
583 phytochemical compounds, such as terpenoids, saponins, flavonoids, glycosides and  
584 carbohydrate, and alkaloids are good antidiabetic metabolites or  $\alpha$ -amylase enzyme  
585 activity inhibitor. Sangeetha and Vedasree (2012) explained, that the ability to inhibit the  
586  $\alpha$ -amylase enzyme was determined by the content of the phenolic compound and protein.  
587 The  $\alpha$ -amylase inhibitor enzyme present in *Pluchea* infusion may be proteinaceous in  
588 nature. Alexandre et al. (2022) informed that phenolic acids have inhibition activity to  $\alpha$ -  
589 amylase enzyme depending their structures. Besides that, capability of phenolic acids to  
590 inhibit  $\alpha$ -amylase enzyme was determined by low half-maximum inhibitory concentration  
591 ( $IC_{50}$ ). There are C=C double bond conjugated with a carbonyl group of phenolic  
592 structures that stabilizes the binding forces to the active site of the  $\alpha$ -amylase. The  
593 hydroxyl groups of them are able to bind by non-covalent interaction, such as hydrogen

Commented [A20]: Implications? Explain

Commented [A21]: What content or what is in the content the influenced the ability to inhibit the enzyme?

Commented [A22]: How will this affect the ability to inhibit the enzyme?

594 binding, cation- $\pi$  interactions, salt bridge interactions, ionic interactions or electrostatic  
595 forces with amino acid residue at the active site in  $\alpha$ -amylase enzyme. ~~Elevated steeping~~  
596 ~~temperature and longer storage period~~ ~~The steeping temperature and storage time can~~  
597 ~~easily cause the~~ removal of the ~~e~~ hydroxyl groups of phenolic compounds that can reduce  
598 ~~their~~ ability of enzyme inhibition. The phenolic acids with a greater number of hydroxyl  
599 groups ~~are exhibits~~ stronger capability to obstruct the  $\alpha$ -amylase enzyme.

**Commented [A23]:** Lines 585 to 595, Either delete or rewrite for better readability and understanding referring to enzyme activity inhibition

#### 600 $\alpha$ -Glucosidase enzyme inhibition activity (GA)

**Commented [A24]:** This part is disorganized. Avoid duplicating statements, observation facts etc

**Formatted:** Centered

601 ~~Alpha~~ $\alpha$ -glucosidase is an important enzyme in carbohydrates digestion, that  
602 catalysis the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and  
603 converts them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et  
604 al., 2014; Proenca et al., 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -  
605 glucosidase enzyme is used to determine ~~their~~ antidiabetics activity. ~~This is supported~~  
606 ~~by~~ Werdani and Widyawati (2018) ~~stated~~, that **Pluchea infusion** has the potential as an  
607 antidiabetic agent. Widyawati et al. (2020) found that brewing fresh **Pluchea infusion** at  
608 95 °C for 5 minutes has an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857 %.

609 ~~The results showed~~, ~~Figure 3b shows~~ that the ability of the **Pluchea leaf infusion**  
610 to inhibit the  $\alpha$ -glucosidase enzyme decreased with increasing steeping temperature and  
611 storage ~~time~~period. Steeping at 95 °C ~~for fresh~~of the un-stored **Pluchea leaf** infusion (~~un-~~  
612 ~~stored~~) obtained the lowest inhibitory ability, i.e.,  $48.32 \pm 1.27$  %, and the highest inhibitory  
613 activity was found at 70 °C ~~steeping temperature for fresh~~ **Pluchea** infusion, which was at  
614  $95.11 \pm 0.70$ % ~~(Figure 3b)~~. The results of a paired T test showed that GA of **Pluchea**  
615 **infusion** was significantly different ~~at both~~between steeping temperature and long storage.  
616 ~~The antidiabetic activity of~~ **Pluchea infusion** ~~Figure 3 further~~ ~~showed~~ shows that the ability

**Formatted:** Font: Not Italic

**Commented [A25]:** Explain

617 of *Pulchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher than the  
618 ability to inhibit the  $\alpha$ -amylase enzyme. Li et al. (2018) informed that flavonoid compounds  
619 have the ability to inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. This is  
620 due to the total flavonoids in steeped *Pluchea* infusion which tended to have the same  
621 pattern as the ability to inhibit the activity of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes.  
622 The statistical analysis using Pearson correlation showed that GA of *Pluchea* infusion  
623 was strongly and negatively correlated with TPC, TTC, DPPH and FRAP  
624 ~~with r was -0.555, -0.715, -0.527 and -0.560, respectively.~~ However, GA was  
625 moderately and positively correlated to TFC, ~~with r was 0.350 and strongly and positively~~  
626 ~~correlated to AA, with r was 0.725.~~ Flavonoid compounds, such as rutin, myricetin,  
627 kaempferol, and quercetin ~~which~~ have antioxidant and antihyperglycemic activities. The  
628 ability to inhibit the action of enzymes from flavonoid compounds is determined by the  
629 position and number of hydroxyl groups and the number of double bonds in rings A and  
630 B and the heterocyclic ring in ring C. The ability to inhibit the  $\alpha$ -glucosidase enzyme from  
631 *Pluchea* infusion was significantly affected by the steeping temperature and long storage.  
632 The capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater than  
633 the  $\alpha$ -amylase enzyme because the mechanism of two enzymes was different, according  
634 to the opinion of McCue et al. (2005). Widyawati et al. (2017) informed that phenolic and  
635 non-phenolic compounds determine the inhibitory activity of the  $\alpha$ -glucosidase enzyme.  
636 The ability of bound phenolic compounds to inhibit  $\alpha$ -glucosidase enzymes was higher  
637 than free phenolic compounds. The presence of polymerization and degradation  
638 reactions, that may be occurred in *Pluchea* infusion during storage, affects the structure  
639 and profile of phenolic and non-phenolic compounds. Asriningtyas et al. (2014) claimed

Formatted: Font: Italic

Commented [A26]: This does not explain explain lines 597-599 with the manner it is written. Include statements that connect the explanation with the observation. Having 'same pattern' is not observed in the figure/graph

Commented [A27]: Interpret/Implications

Commented [A28]: Delete literature citations that are unnecessary to explain the findings

640 that *Pluchea* leaves contain 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid  
641 methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid,  
642 and 1,3,4,5-tetra-*O*-caffeoylquinic acid. Quinic acid is methyl esterified with the number  
643 of caffeic groups in the molecule that determines the activity of inhibiting the  $\alpha$ -  
644 glucosidase enzyme. Analysis of caffeoylquinic acids in *Pluchea* infusion was obtained  
645 that the higher steeping temperature and long storage caused increased concentration  
646 of them, but the  $\alpha$ -glucosidase inhibition activity of them was reduced. Aleixandre et al.  
647 (2022) reported that the simple phenolic acids forming a dipole-dipole interaction of active  
648 site from  $\alpha$ -glucosidase enzyme are effectively inhibiting the enzyme.

649 This study ~~was obtained information~~ showed that the increasing of steeping  
650 temperature and storage ~~time period~~ caused a degradation reaction of polyphenol  
651 compounds to produce simple phenolic compounds, such as gallic acid, (+)-catechin,  
652 myricetin, quercetin, kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic  
653 acid, and 4,5-di-*O*-caffeoylquinic acid, supported the results of total phenolic content and  
654 total tannin content assays. Increased concentration of simple phenolic compounds  
655 determined the ability of these compounds as antioxidant agents, but reduced their  
656 capability as antidiabetic agents.

## 658 CONCLUSION

659 The steeping temperature and storage ~~time period~~ of *Pluchea* infusion significantly  
660 influenced bioactive contents, antioxidant and antidiabetic activities. TPC, TTC, and TFC  
661 were significantly different at various steeping temperature and storage period based on  
662 statistical analysis using a paired ~~T-t~~ test at  $\alpha \leq 0.05$ . ~~There was the difference of t~~The

Corresponding Author: paini@ukwms.ac.id

**Commented [A29]:** Unnecessary because this is not included as one of the derived simple phenolic acids

**Commented [A30]:** Not clear, re-write

**Commented [A31]:** Organize the discussion to explain the observation one at a period. ex:

1) 'Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme decreased with increasing steeping temperature and storage period.' (This must be followed by an explanation or support.)

2) However, GA was moderately and positively correlated to TFC and positively correlated to AA..(This must be followed by implications/support/explanation.)

The ability to inhibit the  $\alpha$ -glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping temperature and long storage.(This can be integrated in 1)

4) The capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater than the  $\alpha$ -amylase enzyme because the mechanism of two enzymes was different, according to the opinion of McCue et al. (2005). (This can be integrated in 1)

The mechanism must be explained -the mechanism of two enzymes was different,

5) Flavonoid compounds, such as rutin, myricetin, kaempferol, and quercetin which have antioxidant and antihyperglycemic activities

6) . Li et al. (2018) informed that flavonoid compounds have the ability to inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. ( May also be integrated in 1)

7) Widyawati et al. (2017) informed that phenolic and non-phenolic compounds determine the inhibitory activity of the  $\alpha$ -glucosidase enzyme. The ability of bound phenolic compounds to inhibit  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. (May also be incorporated in 1).

8) Lines 618 to 629 into 1)

**Commented [A32]:** Reconcile with your discussion

**Commented [A33]:** Suggested conclusion

CONCLUSION

The total phenolic content (TPC) of *Pluchea* infusion at different steeping temperature and storage period generally significantly increased with increasing steeping temperature and storage period. Steeped and stored infusion had significantly higher amounts of phenolic compounds than the samples that were steeped and un-stored. TPC was highest in the store ...

663 phenolic compound profile in ~~fresh~~ ~~the~~ ~~unstored~~ and stored ~~of~~ *Pluchea* infusion ~~and~~ ~~at~~  
664 various steeping temperature. ~~The~~ ~~included~~ simple phenolic compounds ~~were~~ ~~detected~~  
665 ~~in~~ *Pluchea* infusion ~~including~~ ~~such~~ ~~as~~ gallic acid, (+)-catechin, quercetin, myricetin,  
666 kaempferol, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-  
667 caffeoylquinic acid. The results of statistical analysis using a paired ~~T~~ ~~t~~ test at  $\alpha \leq 0.05$   
668 showed that gallic acid and kaempferol of *Pluchea* infusion were insignificantly different  
669 at various steeping temperature and long storage. ~~Nevertheless,~~ ~~T~~ the concentration of  
670 quercetin and 3,5-dicaffeoylquinic acid of *Pluchea* infusion was significantly different of  
671 two treatments except at 70 °C. The (+)-catechin concentration of *Pluchea* infusion was  
672 significantly different at 95 °C, but the myricetin was different concentration at 80 and 95  
673 °C. The 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid compounds from *Pluchea*  
674 infusion were significantly different at 60 °C, however the concentration of 3,4-  
675 dicaffeoylquinic acid was also significantly different at 80 and 95 °C. TPC, TTC and TFC  
676 of *Pluchea* infusion were significantly different at various steeping temperature and  
677 storage period. TPC and TTC significantly increased with increasing steeping  
678 temperature and long storage, but TFC significantly increased at various steeping  
679 temperature and significantly decreased at long storage. The bioactive compounds of  
680 *Pluchea* infusion influenced antioxidant activities (DPPH and FRAP) and antidiabetic  
681 activity (AA and GA). The DPPH was strongly and positively correlated with TPC and  
682 TTC, but it was strongly and negatively correlated with TFC, with coefficient  $r$  0.993,  
683 0.942, and -0.940, respectively. The correlated pattern between FRAP and bioactive  
684 contents of *Pluchea* infusion was similar to it between DPPH and bioactive contents. The  
685 correlated coefficient values ( $r$ ) between FRAP and TPC, TTC and TFC were 0.953, 0.948

686 and -0.826, respectively. The AA and GA were strongly and negatively correlated with  
687 TPC, TTC, DPPH and FRAP, but it was moderately and negatively significant correlated  
688 with TFC. Between the antioxidant activity of DPPH and FRAP and the antidiabetic  
689 activity of AA and GA of Pluchea infusion were strongly and positively correlated with  
690 correlation coefficient (r) values of 0.956 and 0.725, respectively.

691

#### 692 DATA AVAILABILITY

693 Table and figure used to support of this study were included in the article.

694

#### 695 CONFLICT OF INTEREST

696 The authors declare no conflict of interest.

697

#### 698 ACKNOWLEDGEMENTS

699 The authors would like to thank the he Ministry of Education and Culture of the Republic  
700 of Indonesia for fundamental research grant to higher education institutions in 2022

701

#### 702 REFERENCES

- 703 ACAR A, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food  
704 wastes:evaluation of temperature and time effects on quality parameters. Applied Food  
705 Res. 2(100087):1-8.
- 706 ALEIXANDRE A et al. 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -  
707 glucosidase and influence of reaction conditions. Food Chem. 372(131231):1-9.

**Commented [A34]:** Check for authors that may had been deleted in the paper due to revisions done.

708 ALI et al. 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile.  
709 Food Chem. 89(2005):27-36.

710 ALI A. et al. 2018. Impact of storage conditions on the stability of predominant phenolic  
711 constituents and antioxidant activity of dried *piper bettle* extracts. Molecules.23(24):1-13.

712 AL-TEMIMI A, CHOUDHARY R. 2013. Determination of antioxidant activity in different  
713 kinds of plants *in vivo* and *in vitro* by using diverse technical methods. J Nut. Food Sci.  
714 3(1):1-6.

715 AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and  
716 methanol extract and fractions of *Gongronema latifolium* (Asclepidaceae) leaves in  
717 alloxan diabetic rats. J Applied Pharm. Sci. 01(09): 99-102.

718 ARSININGTYAS IS. et al. 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves  
719 of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product  
720 use. Natural Product Res. 28(17):1350-1353.

721 ARYAL S et al. 2019. Total phenolic content, flavonoid content and antioxidant of wild  
722 vegetables from Western Nepal. Plants. 8(96): 1-12.

723 AYELE DT, AKELE ML, MELESE A.T. 2022. Analysis of total phenolic contents,  
724 favonoids, antioxidant and antibacterial activities of *Croton macrostachyus* root extracts.  
725 BMC Chem. 16(30): 1-9.

726 BALIYAN S et al. 2022. Determination of antioxidants by DPPH radical scavenging  
727 activity and quantitative phytochemical analysis of *Ficus religiosa*. Molecules. 27(1326):  
728 1-19.

729 CASTIGLIONI S et al. 2015. Influence of steeping conditions (time, temperature, and  
730 particle size) on antioxidant properties and sensory attributes of some white and green  
731 teas. Intern. J. Food Sci. Nut. Online 1-7.

732 Chan EWC et al. 2022. *Pluchea indica*: An updated review of its botany, uses, bioactive  
733 compounds and pharmacological properties. Pharmaceutical Sciences Asia. 49(1): 77-  
734 85.

735 CHANDRA S et al. 2014. Assessment of total phenolic and flavonoid content, antioxidant  
736 properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit  
737 crops: A Comparative study. Hindawi Publishing Corporation Evidence-Based  
738 Complementary Alter. Med. 2014:1-9 (2014)

739 CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic, flavonoids,  
740 tannin and chlorophyll content of leaves of *Strobilanthes Kunthiana (Neelakurinji)*. J.Med.  
741 Plants Studies. 4(4):282-286.

742 DOBRINAS S, SOCEANU A, POPESCU V, POPOVICI IC, JITARIU D. 2021.  
743 Relationship between total phenolic content, antioxidant capacity, Fe and Cu content from  
744 tea plant samples at different brewing times. Processes. 9(1311):1-11.c

745 FERRARIS Z. 2023. *Pluchea indica* Less.iNaturalist.org.  
746 <https://www.gbif.org/occurrence/4022273660>.

747 FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional  
748 properties and visual appearance of Dampit Robusta coffee leaves tea. Advances in Food  
749 Sci. Sustain. Agric. Agro-industrial Engineering. 4(1):25-32.

750 FU L et al. 2017. Total phenolic contents and antioxidant capacities of herbal and tea  
751 infusions. Intern. J. Molecular Sci. 12:2112-2124.

Corresponding Author: paini@ukwms.ac.id



752 GAO MR et al. 2019. A theoretical and experimental study: the influence of different  
753 standards on the determination of total phenol content in the Folin–Ciocalteu assay. J.  
754 Food Measurement and Characteristic. 13:1349–1356.

755 HUANG D, OU B, PRIOR RL. 2005. The Chemistry behind antioxidant capacity assays.  
756 J. Agric. Food Chem. 53:1841-1856.

757 ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties  
758 of rosehip tea beverage: study by response surface methodology. J. Food Sci. Tech.  
759 54(11):3737–3743.

760 JAYANI NIE et al. 2022. Evaluation of stability and quality characteristics of moringa  
761 (*Moringa oleifera*) herbal tea during storage. Food Res. 6(4):399-406.

762 KIM JM et al. 2020. Effect of storage temperature on the antioxidant activity and catechins  
763 stability of Matcha (*Camellia sinensis*). Food Science and Biotechnology. 29(9):1261–  
764 1271.

765 KILIC et al. 2017. Antioxidant properties of some herbal teas (green tea, senna, corn silk,  
766 rosmery) brewed at different temperatures. Intern. J. Secondary Metabolite. 4(3):142-248  
767 Special Issue 1.

768 KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of  
769 extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves  
770 from different provenances in Thailand. Revista Brasileira de Farmacognosia. 28(2018):  
771 145–150.

772 KOWALSKA J et al. 2021. Influence of tea brewing parameters on the antioxidant  
773 potential of infusions and extracts depending on the degree of processing of the leaves  
774 of *Camellia sinensis*. Molecules.26(4773):1-17.

Corresponding Author: paini@ukwms.ac.id

775 KRUK J, ABOUL-ENAIN BH, DUCHNIK E, MARCHLEWICZ M. 2022. Antioxidative  
776 properties of phenolic compounds and their effect on oxidative stress induced by severe  
777 physical exercise. J. Physiol. Sci. 72(19):1-24.

778 KUMAR N, GOEL N. 2019. Phenolic acids: natural versatile molecules with promising  
779 therapeutic applications. Biotechnology Reports. 24(e00370):1-10.

780 LI K et al. 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the flavonoids  
781 rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity  
782 relationship of its eight flavonoids by a refined assign score method. Chem. Central J.  
783 12(82):1-11.

784 LIN YS. et al. 2020. Effects of storage time and temperature on antioxidants in juice from  
785 *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser. Molecules.  
786 25(3614): 1-12.

787 MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content  
788 and antioxidant activity test of avocado seed extract (*Persea Americana* Mill). Jurnal MIPA  
789 Unsrat Online. 1(1): 5-10. [https://media.neliti.com/media/publications/116214-ID-  
790 penentuan-kandungan-tanin-dan-uji-aktivi.pdf](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf) [In Bahasa Indonesia]

791 MARTINEZ-SOLIS J et al. 2022. Antidiabetic and toxicological effect of the tea infusion  
792 of summer collection from *Annona cherimola* Miller leaves. Plants. 11(3224):1-17.

793 MATHUR R, VIJAYVERGIA R. 2017. Determination of total flavonoid and phenol content  
794 in *Mimusops Elengi* Linn. Intern. J. Pharm. Sci. Res. 8(12): 5282-5285.

795 MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of  
796 sprouted and solid-state bioprocessed soybean. Asia Pacific J. Clinical Nut. 14(2): 145-  
797 152.

Corresponding Author: paini@ukwms.ac.id

798 MORAES-DE-SOUZA RA, OLDONI TLC, REGITANO-D'ARCE MAB, ALENCAR SM.  
799 2008. Antioxidant activity and phenolic composition of herbal infusions consumed in  
800 Brazil. *Cienc Tecnol. Aliment.* 6(1):41-47.

801 MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant  
802 activity: A Review. *Intern. J. Molecular Sci.* 22(3380):1-30.

803 NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus*  
804 *sabdariffa* L.): Effect of dying temperature and brewing conditions on total soluble solid,  
805 phenolic content, antioxidant capacity and sensory quality. *Beverages*.6(2):1-11 (2020).

806 NOREEN H. et al. 2017. Measurement of total phenolic content and antioxidant activity  
807 of aerial parts of medicinal plant *Coronopus didymus*. *Asian Pacific J. Tropical Med.* 10(8):  
808 792–801.

809 NURCHOLIS GW et al. 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity in-  
810 vitro and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum pictum*  
811 (L.). *Current Biochem.* 1(2): 58-65.

812 OH J et al. 2013. Antioxidant and antimicrobial activities of various leafy herbal teas. *Food*  
813 *Control.* 31(2013):403-409.

814 PROENCA C et al. 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an in vitro and in silico  
815 structure–activity relationship study. *J. Enzyme Inhibition Med. Chem.* 32(1): 1216–1228.

816 PUREWAL SS et al. 2022. Unraveling the effect of storage duration on antioxidant  
817 properties, physicochemical and sensorial parameters of ready to serve Kinnow-aml  
818 beverages. *Applied Food Res.* 2(1): 1-8.

819 RAHARJO D, HARYOTO. 2019. Antioxidant activity of mangrove *Sonneratia caseolaris*  
820 L. using the FRAP method. *ISETH.* 2019: 623-629.

Corresponding Author: paini@ukwms.ac.id

821 RAMPHINWA ML et al. 2023. Eco-physiological response of secondary metabolites of  
822 teas: review of quality attributes of herbal tea. *Frontiers in Sustain. Food Systems*.  
823 7(990334):1-14.

824 REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids.  
825 *Czech J. Food Sci.* 30(2):171-177.

826 RUAN J et al. 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea*  
827 *indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel  
828 column chromatography fractions. *Molecules*. 24(2784):1-20.

829 RUSITA YD et al. 2019. Comparison of tannin concentration on fresh and dried tea leaf  
830 (*Camelia sinensis* L.) extract using UV-visible spectrophotometric method. 1st  
831 International Conference of Health, Science & Technology (ICOHETECH) 2019 E-ISBN:  
832 978-623-92207-0-9. November 16<sup>th</sup>, 2019.LPPM Universitas Duta Bangsa Surakarta,  
833 Surakarta.

834 SANGEETHA R, VEDASREE N. 2012. In Vitro  $\alpha$ -Amylase inhibitory activity of the leaves  
835 of *Thespesia populnea*. *Intern. Scholarly Res.Network*. 2012: 1-4 (2012)

836 SHRAIM AM et al. 2021. Determination of total flavonoid content by aluminum chloride  
837 assay: A critical evaluation. *LWT Food Sci. Tech.* 150(111932):1-11.

838 SILVA-RAMIREZ KA et al. 2020. Effect of drying and steeping temperature on the  
839 phenolic content, antioxidant activity, aromatic compounds and sensory properties of  
840 *Culila polyantha* Benth. *Infusions. Processes*. 8(1378):2-20.

841 SRISOOK K et al. 2012. Antioxidant and anti-inflammatory activities of hot water extract  
842 from *Pluchea indica* Less. herbal tea. *J. Med. Plants Res.* 6(23):4077-4081.

Corresponding Author: paini@ukwms.ac.id

843 SURIYAPHAN O. 2014. Nutrition, health benefits and applications of *Pluchea indica* (L)  
844 Less leaves. Pharm. Sci. Asia. 41:1–10.

845 SU D et al. 2019. Impact of thermal processing and storage temperature on phenolic  
846 profile and antioxidant activity of different varieties of lychee juice. LWT-Food Tech.  
847 116(108578):1-9.

848 THANAJIRUSCHAYA P et al. 2010. Effect of storage ~~time~~[time period](#) and temperature on  
849 antioxidant components and properties of milled rice. Khon Kaen Univ. Res. J.15(9):843-  
850 851.

851 VONGSAK B et al. 2018. Comparison of active constituents, antioxidant capacity, and  
852 αglucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. Food  
853 Biosci. 25: 68–73.

854 WANG SC. et al. 2022. Effect of brewing conditions on polyphenols in the dark tea  
855 (*Camellia sinensis* L.) infusions: content, composition and antioxidant activities. Food  
856 Sci.Tech. 42(e36322):1-9.

857 WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica*  
858 Less as functional beverage in diabetic patients. ASSEHR. 98:164-167.

859 WIDYAWATI PS et al. 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract  
860 as antioxidant and antiwarmed over flavor (WOF) of duck meat. International Congress  
861 on Challenges of Biotechnological Research in Food and Health. November 15<sup>th</sup>  
862 2014.Agricultural Technology Faculty, Slamet Riyadi University, Surakarta.

863 WIDYAWATI PS et al. 2016. The physicochemical and antioxidant properties of *Pluchea*  
864 *indica* Less drink in tea bag packaging. IJFNS. 5: 13–120.

Corresponding Author: paini@ukwms.ac.id

865 WIDYAWATI PS et al. 2017. In vitro antioxidant capacities and antidiabetic properties of  
866 *Pluchea* leaves. IJPPS. 9(8): 203-208.

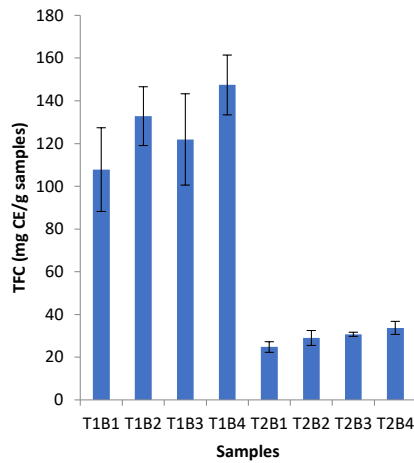
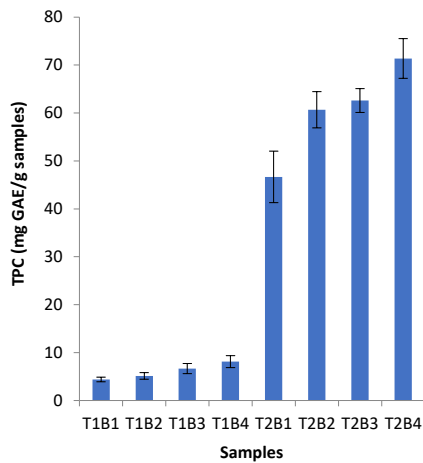
867 WIDYAWATI PS et al. 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica*  
868 Less beverage. CJFST.12(4): 125-139.

869 WIDYAWATI PS ET AL. 2022. The effect of  $\kappa$ -carrageenan proportion and hot water  
870 extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of stink  
871 lily (*Amorphophallus muelleri*) wet noodles. Molecules. 27(5062):1-16.

872 XU P, CHEN L, WANG Y. 2019. Effect of storage [time period](#) on antioxidant activity and  
873 inhibition on  $\alpha$ -amylase and  $\alpha$ -glucosidase of white tea. Food Sci. Nut. 7(2): 636-644.

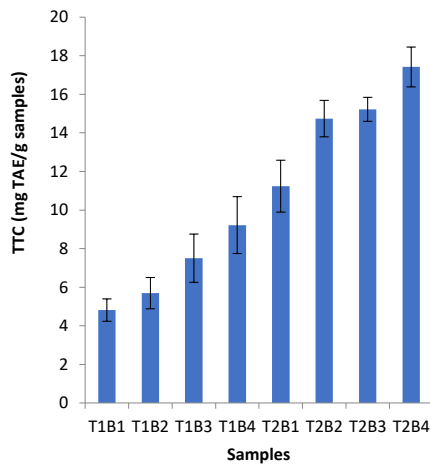
874 ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction [time period](#) on  
875 caffeine in coffee. Lantanida J. 6(2):103-202. [https://jurnal.ar-](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811)  
876 [raniry.ac.id/index.php/lantanida/article/view/3811](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811). [In Bahasa Indonesia]

877 ZHANG Y et al. 2021. Effect of storage conditions and [time period](#) on the polyphenol  
878 content of wheat flours. Processes. 9(248):1-11.



(a)

(b)



(c)

Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping temperature and storage time period (a) Total phenolic content (b) Total flavonoid content (c) Total tannin content. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-

Formatted: Left, Indent: Left: 0.5", Hanging: 0.69", Line spacing: single

888  
889  
890  
891  
892  
893  
894

stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$ .

Commented [A35]:



Table 1. Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperature and storage time period

Phenolic Compounds	Steeping Temperature (°C)	Mean±SD Un-stored	Mean±SD Stored	Mean difference ±SD	Sig (2-tailed)
Gallic Acid (µg/g samples)	60	0.2132±0.0027	0.2364±0.0015	0.0375±0.0175	0.2030
	70	0.2157±0.0013	0.2324±0.0214	0.0167±0.0227	0.4870
	80	0.2234±0.0122	0.2347±0.0078	0.0386±0.0264	0.2870
	95	0.2316±0.0104	0.2402±0.0169	0.0086±0.1990	0.8500
(+)-Catechin (µg/g samples)	60	0.3425±0.0110	0.5085±0.0111	-0.1576±0.0885	0.241
	70	0.3260±0.0265	0.5448±0.0006	-0.2188±0.0259	0.053
	80	0.3240±0.0222	0.5023±0.0773	-0.1451±0.0248	0.077
	95	0.4039±0.0320	0.5995±0.0372	-0.2049±0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756±0.1234	1.4762±0.0271	-1.2887±0.3222	0.111
	70	0.2587±0.0160	1.4245±0.2526	-1.1657±0.2695	0.103
	80	0.4175±0.0104	1.4570±0.0925	-1.0391±0.0841	0.036*
	95	0.8786±0.0434	2.6138±0.0695	-1.735±0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220±0.0268	0.6220±0.0706	-0.5999±0.9733	0.544
	70	0.1530±0.0511	1.0708±0.0289	-0.9177±0.0222	0.011*
	80	0.3666±0.0103	0.8629±0.0815	-0.1082±0.4462	0.790
	95	0.6559±0.0570	2.0230±0.0573	-1.4123±0.3203	0.101
Kaempferol (µg/g samples)	60	0.1394±0.0202	0.3675±0.0183	-0.3207±0.1122	0.154
	70	0.0514±0.0037	0.3726±0.0944	0.3213±0.0907	0.125
	80	0.3699±0.0924	0.7966±0.0366	-0.4267±0.2727	0.271
	95	0.5913±0.0239	0.9478±0.0287	-0.3565±0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103±0.0628	2.4863±0.0270	-1.8760±0.2074	0.050*
	70	0.6271±0.0099	2.3403±0.0325	-1.7131±0.3152	0.082
	80	0.7967±0.03060	2.6278±0.0211	-1.8311±0.0095	0.002*
	95	1.5386±0.0668	4.0211±0.0851	-2.4825±0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635±0.0628	0.9449±0.0501	-0.2814±0.4458	0.536
	70	0.6162±0.0099	0.9485±0.0794	-0.3323±0.0301	0.041*
	80	0.6601±0.0306	0.9099±0.0387	-0.2498±0.3127	0.461
	95	0.6642±0.0668	1.3156±0.0166	-0.6514±0.2666	0.179

Corresponding Author: paini@ukwms.ac.id

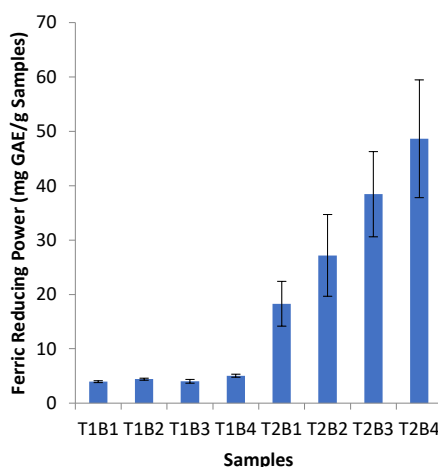
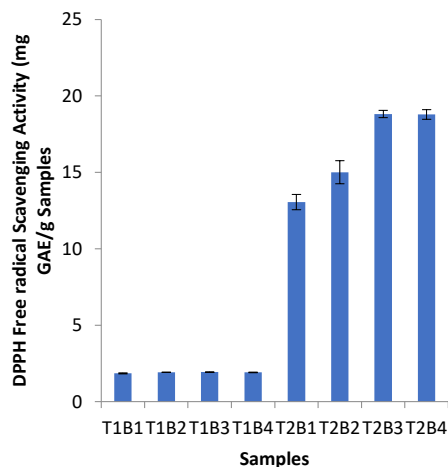
4,5-di-O-Caffeoylquinic acid ( $\mu\text{g/g}$ samples)	60	0.4906 $\pm$ 0.0060	1.1842 $\pm$ 0.0120	-0.6886 $\pm$ 0.2723	0.018*
	70	0.4807 $\pm$ 0.0034	1.0089 $\pm$ 0.0736	-0.5281 $\pm$ 0.0702	0.060
	80	0.5299 $\pm$ 0.0053	1.2382 $\pm$ 0.1435	-0.7082 $\pm$ 0.1489	0.094
	95	1.0018 $\pm$ 0.0526	1.3797 $\pm$ 0.2170	-0.3086 $\pm$ 0.3086	0.333

896 Note : Data were expressed as mean  $\pm$ standard deviation (n=2). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-  
897 steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C,  
898 stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped  
899 at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature,  
900 calculated using a paired T test at  $\alpha \leq 0.05$ . \*  $\alpha \leq 0.05$ .  
901

Formatted: Line spacing: single

Commented [A36]: Improve

902



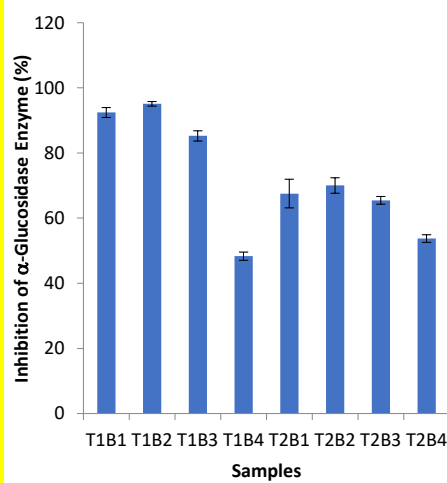
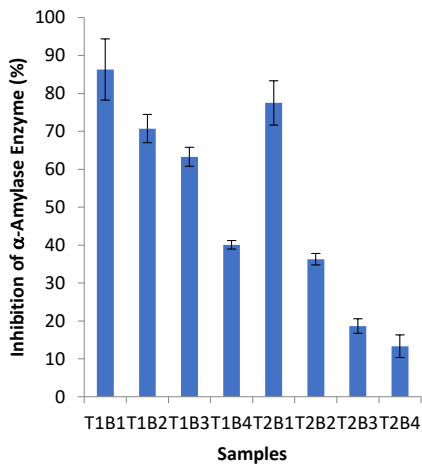
(a)

(b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage ~~time~~ time period (a) DPPH (b) FRAP. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T2B2-steeped at 70 °C, stored for 5 years; T2B3-steeped at 80 °C, stored for 5 years; T2B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$ .

Formatted: Justified, Indent: Left: 0.5", Hanging: 0.69", Line spacing: single

Commented [A37]: Improve



(a)

(b)

**Figure 3.** Antidiabetic activity of pluchea tea at different steeping temperature and storage ~~time~~ period (a)  $\alpha$ -amylase (b)  $\alpha$ -glucosidase. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$ .

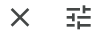
Formatted: Indent: Left: 0.44", Hanging: 0.75"

Formatted: Justified, Indent: Left: 0.44", Hanging: 0.75", Line spacing: single, Tab stops: 1.19", Left

Commented [A38]: Improve



Search pjs@stii.dost.gov.ph



99+

Compose

Mail

Inbox

1,875

Chat

Starred

Meet

Snoozed

Sent

Drafts

4

More

Labels



**Paini Sri Widyawati** <paini@ukwms.ac.id>

to Philippine

Dear Editor

Greetings,

I revised my manuscript and sent it again on February 27th 2024.

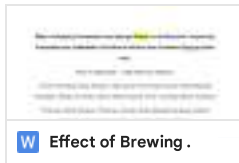
Here I send again this revision.

Thanks for attention

Regards

Paini Sri Widyawati

One attachment • Scanned by Gmail



1 **Effect of Steeping Temperature and Storage [TimePeriod](#) on the Bioactive**  
2 **Compounds, Antioxidant and Antidiabetic Activities of Infusion from Powdered**  
3 ***Pluchea Indica Less***

4 Painsi Sri Widyawati<sup>1)</sup>, Yufita Ratnasari Wilianto<sup>2)</sup>

5 <sup>1)</sup>Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala  
6 Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

7 <sup>2)</sup>Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic  
8 University, Kalisari Street Number 1, Surabaya 60272, Indonesia

9 Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea*  
10 *indica Less*, storage [timeperiod](#)

11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
Corresponding Author: [paini@ukwms.ac.id](mailto:paini@ukwms.ac.id)

21 ABSTRACT

22 This study was done to determine the effects of steeping temperature and storage  
23 ~~timeperiod~~ on the bioactive contents, antioxidant and antidiabetic activities of *Pluchea*  
24 ~~leaf infusion~~. The research used a randomized block design with two factors, i.e., steeping  
25 temperature (T) and storage ~~timeperiod~~ (B). The ~~variety of the Pluchea leaf blades were~~  
26 ~~exposed to 4~~ steeping temperatures ~~included of~~ 60 (T1), 70 (T2), 80 (T3), and 95 (T4)  
27 (°C) with the storage ~~timeperiod-period~~ of 0 (B1) and 5 (B2) ~~(year)~~. ~~The research~~  
28 ~~resultedresulting in~~ 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2,  
29 T4B1, T4B2). Statistical analysis using a paired ~~t-T~~ test at  $\alpha \leq 0.05$  showed that  
30 treatments significantly ~~affected influenced~~ the bioactive contents (total phenol (TPC),  
31 total tannin (TTC), total flavonoid (TFC)), antioxidant [(DPPH scavenging activity (DPPH)  
32 and ferric reducing antioxidant power (FRAP)] ~~potential~~ and antidiabetic [( $\alpha$ -amylase  
33 (AA) and  $\alpha$ -glucosidase (GA) ~~inhibitorsinhibition~~)] ~~activities-properties~~ of the *Pluchea* leaf  
34 ~~infusionsamples~~. TFC decreased during storage period but significantly increased at  
35 higher steeping temperature. The AA and GA of *Pluchea* infusion increased until 70 °C  
36 ~~of the steeping temperature, but decreased until 95 °C~~. ~~The bioactive contents influenced~~  
37 ~~antioxidant and antidiabetic activities~~. TFC was decreased for storage time and significant  
38 ~~increased at higher steeping temperature~~. The AA and GA of *Pluchea* infusion increased  
39 ~~until 70 °C of the steeping temperature, but decreased until 95 °C~~. The AA and GA were  
40 strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was  
41 moderately and negatively correlated with TFC. ~~Between-T~~ the antioxidant activity of  
42 ~~DPPH and FRAP~~ and the antidiabetic activity of AA and GA of *Pluchea* infusion were  
43 strongly and positively correlated. ~~with correlation coefficient (r) values of 0.956 and~~

**Commented [A1]:** Describe treatment effects on total phenolics, tannins, antioxidant, and antidiabetic in one brief sentence each and indicate statistical significance.

**Commented [A2]:** State briefly results of the correlation analysis.

44 0.725, respectively. The treatments gave different effect of simple phenolic compounds  
45 derived from *Pluchea* leaf infusion at different steeping temperatures and storage  
46 included, such as gallic acid, kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-  
47 caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid of  
48 *Pluchea* infusion at different steeping temperature and long storage. To obtain high  
49 antioxidant activity, *Pluchea* infusion selected was stored and steeped at high  
50 temperature, however high antidiabetic activity obtained was fresh *Pluchea* infusion and  
51 steeped at low temperature.

Formatted: Font: Italic

## 52

### 53 INTRODUCTION

54 *Pluchea* herbal tea is a product of dried *Pluchea* leaf processing introduced by  
55 world people (Srisook et al., 2012; Widyawati et al., 2016) because of the efficacy of the  
56 active components in *Pluchea* leaves, as a herbal plant that has been widely used for  
57 traditional medicine and food (Chan et al., 2022). *Pluchea* leaves are composed many  
58 nutrients and bioactive compounds useful to body health. The nutrient compositions in  
59 the *Pluchea* leaves include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates,  
60 calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive compounds is comprised, i.e.,  
61 chlorogenic acid, caffeic acid, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-  
62 caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-  
63 caffeoylquinic acid, quercetin, myricetin, kaempferol, total anthocyanin,  $\beta$ -carotene, and  
64 total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et  
65 al., 2022, Chan et al., 2022).

Corresponding Author: paini@ukwms.ac.id



66 Steeping process of *Pluchea leaves* can be performed with fresh or dry leaves  
67 ~~infusion by~~ in hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et  
68 al., 2020; Jayani et al., 2022). In Asia ~~area~~, especially in Indonesia, people usually  
69 consume the *Pluchea infusion* ~~with brewing of~~ steeping 2 g of powdered *Pluchea*  
70 leaves in tea bag ~~by~~ in 100 mL of hot ~~water~~ or boiling water. ~~Each tea bag contained 2 g~~  
71 ~~of *Pluchea* leaf powder is steeped with 100 mL hot water or boiling water.~~ Widyawati et  
72 al. (2016) claimed that steeping of 2 g of *Pluchea leaf powder* at 95 °C for 5 minutes  
73 ~~results exhibits~~ total phenolic ~~content, and~~ total flavonoid contents, the ability to scavenge  
74 DPPH free radicals, and the capability ~~of to~~ reduce ferric ions ~~at~~ 9.3 mg gallic acid  
75 equivalent (GAE)/g samples, 22.0 mg gallic acid equivalent (GAE)/g samples, 27.2 mg  
76 gallic acid equivalent (GAE)/g samples, and 10.2 mg gallic acid equivalent (GAE)/g  
77 samples, respectively. Werdani and Widyawati (2018) reported that drinking of *Pluchea*  
78 *leaf powder infusion* in the morning and evening regularly (2 g/100 mL) can decline blood  
79 sugar levels.

80 The steeping of *Pluchea herbal tea* with hot water at 95 °C for 5 minutes certainly  
81 determines the stability and amount of extracted bioactive compounds, that influences  
82 the biological activity, especially antioxidant and antidiabetic activities. Silva-Ramirez et  
83 al. (2020) reported that the infusion process can influence the ~~if~~ content and composition  
84 of the bioactive compounds and antioxidant activity of tea. Acar et al. (2022) informed  
85 that infusion quality of *herbal* tea extract depends on several factors, i.e., ~~time~~ ~~storage~~  
86 and temperature. Polyphenol profile and antioxidant properties of *herbal* tea infusion  
87 decline with an increase in steeping/brewing and storage temperatures, and longer  
88 exposure ~~time~~ ~~periods~~.

89 Several studies have mentioned the effect of steeping temperature ~~to on the~~  
90 bioactive compound contents and antioxidant activity, such as some white and green teas  
91 are effective with hot water at 90 °C for 7 min (Castiglioni et al., 2015), ~~on~~ roseship tea is  
92 effectively ~~at infusion~~ ~~timeperiod~~ around 6-8 min at temperatures of 84-86 °C (Ilyasoglu  
93 and Arpa, 2017), ~~on the caffeine content extracted the coffee at the~~ brewing temperature  
94 ~~of coffee influences the caffeine content extracted~~ (Zarwinda and Sartika, 2018), ~~and the~~  
95 ~~steeping the high total phenol content and antioxidant activity~~ of dark tea at 92 °C for 27  
96 min ~~results the highest total phenol content and antioxidant activity~~ (Wang et al., 2022).  
97 The study of the effect of steeping temperature to *Pluchea* infusion was carried out to  
98 afford information about ~~the most efficient~~ preparation of *powdered Pluchea leaves* ~~most~~  
99 ~~efficiently~~ to get higher ~~the~~ bioactive compounds, antioxidant and antidiabetic activities.

100 ~~On the other hand, storage~~ ~~Storage~~ ~~timeperiod~~ *tea* usually for several months until  
101 ~~years~~ of *Pluchea* herbal tea also affects the levels of the bioactive compounds and  
102 biological activity ~~because this~~ ~~herbal tea~~ usually is stored for a several months until years  
103 (Jayani et al., 2022). Tea or herbal tea is generally stored in ambient temperature and  
104 packed in tea bag or ~~Alu~~ foil standing proud or a combination of both. Many researchers  
105 ~~informed~~ ~~reported~~ that storage ~~timeperiod~~ decreases the bioactive compounds,  
106 antioxidant and antidiabetic activities, i.e., juice from *Momordica charantia* L. (Lin et al.,  
107 2020), dried *Piper bettle* extracts (Ali et al., 2018), white tea (Xu et al., 2019), kinnow-  
108 amla beverages (Purewal et al., 2022), whole wheat flour (Zhang et al., 2021).

109 Therefore, *this research studied the effect of steeping temperature and storage*  
110 *timeperiod on the bioactive compounds [(total phenolic content (TPC), total flavonoid*  
111 *content (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging*

Commented [A3]: IS THIS ALUMINUM? If so, replace Alu with aluminum

Commented [A4]: Do you mean standing pouch?

112 activity (DPPH), ferric reducing antioxidant power (FRAP)] and antidiabetic activities [( $\alpha$ -  
113 amylase (AA) and  $\alpha$ -glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea*  
114 leaves. The study was done to determine total phenolic content (TPC), total flavonoid  
115 content (TFC), total tannin content (TTC), DPPH free radical scavenging activity (DPPH),  
116 ferric reducing antioxidant power (FRAP),  $\alpha$ -amylase (AA) and  $\alpha$ -glycosidase (GA)  
117 inhibition activities, and on the phenolic compound profile.

## 119 MATERIALS AND METHODS

### 120 RAW MATERIALS AND PREPARATION

121 The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya,  
122 East Java, Indonesia. The *Pluchea* plants were included in Asteraceae family with  
123 specification according to the GBIF taxon ID number database:3132728 (Ferraris, 2023).  
124 *Pluchea* leaves at 1-6 level of each branch ~~offrom~~ the shoot were collected, sorted,  
125 washed and dried to ~~get a~~ moisture content ~~of~~ around  $11.16 \pm 0.09$  % dry basise  
126 (Widyawati et al., 2022). The ~~powdoring of~~ dried *Pluchea* leaves was ~~done~~ pulverized to  
127 ~~get a~~ 45-mesh size powder. ~~And then, the heating of T~~the *Pluchea* leaf powder was ~~done~~  
128 ~~using a drying~~dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 °C for  
129 10 min to reduce microbial organisms. ~~and Then, 2 g of the powder were~~ packed using  
130 ~~into a paper filter~~ infusion bag, ~~that made from paper filter around 2 g/bag. And then all~~  
131 ~~of samples called~~Packed samples were *Pluchea* herbal tea was stored for 0 (un-stored)  
132 and 5 (stored)years in standing pouch before analysis.

133 In the research, the one tea bag of *Pluchea* herbal tea that stored 0 (B1) and 5  
134 (B2) year, was steeped with 100 mL hot water at various temperatures, including 60 (T1),

135 70 (T2), 80 (T3), and 95 (T4) °C for 5 min with infusion method ~~that obtained~~obtaining 8  
136 treatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2.  
137 After the temperature of *Pluchea* infusion similar to ambient temperature was analyzed  
138 further.

## 140 REAGENTS

141 The ~~compounds~~reagents used ~~to analyze~~in the analyses including include 2,2-  
142 diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid, α-amylase, α-  
143 glucosidase, pNPG (p-nitrophenyl-α-glucopyranoside), (+)-catechin, kaempferol,  
144 myricetin, quercetin, 3,4-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, 3,5-di-O-  
145 caffeoylquinic acid, and (+)-catechin were purchased from Sigma-Aldrich (St. Louis, MO,  
146 USA). Methanol, Folin–Ciocalteu’s Phenol, sodium nitric, aluminum chloride, ferric  
147 chloride, sodium dihydrogen phosphate, sodium phosphate, potassium ferricyanide,  
148 starch, acetic acid, and sodium hydroxide were purchased from Merck (Kenilworth, NJ,  
149 USA). All reagents used were of analytical grade except for distilled water which was  
150 purchased from PT Aqua Industry Surabaya.

## 152 METHODOLOGY

### 153 ANALYSIS OF THE BIOACTIVE COMPOUNDS

#### 154 TOTAL PHENOLIC CONTENT ANALYSIS

155 Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the  
156 technique by Gao et al. (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu’s  
157 phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. And

**Commented [A5]:** Confusing, needs to be re-written eg The unstored samples were steeped in 100 mL distilled water at 60, 70, 80, and 95 °C for 5 min, then immediately were analyzed for the bioactive compounds [(total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC)], antioxidant potential [(DPPH free radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP)] and antidiabetic activities [(α-amylase (AA) and α-glycosidase (GA) inhibition)]. The rest of the samples were stored at (describe storage conditions) and analyze after 5 years..

**Commented [A6]:** Confusing

**Formatted:** Centered

158 then 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5 % was ~~entered added and filled up to 10 mL volume with distilled~~  
159 ~~water and distilled water was added until 10 mL volume~~. The color intensity of solution  
160 was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at  $\lambda$  760 nm  
161 with gallic acid as the reference standard. The total phenolic content was calculated using  
162 the formula:  $y=0.00009x+0.008$  with  $R^2=0.9941$ . The results were expressed as mg gallic  
163 acid equivalent (GAE)/g samples.

Commented [A7]: Specify what color

164

#### 165 TOTAL FLAVONOID CONTENT ASSAY

166 Total flavonoid content (TFC) of the samples was measured based on the reaction  
167 between AlCl<sub>3</sub> and NaNO<sub>2</sub> with ~~an the~~ aromatic ring of flavonoid compounds, especially  
168 flavonol and flavon (Shraim et al., 2021). The reaction between AlCl<sub>3</sub> and flavonoid  
169 compounds resulted ~~in~~ a yellow solution. About 30  $\mu$ L *Pluchea* infusion was mixed with  
170 0.3 mL NaNO<sub>2</sub> 5 % in 10 mL volumetric flask and incubated for 5 min. The mixture was  
171 added with 0.3 mL AlCl<sub>3</sub> 10 % for 5 min. And then, 2 mL NaOH 1 M and distilled water  
172 were added until 10 mL volume. Then, the red solution was produced after NaOH solution  
173 addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800,  
174 Shimadzu, Japan) at  $\lambda$  510 nm with (+)-catechin as the reference standard compound,  
175 and the results were expressed as mg catechin equivalents (CE)/g samples using the  
176 formula:  $y=0.00008x-0.0023$  with  $R^2= 0.9980$ .

177

#### 178 TOTAL TANNIN CONTENT ANALYSIS

179 Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method  
180 (Chandran and Indira, 2016). Approximately 10  $\mu$ L *Pluchea* infusion was added ~~with~~ 1 mL

181 Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flash and incubated for 5 min.  
182 Then, the mixture was added with 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5 % and filled up to 10 mL volume with  
183 distilled water. ~~was added until 10 mL volume.~~ The blue dark color solution ~~that was~~  
184 measured in UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at λ 760 nm with tannic  
185 acid as the reference standard. Calculation of TTC was expressed as mg tannic acid  
186 equivalents (TAE)/g samples used the formula:  $y=0.00009x+0.0021$  with  $R^2=0.9993$

## 188 ANALYSIS OF THE ANTIOXIDANT POTENTIAL

Formatted: Centered

### 189 DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

190 The DPPH free radical scavenging activity (DPPH) was measured by the  
191 spectrophotometric method (Widyawati et al., 2017) to determine the ability of the  
192 phytochemicals antioxidant activity of the *Pluchea* leaf infusion to ~~doner donate~~  
193 hydrogen atom to the nitrogen atom in DPPH resulting in the formation of -DPPH-H  
194 compound with exhibiting a yellow-colored solution. About 25 μL *Pluchea* leaf infusion  
195 was ~~entered poured~~ into reaction tube ~~and into which was added~~ added 3 mL DPPH  
196 solution (4 mg/100 mL). ~~And then the solution was~~After incubationed for 15 min in a dark  
197 room, ~~the and~~ absorbance was measured by a spectrophotometer (Spectrophotometer  
198 UV-Vis 1800, Shimadzu, Japan) at λ. 517 nm. The reference standard compound was  
199 gallic acid and the results of analysis were expressed as mg gallic acid equivalents  
200 (GAE)/g samples that calculated using formula:  $y=0.146x+1.7896$  with  $R^2=0.9975$ .

### 202 FERRIC REDUCING POWER ANALYSIS

203 Ferric reducing power (FRAP) was determined following the method used by  
204 Widyawati et al. (2014) method. Approximately 10  $\mu$ L of samples were added 2.5 mL  
205 phosphate buffer pH 6.6 and 2.5 mL and 1% potassium ferricyanide 1% in the reaction  
206 tube. And then mixture was shaken and ~~incubation~~ incubated for 20 min at 50 °C. Finally,  
207 2.5 mL chloroacetic acid 10% (w/v) was added. Into the 2.5 mL supernatant was added  
208 2.5 mL distilled water, 0.5 mL ferric chloride 0.1% (w/v) and incubated for 10 min.  
209 Potency of the samples reducing iron (III) to iron (II) ion was ~~signed~~ indicated by the  
210 intensity of blue color formed that was measured using UV-Vis spectrophotometer  
211 (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  700 nm. Intensity of the blue  
212 color indicated higher reducing capacity. The reducing power expressed as mg gallic acid  
213 equivalent (GAE)/g samples was calculated using the formula:  $y=0.0002x+0,0256$  with  
214  $R^2=0,9906$ .

215

#### 216 $\alpha$ -AMYLASE ENZYME INHIBITION CAPACITY ASSAY

217 In vitro inhibition of  $\alpha$ -amylase enzyme (AA) followed the procedure as described  
218 by Widyawati et al. (2020). Each 500  $\mu$ L of samples, was mixed with starch 1 % (w/v) and  
219 sodium acetate buffer pH 5, ~~were mixed. Then, into each~~ 250  $\mu$ L of the mixture and was  
220 added an  $\alpha$ -amylase solution (0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in  
221 50 mL of 0.2 M sodium acetate pH 5). Mixture was shaken and into which was added 2  
222 mL sodium hydroxide 1M. Before the analysis, this mixture was incubated at 37 °C for 10  
223 min. Then, the capacity of the  $\alpha$ -amylase enzyme to hydrolyzed the starch to release  
224 glucose was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800,  
225 Shimadzu, Japan) that could be analyzed based on absorbance at  $\lambda$  540 nm. The

226 inhibition percentage of  $\alpha$ -amylase was assessed using the formula:  $(ACb - ACa) - (As$   
227  $- Ab) (ACb - ACa) \times 100 \%$ . Where, ACb is the absorbance of 100 % enzyme activity  
228 (solvent with the enzyme), ACa is the absorbance of 0 % enzyme activity (solvent without  
229 the enzyme), As is the absorbance of test sample with enzyme, Ab is absorbance of test  
230 sample without enzyme.

231

#### 232 $\alpha$ -GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

233 The analysis of the  $\alpha$ -glycosidase inhibitor activity (GA) was done by Widyawati et  
234 al. (2020) method with slight modification. About 150  $\mu$ L samples ~~contained~~ containing  
235 100  $\mu$ L *Pluchea* infusion and 50  $\mu$ L pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M  
236 at pH 7) were reacted with 50  $\mu$ L  $\alpha$ -glycosidase 2 mM (0.0833 unit/mL), and then the  
237 mixture was incubated at 37 °C for 15 min. ~~Finally, the~~The reaction was stopped with the  
238 addition of 1000  $\mu$ L sodium carbonate 0.2 M. The residue of this enzyme hydrolyzed p-  
239 nitrophenyl- $\alpha$ -D-glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The

240 ~~inhibition~~ activity of ~~steeping the~~ *Pluchea* ~~tea-infusion to enzyme~~ was measured by UV-  
241 vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at  $\lambda$  405 nm.

242 The inhibition percentage of  $\alpha$ -glycosidase was calculated using formula:  $(ACb - ACa) -$   
243  $(As - Ab) (ACb - ACa) \times 100 \%$ . Where, ACb is the absorbance of 100 % enzyme activity  
244 (solvent with enzyme), ACa is the absorbance of 0 % enzyme activity (solvent without  
245 enzyme), As is the absorbance of test sample with enzyme, Ab is the absorbance of test  
246 sample without enzyme.

247

#### 248 HPLC ANALYSIS OF PHENOLICS

Corresponding Author: paini@ukwms.ac.id

Commented [A8]: Confusing. Rewrite



249 The phenolic compounds of the samples were analyzed by HPLC based on  
250 Kongkiatpaiboon et al. (2018) method with modifications. Each *Pluchea* infusion was  
251 sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter  
252 syringe (Whatmann, 0.2 µm, NYL). About 20 µL of sample was injected in an HPLC  
253 (LC20AD series, Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC  
254 LC-20AD pump, CTO-30A column oven, CBM-20A/20 Alite system controller, and SPD-  
255 20A/20 AV UV-Vis detector. Separation of phenolic compounds in samples was carried  
256 out using a Shim-pack VP-ODS C18 column (ID 5 µm × 50 mm × 4.6 mm) with a GVP-  
257 ODS Cartridge guard column (2 pieces) (ID 10 mm × 4.6 mm). ~~Analytical conditions:~~ The  
258 mobile phase used consisted of a solution of (A) 0.5 % acetic acid in water and (B)  
259 absolute methanol. Analysis was carried out using a gradient system in the following  
260 order: initial conditions of 10 % B in A to 50 % B in A were maintained for 40 minutes;  
261 then 100 % B was maintained for 20 minutes. Next the column was re-equilibrated with  
262 10 % B in A maintained for 10 minutes before analysis of the next sample. The sample  
263 flow rate was set at 1.0 ml/min with a controlled temperature at 40 °C. Detection was used  
264 at a wavelength of 280 nm. The reference standard used were gallic acid, (+)-catechin,  
265 myricetin, quercetin, kaempferol, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and  
266 4,5-dicaffeoylquinic acid. All of the reference standard was dissolved in distilled water  
267 and prepared similar to the samples before injected in HPLC.

268

## 269 EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

270 The research design used a randomized block design with two factors, i.e., the  
271 steeping temperature (T) and the storage ~~time~~ timeperiod (B). *Pluchea* leaf blades were

272 subjected to 4 steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95  
273 °C (T4), and the storage ~~timeperiod~~ of 0 year /~~fresh-un-stored~~ (B1), and 5 year/stored  
274 (B2). ~~The research resultedresulting in~~ 8 treatment combinations (T1B1, T1B2, T2B1,  
275 T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic was repeated two  
276 ~~timeperiods~~. The data of samples were analyzed by ANOVA at  $\alpha \leq 0.05$ , and continued  
277 analysis using a paired T test at  $\alpha \leq 0.05$ . treatment means of specific phenolic  
278 compounds that were identified were expressed as the mean  $\pm$  SD. The analysis used  
279 SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

**Commented [A9]:** Were all analyses replicated 2 periods only? What do you mean by 'continued analysis using a paired t-test at  $\alpha \leq 0.05$ '? Rewrite this part of the paragraph.

## 281 RESULTS AND DISCUSSIONS

282 *Pluchea* leaf infusion is produced by young *Pluchea* leaf from 1-6 level on each  
283 branch the shoot (Widyawati et al., 2022), that is steeped at 95 °C for 5 min, has many  
284 biological activities, such as antioxidant activity (Widyawati et al., 2016), antidiabetic  
285 activity (Werdani and Widyawati, 2018), anti-inflammatory (Srisook et al., 2015). The  
286 chemical constituents in *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols,  
287 cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2  
288 g/100 mL steeping *Pluchea* tea has total phenolic content 9.3 mg gallic acid equivalents  
289 (GAE)/g samples, total flavonoid content 22.0 mg catechin equivalents (CE)/g samples,  
290 DPPH free radical scavenging activity 27.2 mg gallic acid equivalents (GAE)/g samples,  
291 and ferric reducing power 10.2 mg gallic acid equivalents (GAE)/g samples (Widyawati et  
292 al., 2016). Previous research has informed related to the composition of phytochemical  
293 compounds in *Pluchea* leaves, such as phenolic acids such as chlorogenic acids, caffeic  
294 acids, 3-O-caffeoylquinic acids, 4-O-caffeoylquinic acids, 5-O-caffeoylquinic acids, 3,4-

295 di-O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic  
296 acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin;  $\beta$ -  
297 carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al.,  
298 2019; Chan et al., 2022; Widyawati et al., 2022). Presence of phytochemical compounds  
299 in herbal product were influenced by environmental factors, i.e., temperature, light  
300 exposure, oxygen level, pH and moisture. The structure of phytochemical compounds in  
301 herbal tea is very sensitive of the surrounding changes. The effect arising from these  
302 changes causes the structure of the phytochemical molecule to be degraded to produce  
303 smaller size molecules or to combine to produce larger size molecules (Ali et al., 2018;  
304 Jayani et al. 2022, Ramphinwa et al., 2023). Therefore, this study was conducted to  
305 determine the effect of steeping temperature and storage ~~time~~period of *Pluchea* tea on  
306 levels of the bioactive compounds, antioxidant and antidiabetic properties and phenolic  
307 compound profile.

**Commented [A10]:** Delete this part. Information in here are already found in the Introduction section.

## 309 BIOACTIVE COMPOUNDS

### 310 Phenolics Compounds

**Formatted:** Centered

311 The bioactive compounds are active compounds in plants that are essential to  
312 protect a body health (Nguyen and Chuyen, 2020). These compounds usually have many  
313 biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer,  
314 antimicrobial, antibacterial, anti-cholesterol and so on (Suriyaphan, 2014; Acar et al.,  
315 2022). Phenolic compounds have potential redox properties that can scavenge free  
316 radicals that can cause a number of chronic diseases (Noreen et al., 2017; Aryal et al.,  
317 2019; Acar et al., 2022).

318 The total phenolic content (TPC) of *Pluchea* infusion at different steeping  
319 temperature and storage period generally significantly increased with increasing steeping  
320 temperature and storage period based on paired ~~T-t~~ test at  $\alpha \leq 0.05$  (Figure 1a). Steeped  
321 and stored infusion had significantly higher amounts of phenolic compounds than the  
322 samples that were steeped and un-stored. Further, the highest total phenolic content was  
323 observed in samples infused at 95 °C and stored for 5 years (at 71.38±4.14 mg GAE/g  
324 samples) while the lowest was measured in the un-stored samples and infused at 60 °C  
325 (at mg GAE/g sample). Phenolic content of stored samples that were infused at  
326 different temperatures that then stored were steeped only at 60 and 95 °C also showed a  
327 significant increase in their phenolic content. This implies that the steeping temperature  
328 and the storage periods significantly resulted in the high amounts of the phenolic  
329 compounds of the infusions. Results also indicated that phenolic compounds were  
330 generally greater in the infusion at high steeping temperatures and long storage period  
331 (Figure 1a). This could have been due to that fact that during steeping fresh *Pluchea* tea  
332 had a lower total phenolic content than stored *Pluchea* tea for 5 years, besides that the  
333 higher the steeping temperature also caused the greater the extracted total phenolic  
334 content. The temperature of infusion influenced total phenolic content, it could relate to  
335 This could have been due to the fact that the steeping temperature and storage period  
336 can cause the process of degradation, oxidation, and leaching/release of phenolic  
337 compounds. Phenolic compounds are water soluble and thus soaking in hot water for a  
338 certain period of period as in steeping causes the migration process of more phenolic  
339 compounds to the water because of longer increasing contact exposure between of  
340 phenolic compounds to water (Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al.

341 (2022). Su et al. (2019) reported that temperature treatment can stimulate the release  
342 of phenolic compounds of lychee juice stored at different temperatures of 4 and 45 °C  
343 and different long storage (fresh and 72 hours).  
344 this compounds and water. The same phenomena also occurred in Castiglioni  
345 et al. (2015); Kilic et al. (2017), and Acar et al. (2022).  
346 This occurrence showed that steeping temperature and storage period caused the  
347 process of degradation and oxidation of phenolic compounds. Su et al. (2019) reported  
348 that temperature treatment can stimulate the release of phenolic compounds and  
349 increase antioxidant activity of lychee juice stored at different temperatures of 4 and 45  
350 °C and different long storage (fresh and 72 hours). Hydrogen bonding is affected by  
351 Temperature treatment because the degrades (or hydrolyzes) the hydrogen bond  
352 between phenolic compounds and proteins can be degraded that the measured levels  
353 resulting in an increase of phenolic compounds when exposed to are higher  
354 temperatures. The phenomena were supported by (Ali et al. (2018); Jayani et al. (2022),  
355 and Ramphinwa et al. (2023). Zhang et al. (2021) reported that phenolic compounds  
356 present in plants are not completely stable, but are easily degraded during storage after  
357 harvest. Reblova (2012) claimed that antioxidant compounds can be slowly degraded  
358 with increasing temperature. Besides that, Fibrianto et al. (2021) also stated that the  
359 brewing temperature has an effect on the extracted antioxidant compounds, such as  
360 alkaloids, catechins and tannins. Thus, there is an assumption that temperature and  
361 storage caused the degradation, oxidation and hydrolysis of the phenolic compounds that  
362 the phenolic compounds in *Pluchea* infusion are degraded due to oxidation and hydrolysis  
363 because of temperature and storage time period and can be easily extracted during

364 steeping, thus resulting in the increased amount of the phenolic content  
365 compounds as the at higher steeping temperature and longer storage increase period.

366 Based on using of a reference standard could be informed that Simple phenolic  
367 compounds identified in steeped and stored in *Pluchea leaf* infusion, including gallic  
368 acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-O-caffeoylquinic acids,  
369 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic acids was showed in Table 1.

370 The treatment effects results of statistical analysis using a paired T test at  $\alpha \leq 0.05$  showed  
371 that gallic acid and kaempferol contents of *Pluchea* infusion were insignificantly different  
372 at various steeping temperature and long storage periods. Nevertheless, the The  
373 concentration of quercetin and 3,5-di-O-caffeoylquinic acid of the un-stored and stored  
374 *Pluchea* infusion was significantly different from the rest of the samples between of two  
375 treatments except at 70 °C. The while (+)-catechin concentration of *Pluchea* infusion was  
376 only significantly different at 95 °C, but the myricetin content was significantly different  
377 different concentration at 80 and 95 °C. The 3,4-di-O-caffeoylquinic acid content showed  
378 significace difference at 60, 80 and 95 °C and while 4,5-O-dicaffeoylquinic acid  
379 compounds content from *Pluchea* infusion were was only significantly different at 60 °C,  
380 however the concentration of 3,4 dicaffeoylquinic acid was also significantly different at  
381 80 and 95 °C.

382 Based on the analysis of concentration of Results further showed simple phenolic  
383 compounds showed that gallic acids and kaempferol were relatively stable phenolic acid  
384 because of as reflected by the insignificant changes when exposed no changes at to the  
385 different steeping temperature and storage time period, with concentration about 0.24 ±  
386 0.00 to 0.24 ± 0.02 µg/g samples and 0.14 ± 0.02 to 0.95 ± 0.03 µg/g samples, respectively.

387 ~~However, myricetin~~Myricetin, (+)-catechin and 3,4-di-O-caffeoylquinic acid showed a  
388 drastic ~~increasing~~ increase at higher steeping temperature and longer storage period  
389 ~~implying -It's meant~~ that these compounds tended to be relatively labile. Quercetin, 3,5-  
390 di-O-caffeoylquinic acid and 4,5-di-O-caffeoylquinic acid underwent moderate changes  
391 ~~compared to the other two groups of phenolic acids.~~ Therefore, myricetin, (+)-catechin  
392 and 3,4-di-O-caffeoylquinic acid were easier to dissolve or degraded to form simple  
393 phenolic compounds at higher steeping temperature and storage ~~time~~period. ~~can cause~~  
394 ~~macromolecules of three phenolic acids in herbal tea convenient degradable to form~~  
395 simple phenolic compounds for storage, as explained by (Su et al. (2019), Ali et al. (2018);  
396 Jayani et al. (2022); Ramphinwa et al. (2023), and Zhang et al. (2021). Degradable  
397 polyphenol compounds have a simple structure and free hydroxyl groups that can react  
398 with Folin-Ciocalteu's Phenol reagent, resulting complex blue solution that can detected  
399 as total phenolic content.

**Commented [A11]:** Does the negative sign mean an increase or decrease

400 **Flavonoid Content (TFC)**

401 **Flavonoids are the major phenolic compounds that have potential chemical and**  
402 **biological activities, such as** radical scavenging and antimicrobial activities (Ayele et al.,  
403 2022; Chandra et al., 2014) **that can** protect the human body from the oxidative stress  
404 caused many degenerative diseases, especially cancer, cardiovascular problems and  
405 ageing (Mathur and Vijayvergia, 2017). **The total flavonoid content of steeped *Pluchea***  
406 **infusion decreased with longer storage period. Un-stored samples exhibited higher**  
407 **flavonoid content than the stored samples. The statistical analysis using a paired T test**  
408 **at  $\alpha= 0.05$  showed that total flavonoid content of *Pluchea* infusion was significantly**  
409 **different between ~~two treatments~~the steeped un-stored and steeped stored samples**

**Commented [A12]:** What does the negative (-) sign implies? What is your basis of classifying the simple phenolic compounds as relatively labile, moderate?

**Formatted:** Centered

410 (Figure 1b). The highest total flavonoid content was exhibited by ~~fresh the un-stored~~  
411 ~~samples steeped at 95 °C at~~ about 147.42±14.03 mg CE/g samples. Total flavonoid  
412 content was significantly lower in the stored ~~samples regardless of steeping temperature~~  
413 ~~than those of the un-stored around 24.75±2.47 to 33.71±3.06 mg CE/g samples~~ implying  
414 that the increase in the flavonoid content of the infusion was affected primarily by the  
415 steeping temperature.

Commented [A13]: cite similar studies to support your findings

#### 416 Tannin Content (TTC)

Formatted: Centered

417 Tannins are bioactive compounds that provide properties, such as astringent, anti-  
418 diarrheal, antibacterial and antioxidant (Malangngi et al., 2012). Generally, results  
419 indicated that the total tannin content of *Pluchea* infusion significantly increased with  
420 increasing steeping temperature and storage period (Figure 1c). Among, the un-stored  
421 steeped samples, the tannin content was significantly lowest in ~~the~~ samples infused at 60  
422 °C ~~at~~ about 4.81±0.58 to 17.42±1.04 mg TAE/g samples, ~~which is was~~ significantly  
423 different ~~lower~~ from ~~that of~~ the lowest tannin content of the stored samples. Among the  
424 stored and steeped samples, the highest tannin content was observed at samples  
425 steeped at 95 °C about 17.42 ± 1.04 mg TAE/g samples and was significantly different  
426 from that of the highest tannin content of the un-stored steeped samples at 95 °C about  
427 9.22 ± 1.48 mg TAE/g samples. ~~Indicating that the tannin content was~~ primarily affected  
428 ~~by both high steeping temperature and long storage period~~ ~~than high steeping~~  
429 ~~temperature and that the presence of high tannin content was primarily brought about by~~  
430 ~~long storage period.~~ Kowalska et al. (2021) informed that ~~the~~ the condensation of catechins  
431 to tannins ~~of polyphenolic compounds~~ is a dominant process ~~occurred~~ ~~occurring~~ in tea  
432 leaves that is accelerated during maceration of raw ~~material~~ tea leaves (Kowalska et al.



433 (2021) could have had contributed to the observed increase in the tannin content in the  
434 treated samples. However, the high temperature can degrade polyphenolic compounds  
435 to form simple phenolic compounds that is essential to body health. The results showed,  
436 that the higher the brewing temperature and the longer the storage time caused the tannin  
437 compound to degrade to result catechin compounds. This phenomenon is in line with the  
438 increase in total phenol levels and the concentration of (+) catechin compounds. Ali et al.  
439 (2018) said that pH, storage temperature, chemical structure and concentration, light,  
440 oxygen, enzymes and metal ions affect the presence of bioactive compounds in the  
441 material. Nevertheless,

442 Although, high temperature and long storage period can cause the degradation of  
443 tannins to catechins, Rusita et al. (2019) emphasized that tannins are a polar  
444 thermostable complex compounds, that is are resistant to heating, indicating that even  
445 with the exposure to high temperature, the tannins still remained high in the treated  
446 samples as a result the tannin content in *Pluchea* tea increases with increasing steeping  
447 temperature and storage time period, this is caused tannins are thermostable complex  
448 compounds.

#### 450 ANTIOXIDANT ACTIVITY

451 Antioxidant activity is capability of compounds to inhibit the oxidation of  
452 macromolecules from biological target that involve in oxidative chain reactions (Ali et al.,  
453 2005; Oh et al., 2013). The antioxidant activity assay was done in this research using  
454 DPPH Free Radical Scavenging Activity (DPPH) and ferric reducing power (FRAP)  
455 methods. The phenolic compounds are an active antioxidant that have antioxidant

456 capability ~~that depends~~ on their redox properties. The structure of phenolic compounds  
457 determine ~~the~~ effectivity to ~~doner donate~~ hydrogen atom which is negatively correlated  
458 with the O-H phenolic bond strength. The higher antioxidant power of phenolic  
459 compounds is caused ~~by~~ the weaker O-H phenolic bond (Kruk et al., 2022). The  
460 mechanism of phenolic compounds ~~is involved~~ as antioxidants ~~through depends on their~~  
461 the ability to donate hydrogen atom ~~ands~~, transfer electrons, ~~and as~~ reducing agents and  
462 singlet oxygen quenchers (Ali et al., 2005; Huang et al. 2005).

Commented [A14]: what do you mean? rewrite

#### 464 DPPH Free Radical Scavenging Activity

Formatted: Centered

465 DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate  
466 antioxidant activity because this method ~~is simple~~ that is suitable to measure the donating  
467 hydrogen atoms capability of ~~herbal infusion~~. This reaction can cause the purple color of  
468 ~~DPPH to change to yellow color~~ (Munteanu and Apetrei, 2021; Baliyan et al., 2022).  
469 ~~Figure 2a shows that the free radical scavenging property of the stored and steeped~~  
470 ~~samples were significantly higher than the un-stored steeped samples. The result of~~  
471 ~~DPPH assay~~ It can also be observed ~~indicates~~ that the ~~free radical scavenging property~~  
472 ~~DPPH values accrued~~ was significantly different among the stored and steeped samples  
473 ~~but insignificant among the un-stored and steeped samples at higher steeping~~  
474 ~~temperature and longer storage timeperiod. Statistical analysis by ANOVA using a paired~~  
475 ~~T test at  $\alpha \leq 0.05$  proven that the higher the steeping temperature of fresh *Pluchea*~~  
476 ~~infusion (T1B1, T2B1, T3B1, and T4B1) was consistent the ability to DPPH free radicals~~  
477 ~~scavenging activity, whereas the stored *Pluchea* infusion resulted in the higher activity~~  
478 ~~and the values went up as rising of the infusion temperature. *Pluchea* infusion stored at~~

479 room temperature for 5 years resulted in the high DPPH-free radical scavenging activity  
480 by more than 100 %. Steeping at higher temperatures significantly increased the DPPH  
481 free radical scavenging activity in stored *Pluchea* infusion by around 15 to 25 %. Steeping  
482 at 80-95 °C in stored *Pluchea* infusion insignificantly affected the free radical scavenging  
483 property of the bioactive compounds (Figure 2a). This implies that that the higher free  
484 radical scavenging property was primarily affected by the storage period than steeping  
485 temperature. During the storage process it is possible to form complex phenolic  
486 compounds which provide a high ability to scavenge DPPH-free radicals  
487 (Thanajiruschaya et al., 2010)

488 Scavenging The scavenging activity of DPPH free radicals of the the samples was  
489 strongly and positively correlated with total with total phenolic and tannin contents levels,  
490 but inversely to with total flavonoid levels. Based on Pearson correlation at Table 2, the  
491 correlated coefficient values (r) between DPPH and TPC, TTC and TFC were 0.993,  
492 0.942, and 0.940, respectively. During the storage process it is possible to form complex  
493 phenolic compounds which provide a high ability to scavenge DPPH free radicals  
494 (Thanajiruschaya et al., 2010). This research study also demonstrated that longer storage  
495 time period and higher infusion temperature produced many simple phenolic compounds  
496 with free hydroxyl groups capable to donor hydrogen atom to DPPH free radical. Many  
497 phenolic acids, such as gallic acids, (+)-catechins, myricetins, kaempferols, quercetins,  
498 3,5-di-O-caffeoylquinic acids, 3,4-di-O-caffeoylquinic acids, 4,5-di-O-caffeoylquinic acids  
499 have established potential antioxidant activity (Kumar and Goel, 2019) (Table 1). Kruk  
500 et al (2022) informed that the capability of phenolic compounds to donor hydrogen atom  
501 depends on chemical structure, number and position of hydroxyl groups attached to a

Commented [A15]:

Commented [A16R15]: Clarify on how you were able to come up with free radical scavenging activity by more than 100 %. Steeping temperatures significantly increased the free radical scavenging activity in stored *Pluchea* infusion by around 15 to 25 %

Commented [A17]: Explain/interpret this observation based on the data that you were able to obtain.

502 benzene ring, a double bond between C2 and C3 rings and a carbonyl group (C=O) on  
503 the C ring at C4. The effectivity of antioxidant compounds donor hydrogen atom is  
504 determined by O-H bond dissociation energy.

505 The DPPH-free radical scavenging property observed in the study was not in  
506 consistent with the results of the study by Moraes-de-Souza et al. (2008). The research  
507 shows that total phenolic content of herbal infusion is low correlated with DPPH-free  
508 radical scavenging activity. However, Dobrinas et al. (2021) informed that total phenolic  
509 content of tea infusion is positively and significantly correlated with the free radical  
510 scavenging property/inhibitor activity of DPPH of tea infusion.

511

#### Ferric Reducing Antioxidant Power (FRAP)

Formatted: Centered

512 FRAP is an analysis of antioxidant power of the phytochemical compounds based  
513 on the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic  
514 acid, and ferric chloride to produce a color complex, that can be measured at  $\lambda$  700 nm  
515 (Fu et al., 2011; Al-Temimi and Choudhary, 2013). The principle of the assay measures  
516 that is based of the ability of antioxidant compounds to reduce iron ions of potassium  
517 ferrocyanide ( $\text{Fe}^{3+}$ ) to be potassium ferrocyanide ( $\text{Fe}^{2+}$ ). Potassium ferrocyanide reacts  
518 with ferric chloride to form a ferric-ferrous complex and results green color solution  
519 (Widyawati et al., 2017; Raharjo and Haryoto, 2019).

520 The results showed that the ferric reducing antioxidant power (FRAP) increased  
521 with at higher steeping temperature and longer storage time period. The lowest FRAP was  
522 observed in the un-stored samples which was steeped at 60 °C at  $3.95 \pm 0.17$  mg gallic  
523 acid equivalents (GAE)/g samples, and the highest was owned exhibited by in *Pluchea*

525 infusion which was stored for 5 years at 95 °C at 48.63 ±10.83 mg gallic acid equivalents  
526 (GAE)/g samples (Figure 2b). FRAP increased significantly as steeping temperature was  
527 increased. FRAP of the samples stored for 5 years was also significantly higher than the  
528 un-stored samples at  $\alpha \leq 0.05$ . Based on Pearson correlation, the FRAP of *Pluchea*  
529 infusion was strongly and positively significant correlated with the DPPH, TPC and TTC,  
530 but inversely to TFC. The correlated coefficient values (r) between FRAP and DPPH,  
531 TPC, TTC and TFC were 0.956, 0.953, 0.948 and -0.826, respectively.

532 This case was is in contrast to with the study on the antioxidant activity of DPPH  
533 and FRAP on of matcha, because The the longer storage time period reduces the levels  
534 of catechin content due to the catechins, such as epigallocatechin gallat (EGCG),  
535 epicatechin gallat (ECG), epigallocatechin (EGC), and epicatechin (EC) which are  
536 bioactive compounds that have high antioxidant activity (Kim et al. 2020), and also the  
537 case of the effect of temperature and storage time in betel (*Piper bettle* L.) extract. Light  
538 and temperature influence degradation of phenolic compounds of betel that determine  
539 antioxidant activity. Different structure of phenolic compounds determines their stability  
540 to degrade accelerating of light and temperature. Hydroxychavicol is the best stability of  
541 phenolic compounds of betel compared with eugenol, isoeugenol and allyl pyrocatechol  
542 (Ali et al., 2018). Thanajiruschaya et al. (2010) revealed that the antioxidant activity of  
543 rice stored at high temperatures is greater than that stored at low temperatures. The ferric  
544 reducing capability of *Pluchea* could have due infusion corresponded to the presence to  
545 of simple phenolic acid values that have the ability to transfer electron from their free  
546 hydroxyl groups of, presence of them in samples could accrue antioxidant activity  
547 because of ability of the electron transfer from free hydroxyl groups of phenolic acids.

Commented [A18]: Relate these with Figure 2b. Rewrite

548 [The FRAP of \*Pluchea\* infusion was strongly and positively significant correlated with the](#)  
549 [DPPH, TPC and TTC, but inversely to TFC.](#)

## 550 ANTIDIABETIC ACTIVITY

551  **$\alpha$ -Amylase enzyme inhibition activity (AA)**

Formatted: Centered

552 Antidiabetic activity is a measure of the potency of phenolic compounds to regulate  
553 the uptake of glucose by the cells from the blood through the mediation of 2-degestive  
554 enzymes i.e.,  $\alpha$ -amylase and  $\alpha$ -glucosidase, which are involved the control of dietary  
555 carbohydrate digestion and release in the postprandial blood glucose in human body (Fu  
556 et al., 2017). The phenolic compounds have the capability to bind with the protein  
557 component of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Martinez-Solis et al., 2022)  
558 resulting in the reduced activity of the enzymes. The results showed, that ~~the lower~~  
559 ~~steeping *Pluchea* leaf infusion~~ was able to inhibit the action of the  $\alpha$ -amylase enzymes  
560 (Figure 3a). The *Pluchea* infusion ~~had very good activity, exhibited a good  $\alpha$ -mylase~~  
561 ~~enzyme inhibition activity~~ of more than 50 % and even almost 100 % ~~for fresh in the un-~~  
562 ~~stored *Pluchea* infusion which steeped was brewed~~ at 60, 70 and 80 °C with highest at  
563 60 °C, and in stored *Pluchea* leaf infusion which was steeped at 60 °C. ~~Whereas The~~  
564 ~~stored fresh *Pluchea* leaf infusion steeped at 70, 80 and 95 °C for 5 minutes had lower~~  
565 ~~enzyme inhibition activity an activity of of less than 50 % with lowest at 95 °C, inhibiting the~~  
566  ~~$\alpha$ -amylase enzyme of less than 50 %, which was equal to 40.08±1.12 %.~~ Widyawati et al.  
567 (2017) ~~detected found that~~ the ability to inhibit the  $\alpha$ -amylase enzyme ~~from in fresh un-~~  
568 ~~stored *Pluchea* infusion steeped at 95 °C for 5 minutes~~ ~~by~~ was also low at 28.79 %.  
569 Increasing the steeping temperature and storage ~~time period~~ reduced the ability ~~to of the~~  
570 ~~phytochemicals in the *Pluchea* infusions to~~ inhibit the  $\alpha$ -amylase enzyme activity. ~~The~~

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Commented [A19]: Why?/Explain

571 results of the analysis based on a paired T test at  $\alpha \leq 0.05$  showed, that the steeping  
572 temperature and storage timeperiod had a significant effect on the ability to inhibit the  $\alpha$ -  
573 amylase enzyme. Based on Pearson correlation, the Table 2 further shows that the AA of  
574 *Pluchea* infusion was strongly and negatively significant correlated with TPC, TTC, DPPH  
575 and FRAP, but it was moderately and negatively significant correlated with TFC. The  
576 correlated coefficient values ( $r$ ) between AA and TPC, TTC, DPPH, FRAP and TFC were  
577 0.708, -0.857, -0.696, -0.806 and 0.429, respectively.

578 This inhibitory activity was thought to be contributed by other bioactive compounds,  
579 besides phenolics which are sensitive to steeping temperature and storage timeperiod. Li  
580 et al. (2018) stated that there are flavonoid compounds that contribute to the ability to  
581 inhibit the  $\alpha$ -amylase enzyme. Flavonoid compounds with a hydroxyl structure at C-4' in  
582 ring B are more effective than C-6 in ring A. Akah et al. (2011) informed reported that the  
583 phytochemical compounds, such as terpenoids, saponins, flavonoids, glycosides and  
584 carbohydrate, and alkaloids are good antidiabetic metabolites or  $\alpha$ -amylase enzyme  
585 activity inhibitor. Sangeetha and Vedesree (2012) explained, that the ability to inhibit the  
586  $\alpha$ -amylase enzyme was determined by the content of the phenolic compound and protein.  
587 The  $\alpha$ -amylase inhibitor enzyme present in *Pluchea* infusion may be proteinaceous in  
588 nature. Alexandre et al. (2022) informed that phenolic acids have inhibition activity to  $\alpha$ -  
589 amylase enzyme depending their structures. Besides that, capability of phenolic acids to  
590 inhibit  $\alpha$ -amylase enzyme was determined by low half-maximum inhibitory concentration  
591 ( $IC_{50}$ ). There are C=C double bond conjugated with a carbonyl group of phenolic  
592 structures that stabilizes the binding forces to the active site of the  $\alpha$ -amylase. The  
593 hydroxyl groups of them are able to bind by non-covalent interaction, such as hydrogen

Commented [A20]: Implications? Explain

Commented [A21]: What content or what is in the content the influenced the ability to inhibit the enzyme?

Commented [A22]: How will this affect the ability to inhibit the enzyme?

594 binding, cation- $\pi$  interactions, salt bridge interactions, ionic interactions or electrostatic  
595 forces with amino acid residue at the active site in  $\alpha$ -amylase enzyme. ~~Elevated steeping~~  
596 ~~temperature and longer storage period~~ ~~The steeping temperature and storage time can~~  
597 ~~easily cause the~~ removal of the ~~e~~ hydroxyl groups of phenolic compounds that can reduce  
598 ~~their~~ ability of enzyme inhibition. The phenolic acids with a greater number of hydroxyl  
599 groups ~~are exhibits~~ stronger capability to obstruct the  $\alpha$ -amylase enzyme.

**Commented [A23]:** Lines 585 to 595, Either delete or rewrite for better readability and understanding referring to enzyme activity inhibition

#### 600 $\alpha$ -Glucosidase enzyme inhibition activity (GA)

**Commented [A24]:** This part is disorganized. Avoid duplicating statements, observation facts etc

**Formatted:** Centered

601 ~~Alpha~~ $\alpha$ -glucosidase is an important enzyme in carbohydrates digestion, that  
602 catalysis the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and  
603 converts them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et  
604 al., 2014; Proenca et al., 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -  
605 glucosidase enzyme is used to determine ~~their~~ antidiabetics activity. ~~This is supported~~  
606 ~~by~~ Werdani and Widyawati (2018) ~~stated~~, that *Pluchea* infusion has the potential as an  
607 antidiabetic agent. Widyawati et al. (2020) found that brewing fresh *Pluchea* infusion at  
608 95 °C for 5 minutes has an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857 %.

609 ~~The results showed~~, Figure 3b shows that the ability of the *Pluchea* leaf infusion  
610 to inhibit the  $\alpha$ -glucosidase enzyme decreased with increasing steeping temperature and  
611 storage ~~time~~period. Steeping at 95 °C ~~for fresh~~ of the un-stored *Pluchea* leaf infusion (~~un-~~  
612 ~~stored~~) obtained the lowest inhibitory ability, i.e.,  $48.32 \pm 1.27$  %, and the highest inhibitory  
613 activity was found at 70 °C ~~steeping temperature for fresh~~ *Pluchea* infusion, which was at  
614  $95.11 \pm 0.70$ % (Figure 3b). The results of a paired T test showed that GA of *Pluchea*  
615 infusion was significantly different ~~at both~~ between steeping temperature and long storage.  
616 ~~The antidiabetic activity of~~ *Pluchea* infusion Figure 3 further ~~showed~~ shows that the ability

**Formatted:** Font: Not Italic

**Commented [A25]:** Explain



617 of *Pulchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher than the  
618 ability to inhibit the  $\alpha$ -amylase enzyme. Li et al. (2018) informed that flavonoid compounds  
619 have the ability to inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. This is  
620 due to the total flavonoids in steeped *Pluchea* infusion which tended to have the same  
621 pattern as the ability to inhibit the activity of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes.  
622 The statistical analysis using Pearson correlation showed that GA of *Pluchea* infusion  
623 was strongly and negatively correlated with TPC, TTC, DPPH and FRAP  
624 ~~with r was -0.555, -0.715, -0.527 and -0.560, respectively.~~ However, GA was  
625 moderately and positively correlated to TFC, ~~with r was 0.350 and strongly and positively~~  
626 ~~correlated to AA, with r was 0.725.~~ Flavonoid compounds, such as rutin, myricetin,  
627 kaempferol, and quercetin ~~which~~ have antioxidant and antihyperglycemic activities. The  
628 ability to inhibit the action of enzymes from flavonoid compounds is determined by the  
629 position and number of hydroxyl groups and the number of double bonds in rings A and  
630 B and the heterocyclic ring in ring C. The ability to inhibit the  $\alpha$ -glucosidase enzyme from  
631 *Pluchea* infusion was significantly affected by the steeping temperature and long storage.  
632 The capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater than  
633 the  $\alpha$ -amylase enzyme because the mechanism of two enzymes was different, according  
634 to the opinion of McCue et al. (2005). Widyawati et al. (2017) informed that phenolic and  
635 non-phenolic compounds determine the inhibitory activity of the  $\alpha$ -glucosidase enzyme.  
636 The ability of bound phenolic compounds to inhibit  $\alpha$ -glucosidase enzymes was higher  
637 than free phenolic compounds. The presence of polymerization and degradation  
638 reactions, that may be occurred in *Pluchea* infusion during storage, affects the structure  
639 and profile of phenolic and non-phenolic compounds. Asriningtyas et al. (2014) claimed

Formatted: Font: Italic

Commented [A26]: This does not explain explain lines 597-599 with the manner it is written. Include statements that connect the explanation with the observation. Having 'same pattern' is not observed in the figure/graph

Commented [A27]: Interpret/Implications

Commented [A28]: Delete literature citations that are unnecessary to explain the findings

640 that *Pluchea* leaves contain 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid  
641 methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid,  
642 and 1,3,4,5-tetra-*O*-caffeoylquinic acid. Quinic acid is methyl esterified with the number  
643 of caffeic groups in the molecule that determines the activity of inhibiting the  $\alpha$ -  
644 glucosidase enzyme. Analysis of caffeoylquinic acids in *Pluchea* infusion was obtained  
645 that the higher steeping temperature and long storage caused increased concentration  
646 of them, but the  $\alpha$ -glucosidase inhibition activity of them was reduced. Aleixandre et al.  
647 (2022) reported that the simple phenolic acids forming a dipole-dipole interaction of active  
648 site from  $\alpha$ -glucosidase enzyme are effectively inhibiting the enzyme.

649 This study ~~was obtained information~~ showed that the increasing of steeping  
650 temperature and storage ~~time period~~ caused a degradation reaction of polyphenol  
651 compounds to produce simple phenolic compounds, such as gallic acid, (+)-catechin,  
652 myricetin, quercetin, kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic  
653 acid, and 4,5-di-*O*-caffeoylquinic acid, supported the results of total phenolic content and  
654 total tannin content assays. Increased concentration of simple phenolic compounds  
655 determined the ability of these compounds as antioxidant agents, but reduced their  
656 capability as antidiabetic agents.

## 658 CONCLUSION

659 The steeping temperature and storage ~~time period~~ of *Pluchea* infusion significantly  
660 influenced bioactive contents, antioxidant and antidiabetic activities. TPC, TTC, and TFC  
661 were significantly different at various steeping temperature and storage period based on  
662 statistical analysis using a paired ~~T-t~~ test at  $\alpha \leq 0.05$ . ~~There was the difference of t~~The

Corresponding Author: paini@ukwms.ac.id

**Commented [A29]:** Unnecessary because this is not included as one of the derived simple phenolic acids

**Commented [A30]:** Not clear, re-write

**Commented [A31]:** Organize the discussion to explain the observation one at a period. ex:

1) 'Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme decreased with increasing steeping temperature and storage period.' (This must be followed by an explanation or support.)

2) However, GA was moderately and positively correlated to TFC and positively correlated to AA..(This must be followed by implications/support/explanation.)

The ability to inhibit the  $\alpha$ -glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping temperature and long storage.(This can be integrated in 1)

4) The capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater than the  $\alpha$ -amylase enzyme because the mechanism of two enzymes was different, according to the opinion of McCue et al. (2005). (This can be integrated in 1)

The mechanism must be explained -the mechanism of two enzymes was different,

5) Flavonoid compounds, such as rutin, myricetin, kaempferol, and quercetin which have antioxidant and antihyperglycemic activities

6) . Li et al. (2018) informed that flavonoid compounds have the ability to inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. ( May also be integrated in 1)

7) Widyawati et al. (2017) informed that phenolic and non-phenolic compounds determine the inhibitory activity of the  $\alpha$ -glucosidase enzyme. The ability of bound phenolic compounds to inhibit  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. (May also be incorporated in 1).

8) Lines 618 to 629 into 1)

**Commented [A32]:** Reconcile with your discussion

**Commented [A33]:** Suggested conclusion

## CONCLUSION

The total phenolic content (TPC) of *Pluchea* infusion at different steeping temperature and storage period generally significantly increased with increasing steeping temperature and storage period. Steeped and stored infusion had significantly higher amounts of phenolic compounds than the samples that were steeped and un-stored. TPC was highest in the store

663 phenolic compound profile in ~~fresh~~ ~~the~~ ~~unstored~~ and stored ~~of~~ *Pluchea* infusion ~~and~~ ~~at~~  
664 various steeping temperature. ~~The~~ ~~included~~ simple phenolic compounds ~~were~~ ~~detected~~  
665 ~~in~~ *Pluchea* infusion ~~including~~ ~~such~~ ~~as~~ gallic acid, (+)-catechin, quercetin, myricetin,  
666 kaempferol, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-  
667 caffeoylquinic acid. The results of statistical analysis using a paired ~~T~~ ~~t~~ test at  $\alpha \leq 0.05$   
668 showed that gallic acid and kaempferol of *Pluchea* infusion were insignificantly different  
669 at various steeping temperature and long storage. ~~Nevertheless,~~ ~~T~~ the concentration of  
670 quercetin and 3,5-dicaffeoylquinic acid of *Pluchea* infusion was significantly different of  
671 two treatments except at 70 °C. The (+)-catechin concentration of *Pluchea* infusion was  
672 significantly different at 95 °C, but the myricetin was different concentration at 80 and 95  
673 °C. The 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid compounds from *Pluchea*  
674 infusion were significantly different at 60 °C, however the concentration of 3,4-  
675 dicaffeoylquinic acid was also significantly different at 80 and 95 °C. TPC, TTC and TFC  
676 of *Pluchea* infusion were significantly different at various steeping temperature and  
677 storage period. TPC and TTC significantly increased with increasing steeping  
678 temperature and long storage, but TFC significantly increased at various steeping  
679 temperature and significantly decreased at long storage. The bioactive compounds of  
680 *Pluchea* infusion influenced antioxidant activities (DPPH and FRAP) and antidiabetic  
681 activity (AA and GA). The DPPH was strongly and positively correlated with TPC and  
682 TTC, but it was strongly and negatively correlated with TFC, with coefficient  $r$  0.993,  
683 0.942, and -0.940, respectively. The correlated pattern between FRAP and bioactive  
684 contents of *Pluchea* infusion was similar to it between DPPH and bioactive contents. The  
685 correlated coefficient values ( $r$ ) between FRAP and TPC, TTC and TFC were 0.953, 0.948

686 and -0.826, respectively. The AA and GA were strongly and negatively correlated with  
687 TPC, TTC, DPPH and FRAP, but it was moderately and negatively significant correlated  
688 with TFC. Between the antioxidant activity of DPPH and FRAP and the antidiabetic  
689 activity of AA and GA of Pluchea infusion were strongly and positively correlated with  
690 correlation coefficient (r) values of 0.956 and 0.725, respectively.

691

#### 692 DATA AVAILABILITY

693 Table and figure used to support of this study were included in the article.

694

#### 695 CONFLICT OF INTEREST

696 The authors declare no conflict of interest.

697

#### 698 ACKNOWLEDGEMENTS

699 The authors would like to thank the he Ministry of Education and Culture of the Republic  
700 of Indonesia for fundamental research grant to higher education institutions in 2022

701

#### 702 REFERENCES

- 703 ACAR A, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food  
704 wastes:evaluation of temperature and time effects on quality parameters. Applied Food  
705 Res. 2(100087):1-8.
- 706 ALEIXANDRE A et al. 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -  
707 glucosidase and influence of reaction conditions. Food Chem. 372(131231):1-9.

**Commented [A34]:** Check for authors that may had been deleted in the paper due to revisions done.

Corresponding Author: paini@ukwms.ac.id

708 ALI et al. 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile.  
709 Food Chem. 89(2005):27-36.

710 ALI A. et al. 2018. Impact of storage conditions on the stability of predominant phenolic  
711 constituents and antioxidant activity of dried *piper betle* extracts. Molecules.23(24):1-13.

712 AL-TEMIMI A, CHOUDHARY R. 2013. Determination of antioxidant activity in different  
713 kinds of plants *in vivo* and *in vitro* by using diverse technical methods. J Nut. Food Sci.  
714 3(1):1-6.

715 AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and  
716 methanol extract and fractions of *Gongronema latifolium* (Asclepidaceae) leaves in  
717 alloxan diabetic rats. J Applied Pharm. Sci. 01(09): 99-102.

718 ARSININGTYAS IS. et al. 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves  
719 of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product  
720 use. Natural Product Res. 28(17):1350-1353.

721 ARYAL S et al. 2019. Total phenolic content, flavonoid content and antioxidant of wild  
722 vegetables from Western Nepal. Plants. 8(96): 1-12.

723 AYELE DT, AKELE ML, MELESE A.T. 2022. Analysis of total phenolic contents,  
724 favonoids, antioxidant and antibacterial activities of *Croton macrostachyus* root extracts.  
725 BMC Chem. 16(30): 1-9.

726 BALIYAN S et al. 2022. Determination of antioxidants by DPPH radical scavenging  
727 activity and quantitative phytochemical analysis of *Ficus religiosa*. Molecules. 27(1326):  
728 1-19.

729 CASTIGLIONI S et al. 2015. Influence of steeping conditions (time, temperature, and  
730 particle size) on antioxidant properties and sensory attributes of some white and green  
731 teas. Intern. J. Food Sci. Nut. Online 1-7.

732 Chan EWC et al. 2022. *Pluchea indica*: An updated review of its botany, uses, bioactive  
733 compounds and pharmacological properties. Pharmaceutical Sciences Asia. 49(1): 77-  
734 85.

735 CHANDRA S et al. 2014. Assessment of total phenolic and flavonoid content, antioxidant  
736 properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit  
737 crops: A Comparative study. Hindawi Publishing Corporation Evidence-Based  
738 Complementary Alter. Med. 2014:1-9 (2014)

739 CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic, flavonoids,  
740 tannin and chlorophyll content of leaves of *Strobilanthes Kunthiana (Neelakurinji)*. J.Med.  
741 Plants Studies. 4(4):282-286.

742 DOBRINAS S, SOCEANU A, POPESCU V, POPOVICI IC, JITARIU D. 2021.  
743 Relationship between total phenolic content, antioxidant capacity, Fe and Cu content from  
744 tea plant samples at different brewing times. Processes. 9(1311):1-11.c

745 FERRARIS Z. 2023. *Pluchea indica* Less.iNaturalist.org.  
746 <https://www.gbif.org/occurrence/4022273660>.

747 FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional  
748 properties and visual appearance of Dampit Robusta coffee leaves tea. Advances in Food  
749 Sci. Sustain. Agric. Agro-industrial Engineering. 4(1):25-32.

750 FU L et al. 2017. Total phenolic contents and antioxidant capacities of herbal and tea  
751 infusions. Intern. J. Molecular Sci. 12:2112-2124.

Corresponding Author: paini@ukwms.ac.id

752 GAO MR et al. 2019. A theoretical and experimental study: the influence of different  
753 standards on the determination of total phenol content in the Folin–Ciocalteu assay. J.  
754 Food Measurement and Characteristic. 13:1349–1356.

755 HUANG D, OU B, PRIOR RL. 2005. The Chemistry behind antioxidant capacity assays.  
756 J. Agric. Food Chem. 53:1841-1856.

757 ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties  
758 of rosehip tea beverage: study by response surface methodology. J. Food Sci. Tech.  
759 54(11):3737–3743.

760 JAYANI NIE et al. 2022. Evaluation of stability and quality characteristics of moringa  
761 (*Moringa oleifera*) herbal tea during storage. Food Res. 6(4):399-406.

762 KIM JM et al. 2020. Effect of storage temperature on the antioxidant activity and catechins  
763 stability of Matcha (*Camellia sinensis*). Food Science and Biotechnology. 29(9):1261–  
764 1271.

765 KILIC et al. 2017. Antioxidant properties of some herbal teas (green tea, senna, corn silk,  
766 rosmery) brewed at different temperatures. Intern. J. Secondary Metabolite. 4(3):142-248  
767 Special Issue 1.

768 KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of  
769 extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves  
770 from different provenances in Thailand. Revista Brasileira de Farmacognosia. 28(2018):  
771 145–150.

772 KOWALSKA J et al. 2021. Influence of tea brewing parameters on the antioxidant  
773 potential of infusions and extracts depending on the degree of processing of the leaves  
774 of *Camellia sinensis*. Molecules.26(4773):1-17.

Corresponding Author: paini@ukwms.ac.id

775 KRUK J, ABOUL-ENAIN BH, DUCHNIK E, MARCHLEWICZ M. 2022. Antioxidative  
776 properties of phenolic compounds and their effect on oxidative stress induced by severe  
777 physical exercise. *J. Physiol. Sci.* 72(19):1-24.

778 KUMAR N, GOEL N. 2019. Phenolic acids: natural versatile molecules with promising  
779 therapeutic applications. *Biotechnology Reports.* 24(e00370):1-10.

780 LI K et al. 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the flavonoids  
781 rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity  
782 relationship of its eight flavonoids by a refined assign score method. *Chem. Central J.*  
783 12(82):1-11.

784 LIN YS. et al. 2020. Effects of storage time and temperature on antioxidants in juice from  
785 *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser. *Molecules.*  
786 25(3614): 1-12.

787 MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content  
788 and antioxidant activity test of avocado seed extract (*Persea Americana* Mill). *Jurnal MIPA*  
789 *Unsrat Online.* 1(1): 5-10. [https://media.neliti.com/media/publications/116214-ID-  
790 penentuan-kandungan-tanin-dan-uji-aktivi.pdf](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf) [In Bahasa Indonesia]

791 MARTINEZ-SOLIS J et al. 2022. Antidiabetic and toxicological effect of the tea infusion  
792 of summer collection from *Annona cherimola* Miller leaves. *Plants.* 11(3224):1-17.

793 MATHUR R, VIJAYVERGIA R. 2017. Determination of total flavonoid and phenol content  
794 in *Mimusops Elengi* Linn. *Intern. J. Pharm. Sci. Res.* 8(12): 5282-5285.

795 MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of  
796 sprouted and solid-state bioprocessed soybean. *Asia Pacific J. Clinical Nut.* 14(2): 145-  
797 152.

Corresponding Author: paini@ukwms.ac.id



798 MORAES-DE-SOUZA RA, OLDONI TLC, REGITANO-D'ARCE MAB, ALENCAR SM.  
799 2008. Antioxidant activity and phenolic composition of herbal infusions consumed in  
800 Brazil. *Cienc Tecnol. Aliment.* 6(1):41-47.

801 MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant  
802 activity: A Review. *Intern. J. Molecular Sci.* 22(3380):1-30.

803 NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus*  
804 *sabdariffa* L.): Effect of dying temperature and brewing conditions on total soluble solid,  
805 phenolic content, antioxidant capacity and sensory quality. *Beverages*.6(2):1-11 (2020).

806 NOREEN H. et al. 2017. Measurement of total phenolic content and antioxidant activity  
807 of aerial parts of medicinal plant *Coronopus didymus*. *Asian Pacific J. Tropical Med.* 10(8):  
808 792–801.

809 NURCHOLIS GW et al. 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity in-  
810 vitro and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum pictum*  
811 (L.). *Current Biochem.* 1(2): 58-65.

812 OH J et al. 2013. Antioxidant and antimicrobial activities of various leafy herbal teas. *Food*  
813 *Control.* 31(2013):403-409.

814 PROENCA C et al. 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an in vitro and in silico  
815 structure–activity relationship study. *J. Enzyme Inhibition Med. Chem.* 32(1): 1216–1228.

816 PUREWAL SS et al. 2022. Unraveling the effect of storage duration on antioxidant  
817 properties, physicochemical and sensorial parameters of ready to serve Kinnow-aml  
818 beverages. *Applied Food Res.* 2(1): 1-8.

819 RAHARJO D, HARYOTO. 2019. Antioxidant activity of mangrove *Sonneratia caseolaris*  
820 L. using the FRAP method. *ISETH.* 2019: 623-629.

Corresponding Author: paini@ukwms.ac.id

821 RAMPHINWA ML et al. 2023. Eco-physiological response of secondary metabolites of  
822 teas: review of quality attributes of herbal tea. *Frontiers in Sustain. Food Systems*.  
823 7(990334):1-14.

824 REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids.  
825 *Czech J. Food Sci.* 30(2):171-177.

826 RUAN J et al. 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea*  
827 *indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel  
828 column chromatography fractions. *Molecules*. 24(2784):1-20.

829 RUSITA YD et al. 2019. Comparison of tannin concentration on fresh and dried tea leaf  
830 (*Camelia sinensis* L.) extract using UV-visible spectrophotometric method. 1st  
831 International Conference of Health, Science & Technology (ICOHETECH) 2019 E-ISBN:  
832 978-623-92207-0-9. November 16<sup>th</sup>, 2019.LPPM Universitas Duta Bangsa Surakarta,  
833 Surakarta.

834 SANGEETHA R, VEDASREE N. 2012. In Vitro  $\alpha$ -Amylase inhibitory activity of the leaves  
835 of *Thespesia populnea*. *Intern. Scholarly Res.Network*. 2012: 1-4 (2012)

836 SHRAIM AM et al. 2021. Determination of total flavonoid content by aluminum chloride  
837 assay: A critical evaluation. *LWT Food Sci. Tech.* 150(111932):1-11.

838 SILVA-RAMIREZ KA et al. 2020. Effect of drying and steeping temperature on the  
839 phenolic content, antioxidant activity, aromatic compounds and sensory properties of  
840 *Culila polyantha* Benth. *Infusions. Processes*. 8(1378):2-20.

841 SRISOOK K et al. 2012. Antioxidant and anti-inflammatory activities of hot water extract  
842 from *Pluchea indica* Less. herbal tea. *J. Med. Plants Res.* 6(23):4077-4081.

Corresponding Author: paini@ukwms.ac.id

843 SURIYAPHAN O. 2014. Nutrition, health benefits and applications of *Pluchea indica* (L)  
844 Less leaves. Pharm. Sci. Asia. 41:1–10.

845 SU D et al. 2019. Impact of thermal processing and storage temperature on phenolic  
846 profile and antioxidant activity of different varieties of lychee juice. LWT-Food Tech.  
847 116(108578):1-9.

848 THANAJIRUSCHAYA P et al. 2010. Effect of storage ~~time~~[time period](#) and temperature on  
849 antioxidant components and properties of milled rice. Khon Kaen Univ. Res. J.15(9):843-  
850 851.

851 VONGSAK B et al. 2018. Comparison of active constituents, antioxidant capacity, and  
852 αglucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. Food  
853 Biosci. 25: 68–73.

854 WANG SC. et al. 2022. Effect of brewing conditions on polyphenols in the dark tea  
855 (*Camellia sinensis* L.) infusions: content, composition and antioxidant activities. Food  
856 Sci.Tech. 42(e36322):1-9.

857 WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica*  
858 Less as functional beverage in diabetic patients. ASSEHR. 98:164-167.

859 WIDYAWATI PS et al. 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract  
860 as antioxidant and antiwarmed over flavor (WOF) of duck meat. International Congress  
861 on Challenges of Biotechnological Research in Food and Health. November 15<sup>th</sup>  
862 2014.Agricultural Technology Faculty, Slamet Riyadi University, Surakarta.

863 WIDYAWATI PS et al. 2016. The physicochemical and antioxidant properties of *Pluchea*  
864 *indica* Less drink in tea bag packaging. IJFNS. 5: 13–120.

Corresponding Author: paini@ukwms.ac.id

865 WIDYAWATI PS et al. 2017. In vitro antioxidant capacities and antidiabetic properties of  
866 *Pluchea* leaves. IJPPS. 9(8): 203-208.

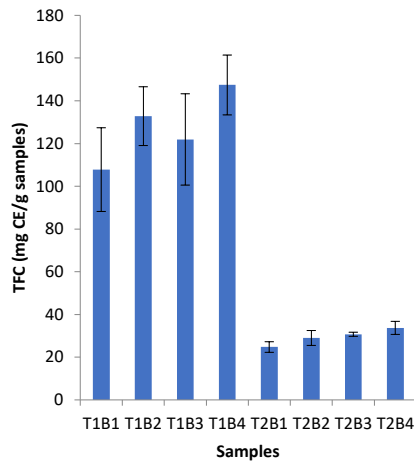
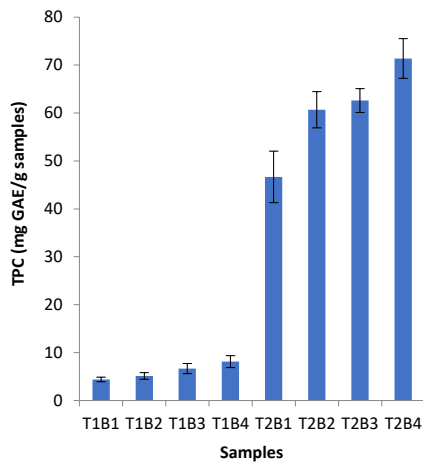
867 WIDYAWATI PS et al. 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica*  
868 Less beverage. CJFST.12(4): 125-139.

869 WIDYAWATI PS ET AL. 2022. The effect of  $\kappa$ -carrageenan proportion and hot water  
870 extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of stink  
871 lily (*Amorphophallus muelleri*) wet noodles. Molecules. 27(5062):1-16.

872 XU P, CHEN L, WANG Y. 2019. Effect of storage [time period](#) on antioxidant activity and  
873 inhibition on  $\alpha$ -amylase and  $\alpha$ -glucosidase of white tea. Food Sci. Nut. 7(2): 636-644.

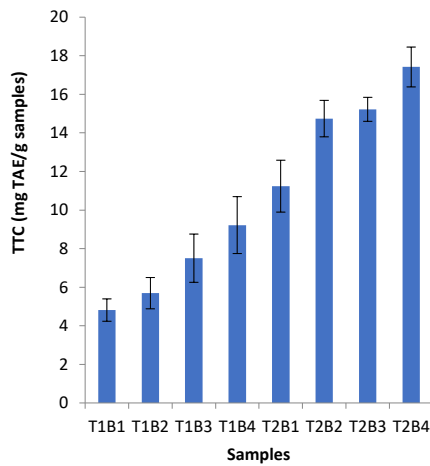
874 ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction [time period](#) on  
875 caffeine in coffee. Lantanida J. 6(2):103-202. [https://jurnal.ar-](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811)  
876 [raniry.ac.id/index.php/lantanida/article/view/3811](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811). [In Bahasa Indonesia]

877 ZHANG Y et al. 2021. Effect of storage conditions and [time period](#) on the polyphenol  
878 content of wheat flours. Processes. 9(248):1-11.



(a)

(b)



(c)

Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping temperature and storage time period (a) Total phenolic content (b) Total flavonoid content (c) Total tannin content. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-

Formatted: Left, Indent: Left: 0.5", Hanging: 0.69", Line spacing: single

888  
889  
890  
891  
892  
893  
894

stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$ .

Commented [A35]:

Table 1. Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperature and storage time period

Phenolic Compounds	Steeping Temperature (°C)	Mean±SD Un-stored	Mean±SD Stored	Mean difference ±SD	Sig (2-tailed)
Gallic Acid (µg/g samples)	60	0.2132±0.0027	0.2364±0.0015	0.0375±0.0175	0.2030
	70	0.2157±0.0013	0.2324±0.0214	0.0167±0.0227	0.4870
	80	0.2234±0.0122	0.2347±0.0078	0.0386±0.0264	0.2870
	95	0.2316±0.0104	0.2402±0.0169	0.0086±0.1990	0.8500
(+)-Catechin (µg/g samples)	60	0.3425±0.0110	0.5085±0.0111	-0.1576±0.0885	0.241
	70	0.3260±0.0265	0.5448±0.0006	-0.2188±0.0259	0.053
	80	0.3240±0.0222	0.5023±0.0773	-0.1451±0.0248	0.077
	95	0.4039±0.0320	0.5995±0.0372	-0.2049±0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756±0.1234	1.4762±0.0271	-1.2887±0.3222	0.111
	70	0.2587±0.0160	1.4245±0.2526	-1.1657±0.2695	0.103
	80	0.4175±0.0104	1.4570±0.0925	-1.0391±0.0841	0.036*
	95	0.8786±0.0434	2.6138±0.0695	-1.735±0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220±0.0268	0.6220±0.0706	-0.5999±0.9733	0.544
	70	0.1530±0.0511	1.0708±0.0289	-0.9177±0.0222	0.011*
	80	0.3666±0.0103	0.8629±0.0815	-0.1082±0.4462	0.790
	95	0.6559±0.0570	2.0230±0.0573	-1.4123±0.3203	0.101
Kaempferol (µg/g samples)	60	0.1394±0.0202	0.3675±0.0183	-0.3207±0.1122	0.154
	70	0.0514±0.0037	0.3726±0.0944	0.3213±0.0907	0.125
	80	0.3699±0.0924	0.7966±0.0366	-0.4267±0.2727	0.271
	95	0.5913±0.0239	0.9478±0.0287	-0.3565±0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103±0.0628	2.4863±0.0270	-1.8760±0.2074	0.050*
	70	0.6271±0.0099	2.3403±0.0325	-1.7131±0.3152	0.082
	80	0.7967±0.03060	2.6278±0.0211	-1.8311±0.0095	0.002*
	95	1.5386±0.0668	4.0211±0.0851	-2.4825±0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635±0.0628	0.9449±0.0501	-0.2814±0.4458	0.536
	70	0.6162±0.0099	0.9485±0.0794	-0.3323±0.0301	0.041*
	80	0.6601±0.0306	0.9099±0.0387	-0.2498±0.3127	0.461
	95	0.6642±0.0668	1.3156±0.0166	-0.6514±0.2666	0.179

Corresponding Author: paini@ukwms.ac.id

4,5-di-O-Caffeoylquinic acid ( $\mu\text{g/g}$ samples)	60	0.4906 $\pm$ 0.0060	1.1842 $\pm$ 0.0120	-0.6886 $\pm$ 0.2723	0.018*
	70	0.4807 $\pm$ 0.0034	1.0089 $\pm$ 0.0736	-0.5281 $\pm$ 0.0702	0.060
	80	0.5299 $\pm$ 0.0053	1.2382 $\pm$ 0.1435	-0.7082 $\pm$ 0.1489	0.094
	95	1.0018 $\pm$ 0.0526	1.3797 $\pm$ 0.2170	-0.3086 $\pm$ 0.3086	0.333

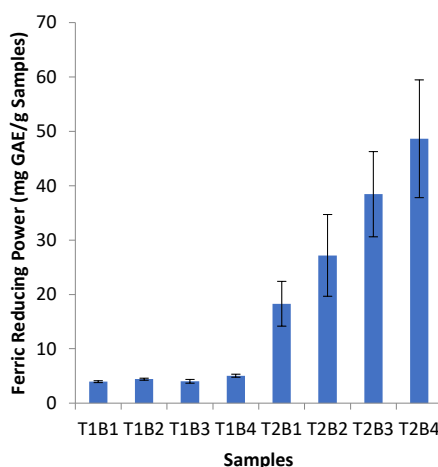
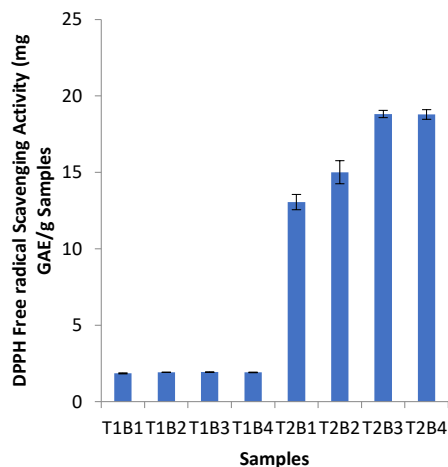
896 Note : Data were expressed as mean  $\pm$ standard deviation (n=2). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-  
897 steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C,  
898 stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped  
899 at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature,  
900 calculated using a paired T test at  $\alpha \leq 0.05$ . \*  $\alpha \leq 0.05$ .  
901

Formatted: Line spacing: single

Commented [A36]: Improve

902





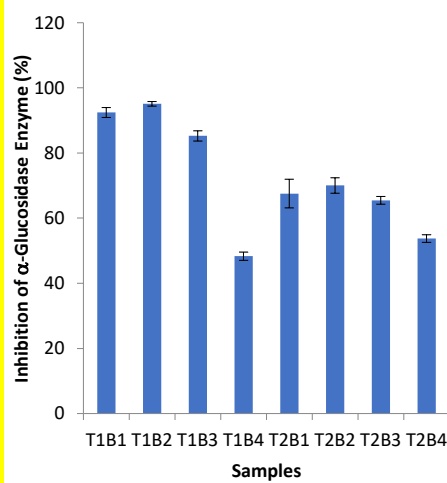
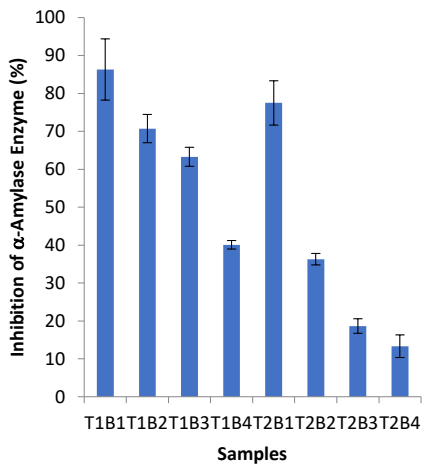
(a)

(b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage ~~time~~ time period (a) DPPH (b) FRAP. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T2B2-steeped at 70 °C, stored for 5 years; T2B3-steeped at 80 °C, stored for 5 years; T2B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$ .

Formatted: Justified, Indent: Left: 0.5", Hanging: 0.69", Line spacing: single

Commented [A37]: Improve



(a)

(b)

**Figure 3.** Antidiabetic activity of pluchea tea at different steeping temperature and storage ~~time~~ period (a)  $\alpha$ -amylase (b)  $\alpha$ -glucosidase. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$ .

Formatted: Indent: Left: 0.44", Hanging: 0.75"

Formatted: Justified, Indent: Left: 0.44", Hanging: 0.75", Line spacing: single, Tab stops: 1.19", Left

Commented [A38]: Improve

6. Final Decision (17-4-2024)

-Correspondence

-Decision Letter

-Document



Paini Sri Widyawati <paini@ukwms.ac.id>

---

**From Caesar Saloma/15 January 2024/ Editorial Decision/ MS 23-158R**

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Wed, Apr 17, 2024 at 2:04 PM

Dear Dr. Widyawati,

We confirm the receipt of your revised Ms 23-158 paper, as well as your point-for-point response to the reviewer's comments. These will be forwarded to the PJS Editor-in-Chief for his consideration and final decision.

Thank you for your sustained contribution to PJS!

Sincerely,  
Ms. CARYL MARIA MINETTE I. ULAY

Editorial Assistant

For Dr. CAESARA. SALOMA  
Editor-in-Chief  
[Quoted text hidden]



Paini Sri Widyawati <paini@ukwms.ac.id>

---

**From Caesar Saloma/15 January 2024/ Editorial Decision/ MS 23-158R**

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Wed, Apr 17, 2024 at 2:32 PM

Dear Ms Caryl

Thanks for attention

Regards

Paini SW

[Quoted text hidden]



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**Final Decision - PJS Paper Ms 23-158**

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Fri, Apr 19, 2024 at 5:00 PM

Dear Dr. Saloma,

Greetings! We are sending the Ms 23-158 documents for your final decision and subsequent communication to the author.

"Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea indica* Less Tea" [Ms 23-158]

Corresponding author

DR. PAINI SRI WIDYAWATI  
Food Technology Study Program  
Agricultural Technology Faculty  
Widya Mandala Surabaya Catholic University  
Surabaya, Indonesia  
[paini@ukwms.ac.id](mailto:paini@ukwms.ac.id)

Reviewer 1

DR. ELIUD M. NJAGI  
Department of Biochemistry, Microbiology, and Biotechnology  
Kenyatta University  
Nairobi, Kenya

[1st evaluation]

Paper secured no affirming commitment from experts

Reviewer 2

DR. WILMA A. HURTADA  
Institute of Human Nutrition and Food  
College of Home Economics  
University of the Philippines Los Baños  
College, Laguna

[1st evaluation]

Paper as presently written is unacceptable for publication; needs extensive revision

[2nd evaluation]

Reconsider only after the comments/recommendations are clarified and/or complied with  
Paper should be published as a research note/short communication

Reviewer 3

DR. DENNIS MARVIN O. SANTIAGO  
Institute of Food Science and Technology  
College of Agriculture and Food Science  
University of the Philippines Los Baños

College, Laguna

[1st evaluation]

Reconsider only after the comments/recommendations are clarified and/or complied with  
Paper should be published as a research note/short communication

[2nd evaluation]

Accept paper for publication

Initial manuscript submitted to PJS: 05 May 2023

Reviewers' comments sent to authors: 20 Sep 2023

1st Revised manuscript sent to PJS: 22 Nov 2023

2nd Revised manuscript sent to PJS: 17 Apr 2024

The folder can be accessed through this link: <https://bit.ly/3UTfrKK>. Thank you!

Sincerely,

Ms. CARYL MARIA MINETTE I. ULAY

Editorial Assistant

--



**Philippine Journal of Science**  
**Science and Technology Information Institute**  
**Department of Science and Technology**  
**DOST Complex, Gen. Santos Ave., Bicutan 1631**  
**Taguig City, Metro Manila, Philippines**  
Telephone no. : 837 - 2191  
Email: [pjs@stii.dost.gov.ph](mailto:pjs@stii.dost.gov.ph); [philjournsci@gmail.com](mailto:philjournsci@gmail.com)  
Website: <https://philjournalsci.dost.gov.ph>  
Scopus: <https://www.scopus.com/sourceid/19700175735>



Paini Sri Widyawati <paini@ukwms.ac.id>

---

## Fwd: Comments on PJS Paper Ms 23-158

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Tue, Apr 23, 2024 at 8:57 AM

Dear Dr. Widyawati,

Greetings! We wish to relay an update regarding the final evaluation of PJS paper Ms 23-158.

We have confirmed that you only provided two revised copies of your manuscript without itemized or point-to-point responses to the reviewer's comments.

Please kindly provide your separate responses to the reviewer's comments in a tabular form as requested by the editor-in-chief.

Thank you for your understanding and compliance.

Sincerely,  
Editorial Assistant

[Quoted text hidden]



1 **Effect of Steeping Temperature and Storage TimePeriod on the Bioactive**

2 **Compounds, Antioxidant and Antidiabetic Activities of Infusion from Powdered**

3 ***Pluchea Indica Less***

4 Painsi Sri Widyawati<sup>1)</sup>, Yufita Ratnasari Wilianto<sup>2)</sup>

5 <sup>1)</sup>Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala  
6 Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

7 <sup>2)</sup>Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic  
8 University, Kalisari Street Number 1, Surabaya 60272, Indonesia

9 Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature,

10 *Pluchea indica Less*, storage timeperiod

11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
Corresponding Author: painsi@ukwms.ac.id

21 ABSTRACT

22 This study was done to determine the effects of steeping temperature and storage  
23 ~~timeperiod~~ on the bioactive contents, antioxidant and antidiabetic activities of *Pluchea*  
24 leaf infusion. The research used a randomized block design with two factors, i.e.,  
25 steeping temperature (T) and storage ~~timeperiod~~ (B). The ~~variety of the Pluchea leaf~~  
26 ~~blades were exposed to 4~~ steeping temperatures ~~included of~~ 60 (T1), 70 (T2), 80 (T3),  
27 and 95 (T4) (°C) with the storage ~~timeperiod-period~~ of 0 (B1) and 5 (B2) (year). ~~The~~  
28 ~~research resulted resulting in~~ 8 treatment combinations (T1B1, T1B2, T2B1, T2B2,  
29 T3B1, T3B2, T4B1, T4B2). Statistical analysis using a paired ~~t-T~~ test at  $\alpha \leq 0.05$   
30 showed that treatments significantly ~~affected influenced~~ the bioactive contents (total  
31 phenol (TPC), total tannin (TTC), total flavonoid (TFC)), antioxidant [(DPPH scavenging  
32 activity (DPPH) and ferric reducing antioxidant power (FRAP))] ~~potential~~ and  
33 antidiabetic [( $\alpha$ -amylase (AA) and  $\alpha$ -glucosidase (GA) ~~inhibitorsinhibition~~)] activities  
34 ~~properties of the Pluchea leaf infusionsamples. TFC decreased during storage period~~  
35 ~~but significantly increased at higher steeping temperature. The AA and GA of Pluchea~~  
36 ~~infusion increased until 70 °C of the steeping temperature, but decreased until 95 °C.~~  
37 ~~The bioactive contents influenced antioxidant and antidiabetic activities. TFC was~~  
38 ~~decreased for storage time and significant increased at higher steeping temperature.~~  
39 ~~The AA and GA of Pluchea infusion increased until 70 °C of the steeping temperature,~~  
40 ~~but decreased until 95 °C.~~ The AA and GA were strongly and negatively correlated with  
41 TPC, TTC, DPPH and FRAP, but it was moderately and negatively correlated with TFC.  
42 ~~Between T~~ the antioxidant activity of DPPH and FRAP and the antidiabetic activity of AA  
43 ~~and GA~~ of *Pluchea* infusion were strongly and positively correlated. ~~with correlation~~

Commented [A1]: Describe treatment effects on total phenolics, tannins, antioxidant, and antidiabetic in one brief sentence each and indicate statistical significance.

44 ~~coefficient (r) values of 0.956 and 0.725, respectively. The treatments gave different~~  
45 ~~effect of simple phenolic compounds derived from *Pluchea* leaf infusion at different~~  
46 ~~steeping temperatures and storage included, such as gallic acid, kaempferol, myricetin,~~  
47 ~~(+)-catechin, quercetin, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and~~  
48 ~~4,5-di-O-caffeoylquinic acid, of *Pluchea* infusion at different steeping temperature and~~  
49 ~~long storage. To obtain high antioxidant activity, *Pluchea* infusion selected was stored~~  
50 ~~and steeped at high temperature, however high antidiabetic activity obtained was fresh~~  
51 ~~*Pluchea* infusion and steeped at low temperature.~~

**Commented [A2]:** State briefly results of the correlation analysis.

**Formatted:** Font: Italic

## 52

### 53 INTRODUCTION

54 *Pluchea* herbal tea is a product of dried *Pluchea* leaf processing introduced by  
55 world people (Srisook et al., 2012; Widyawati et al., 2016) because of the efficacy of the  
56 active components in *Pluchea* leaves, as a herbal plant that has been widely used for  
57 traditional medicine and food (Chan et al., 2022). *Pluchea* leaves are composed many  
58 nutrients and bioactive compounds useful to body health. The nutrient compositions in  
59 the *Pluchea* leaves include protein, fat, ash, insoluble fiber, soluble fiber,  
60 carbohydrates, calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive compounds is  
61 comprised, i.e., chlorogenic acid, caffeic acid, 3-O-caffeoylquinic acid, 4-O-  
62 caffeoylquinic acid, 5-O-caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-  
63 caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, quercetin, myricetin, kaempferol, total  
64 anthocyanin,  $\beta$ -carotene, and total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018;  
65 Ruan et al., 2019; Widyawati et al., 2022, Chan et al., 2022).

66 Steeping process of *Pluchea leaves* can be performed with fresh or dry leaves  
67 ~~infusion by~~in hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et  
68 al., 2020; Jayani et al., 2022). In Asian ~~area~~, especially in Indonesia~~n~~, people usually  
69 consume the *Pluchea infusion* ~~with brewing of~~by steeping 2 g of powdered *Pluchea*  
70 leaves in tea bag ~~by~~in 100 mL of hot ~~water~~or boiling water. ~~Each tea bag contained 2 g~~  
71 ~~of *Pluchea* leaf powder is steeped with 100 mL hot water or boiling water.~~Widyawati et  
72 al. (2016) claimed that steeping of 2 g ~~of *Pluchea* leaf powder~~ at 95 °C for 5 minutes  
73 ~~results~~~~re~~exhibits total phenolic ~~content~~,and total flavonoid contents~~s~~, the ability to  
74 scavenge DPPH free radicals, and the capability ~~of~~to reduce ferric ions ~~at~~ 9.3 mg gallic  
75 acid equivalent (GAE)/g samples~~s~~, 22.0 mg gallic acid equivalent (GAE)/g samples~~s~~, 27.2  
76 mg gallic acid equivalent (GAE)/g samples~~s~~, and 10.2 mg gallic acid equivalent (GAE)/g  
77 samples~~s~~, respectively. Werdani and Widyawati (2018) reported that drinking of *Pluchea*  
78 *leaf powder infusion* in the morning and evening regularly (2 g/100 mL) can decline  
79 blood sugar levels.

80 The steeping of *Pluchea herbal tea* with hot water at 95 °C for 5 minutes certainly  
81 determines the stability and amount of extracted bioactive compounds~~;~~ that influences  
82 the biological activity~~;~~ especially antioxidant and antidiabetic activities. Silva-Ramirez et  
83 al. (2020) reported that the infusion process can influence the~~ir~~ content and composition  
84 of the bioactive compounds and antioxidant activity of tea. Acar et al. (2022) informed  
85 that infusion quality of ~~herbal tea extract~~ depends on several factors, i.e., ~~time~~~~storage~~  
86 and temperature. Polyphenol profile and antioxidant properties of ~~herbal tea infusion~~  
87 decline with an increase in steeping/brewing and storage temperatures~~;~~ and longer  
88 exposure ~~time~~periods.

Formatted: Not Highlight

Formatted: Not Highlight

89 Several studies have mentioned the effect of steeping temperature ~~to-on the~~  
90 bioactive compound contents and antioxidant activity, such as some white and green  
91 teas are effective with hot water at 90 °C for 7 min (Castiglioni et al., 2015), ~~on~~ roseship  
92 tea is effectively at infusion ~~timeperiod~~ around 6-8 min at temperatures of 84-86 °C  
93 (Ilyasoglu and Arpa, 2017), ~~on the caffeine content extracted the coffee at the~~ brewing  
94 temperature ~~of coffee influences the caffeine content extracted~~ (Zarwinda and Sartika,  
95 2018), ~~and the steeping the high total phenol content and antioxidant activity~~ of dark tea  
96 at 92 °C for 27 min ~~results the highest total phenol content and antioxidant activity~~  
97 (Wang et al., 2022). The study of the effect of steeping temperature to **Pluchea** infusion  
98 was carried out to afford information about ~~the most efficient~~ preparation of **powdered**  
99 **Pluchea leaves** ~~most efficiently~~ to get higher ~~the~~ bioactive compounds, antioxidant and  
100 antidiabetic activities.

101 ~~On the other hand, storage~~ ~~Storage timeperiod tea~~ usually for several months  
102 ~~until years~~ of **Pluchea herbal tea** also affects the levels of the bioactive compounds and  
103 biological activity ~~because this herbal tea usually is stored for a several months until~~  
104 ~~years~~ (Jayani et al., 2022). Tea or herbal tea is generally stored in ambient temperature  
105 and packed in tea bag or **Alu foil standing proud** or a combination of both. Many  
106 researchers ~~informed~~ ~~reported~~ that storage ~~timeperiod~~ decreases the bioactive  
107 compounds, antioxidant and antidiabetic activities, i.e., juice from *Momordica*  
108 *charantia* L. (Lin et al., 2020), dried *Piper betlle* extracts (Ali et al., 2018), white tea (Xu  
109 et al., 2019), kinnow-amla beverages (Purewal et al., 2022), whole wheat flour (Zhang  
110 et al., 2021).

Commented [A3]: IS THIS ALUMINUM? If so, replace Alu with aluminum

Commented [A4]: Do you mean standing pouch?

111 Therefore, this research studied the effect of steeping temperature and storage  
112 ~~time period~~ on the bioactive compounds [(total phenolic content (TPC), total flavonoid  
113 content (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging  
114 activity (DPPH), ferric reducing antioxidant power (FRAP)] and antidiabetic activities [( $\alpha$ -  
115 amylase (AA) and  $\alpha$ -glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea*  
116 leaves. ~~The study was done to determine total phenolic content (TPC), total flavonoid  
117 content (TFC), total tannin content (TTC), DPPH free radical scavenging activity  
118 (DPPH), ferric reducing antioxidant power (FRAP),  $\alpha$ -amylase (AA) and  $\alpha$ -glycosidase  
119 (GA) inhibition activities, and on the phenolic compound profile.~~

120

## 121 MATERIALS AND METHODS

### 122 RAW MATERIALS AND PREPARATION

123 The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya,  
124 East Java, Indonesia. The *Pluchea* plants were included in *Asteraceae* family with  
125 specification according to the GBIF taxon ID number database:3132728 (Ferraris,  
126 2023). *Pluchea* leaves at 1-6 level of each branch ~~off from~~ the shoot were collected,  
127 sorted, washed and dried to ~~get a~~moisture content of around  $11.16 \pm 0.09$  % dry basise  
128 (Widyawati et al., 2022). The ~~powdering of~~ dried *Pluchea* leaves was ~~done~~ pulverized to  
129 ~~get a~~ 45-mesh size powder. ~~And then, the heating of T~~the *Pluchea* leaf powder was  
130 ~~done using a drying~~dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120  
131 °C for 10 min to reduce microbial organisms. ~~and Then, 2 g of the powder were packed  
132 using into a paper filter infusion bag, that made from paper filter around 2 g/bag. And~~

133 then all of samples called Packed samples were. *Pluchea* herbal tea was stored for 0  
134 (un-stored) and 5 (stored) years in standing pouch before analysis.

135 In the research, the one tea bag of *Pluchea* herbal tea that stored 0 (B1) and 5  
136 (B2) year, was steeped with 100 mL hot water at various temperatures, including 60  
137 (T1), 70 (T2), 80 (T3), and 95 (T4) °C for 5 min with infusion method that  
138 obtained obtaining 8 treatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1,  
139 T3B2, T4B1, T4B2. After the temperature of *Pluchea* infusion similar to ambient  
140 temperature was analyzed further.

## 142 REAGENTS

143 The compounds reagents used to analyze in the analyses including include 2,2-  
144 diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid,  $\alpha$ -amylase,  $\alpha$ -  
145 glucosidase, pNPG (p-nitrophenyl- $\alpha$ -glucopyranoside), (+)-catechin, kaempferol,  
146 myricetin, quercetin, 3,4-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, 3,5-di-O-  
147 caffeoylquinic acid, and (+)-catechin were purchased from Sigma-Aldrich (St. Louis,  
148 MO, USA). Methanol, Folin-Ciocalteu's Phenol, sodium nitric, aluminum chloride, ferric  
149 chloride, sodium dihydrogen phosphate, sodium phosphate, potassium ferricyanide,  
150 starch, acetic acid, and sodium hydroxide were purchased from Merck (Kenilworth, NJ,  
151 USA). All reagents used were of analytical grade except for distilled water which was  
152 purchased from PT Aqua Industry Surabaya.

## 154 METHODOLOGY

### 155 ANALYSIS OF THE BIOACTIVE COMPOUNDS

**Commented [A5]:** Confusing, needs to be re-written eg  
The unstored samples were steeped in 100 mL distilled  
water at 60, 70, 80, and 95 °C for 5 min, then immediately  
were analyzed for the bioactive compounds [(total  
phenolic content (TPC), total flavonoid content (TFC),  
total tannin content (TTC)], antioxidant potential  
[(DPPH free radical scavenging activity (DPPH), ferric  
reducing antioxidant power (FRAP)] and antidiabetic  
activities [ $\alpha$ -amylase (AA) and  $\alpha$ -glucosidase (GA)  
inhibition]]. The rest of the samples were stored at  
(describe storage conditions) and analyze after 5  
years..

**Commented [A6]:** Confusing

**Formatted:** Centered

156 TOTAL PHENOLIC CONTENT ANALYSIS

157 Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using  
158 the technique by Gao et al. (2019). About 10  $\mu$ L *Pluchea* infusion and 1 mL Folin-  
159 Ciocalteu's phenol reagent 10 % were mixed in 10 mL volumetric flask and incubated  
160 for 5 min. And then 2 mL  $\text{Na}_2\text{CO}_3$  7.5 % was ~~entered-added and filled up to 10 mL~~  
161 ~~volume with distilled water and distilled water was added until 10 mL volume.~~ The  
162 color intensity of solution was measured in the spectrophotometer UV-Vis 1800  
163 (Shimadzu, Japan) at  $\lambda$  760 nm with gallic acid as the reference standard. The total  
164 phenolic content was calculated using the formula:  $y=0.00009x+0.008$  with  $R^2=0.9941$ .  
165 The results were expressed as mg gallic acid equivalent (GAE)/g samples.

Commented [A7]: Specify what color

166

167 TOTAL FLAVONOID CONTENT ASSAY

168 Total flavonoid content (TFC) of the samples was measured based on the  
169 reaction between  $\text{AlCl}_3$  and  $\text{NaNO}_2$  with ~~an-the~~ aromatic ring of flavonoid compounds,  
170 especially flavonol and flavon (Shraim et al., 2021). The reaction between  $\text{AlCl}_3$  and  
171 flavonoid compounds resulted ~~in~~ a yellow solution. About 30  $\mu$ L *Pluchea* infusion was  
172 mixed with 0.3 mL  $\text{NaNO}_2$  5 % in 10 mL volumetric flask and incubated for 5 min. The  
173 mixture was added with 0.3 mL  $\text{AlCl}_3$  10 % for 5 min. And then, 2 mL NaOH 1 M and  
174 distilled water were added until 10 mL volume. Then, the red solution was produced  
175 after NaOH solution addition that was measured by a spectrophotometer  
176 (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  510 nm with (+)-catechin as  
177 the reference standard compound, and the results were expressed as mg catechin  
178 equivalents (CE)/g samples using the formula:  $y=0.00008x-0.0023$  with  $R^2= 0.9980$ .



179  
180 **TOTAL TANNIN CONTENT ANALYSIS**  
181 Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu  
182 method (Chandran and Indira, 2016). Approximately 10  $\mu$ L *Pluchea* infusion was added  
183 with 1 mL Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flask and  
184 incubated for 5 min. Then, the mixture was added with 2 mL  $\text{Na}_2\text{CO}_3$  7.5 % and filled up  
185 to 10 mL volume with distilled water, was added until 10 mL volume. The blue dark  
186 color solution that was measured in UV-Vis spectrophotometer 1800 (Shimadzu, Japan)  
187 at  $\lambda$  760 nm with tannic acid as the reference standard. Calculation of TTC was  
188 expressed as mg tannic acid equivalents (TAE)/g samples used the formula:  
189  $y=0.00009x+0.0021$  with  $R^2=0.9993$

#### 191 ANALYSIS OF THE ANTIOXIDANT POTENTIAL

##### 192 DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

193 The DPPH free radical scavenging activity (DPPH) was measured by the  
194 spectrophotometric method (Widyawati et al., 2017) to determine the ability of the  
195 phytochemicals antioxidant activity of the *Pluchea* leaf infusion to donor-donate  
196 hydrogen atom to the nitrogen atom in DPPH resulting in the formation of -DPPH-H  
197 compound with exhibiting a yellow-colored solution. About 25  $\mu$ L *Pluchea* leaf infusion  
198 was entered-poured into reaction tube and-into which was added added 3 mL DPPH  
199 solution (4 mg/100 mL). And then the solution wasAfter incubationed for 15 min in a  
200 dark room , the-and absorbance was measured by a spectrophotometer  
201 (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$ . 517 nm. The reference

Formatted: Centered

202 standard compound was gallic acid and the results of analysis were expressed as mg  
203 gallic acid equivalents (GAE)/g samples that calculated using formula:  $y=0.146x+1.7896$   
204 with  $R^2=0.9975$ .

205

#### 206 FERRIC REDUCING POWER ANALYSIS

207 Ferric reducing power (FRAP) was determined following the method used by  
208 Widyawati et al. (2014) method. Approximately 10  $\mu$ L of samples were added 2.5 mL  
209 phosphate buffer pH 6.6 and 2.5 mL and 1% potassium ferricyanide 1% in the reaction  
210 tube. And then mixture was shaken and ~~incubation~~ incubated for 20 min at 50 °C.  
211 Finally, 2.5 mL chloroacetic acid 10% (w/v) was added. Into the 2.5 mL supernatant  
212 was added 2.5 mL distilled water, 0.5 mL ferric chloride 0.1% (w/v) and incubated for  
213 10 min. Potency of the samples reducing iron (III) to iron (II) ion was ~~signed~~ indicated by  
214 the intensity of blue color formed that was measured using UV-Vis spectrophotometer  
215 (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  700 nm. Intensity of the blue  
216 color indicated higher reducing capacity. The reducing power expressed as mg gallic  
217 acid equivalent (GAE)/g samples was calculated using the formula:  $y=0.0002x+0,0256$   
218 with  $R^2=0,9906$ .

219

#### 220 $\alpha$ -AMYLASE ENZYME INHIBITION CAPACITY ASSAY

221 In vitro inhibition of  $\alpha$ -amylase enzyme (AA) followed the procedure as described  
222 by Widyawati et al. (2020). Each 500  $\mu$ L of samples, was mixed with starch 1 % (w/v)  
223 and sodium acetate buffer pH 5. ~~were mixed. Then, Into each~~ 250  $\mu$ L of the mixture  
224 ~~and was added an~~  $\alpha$ -amylase solution (0.1 g of this enzyme 12.5 unit/mL) then, was

225 dissolved in 50 mL of 0.2 M sodium acetate pH 5). Mixture was shaken and into which  
226 was added 2 mL sodium hydroxide 1M. Before the analysis, this mixture was incubated  
227 at 37 °C for 10 min. Then, the capacity of the α-amylase enzyme to hydrolyzed the  
228 starch to release glucose was measured by UV-vis spectrophotometer  
229 (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) that could be analyzed based on  
230 absorbance at λ 540 nm. The inhibition percentage of α-amylase was assessed using  
231 the formula:  $(ACb - ACa) - (As - Ab) / (ACb - ACa) \times 100 \%$ . Where, ACb is the  
232 absorbance of 100 % enzyme activity (solvent with the enzyme), ACa is the absorbance  
233 of 0 % enzyme activity (solvent without the enzyme), As is the absorbance of test  
234 sample with enzyme, Ab is absorbance of test sample without enzyme.

235

#### 236 α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

237 The analysis of the α-glycosidase inhibitor activity (GA) was done by Widyawati  
238 et al. (2020) method with slight modification. About 150 μL samples contained  
239 containing 100 μL *Pluchea* infusion and 50 μL pNPG (0.0150 g in 100 mL sodium  
240 phosphate 0.2 M at pH 7) were reacted with 50 μL α-glycosidase 2 mM (0.0833  
241 unit/mL), and then the mixture was incubated at 37 °C for 15 min. Finally, the  
242 reaction was stopped with the addition of 1000 μL sodium carbonate 0.2 M. The residue  
243 of this enzyme hydrolyzed p-nitrophenyl-α-D-glucopyranoside (pNPG) as a substrate to  
244 result p-nitrophenol. The inhibition activity of steeping the *Pluchea* tea infusion to  
245 enzyme was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800,  
246 Shimadzu, Japan) at λ 405 nm. The inhibition percentage of α-glycosidase was  
247 calculated using formula:  $(ACb - ACa) - (As - Ab) / (ACb - ACa) \times 100 \%$ . Where, ACb

Commented [A8]: Confusing. Rewrite

248 is the absorbance of 100 % enzyme activity (solvent with enzyme), A<sub>Ca</sub> is the  
249 absorbance of 0 % enzyme activity (solvent without enzyme), A<sub>s</sub> is the absorbance of  
250 test sample with enzyme, A<sub>b</sub> is the absorbance of test sample without enzyme.

251

## 252 HPLC ANALYSIS OF PHENOLICS

253 The phenolic compounds of the samples were analyzed by HPLC based on  
254 Kongkiatpaiboon et al. (2018) method with modifications. Each *Pluchea* infusion was  
255 sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter  
256 syringe (Whatmann, 0.2 µm, NYL). About 20 µL of sample was injected in an HPLC  
257 (LC20AD series, Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence  
258 UFLC LC-20AD pump, CTO-30A column oven, CBM-20A/20 Alite system controller,  
259 and SPD-20A/20 AV UV-Vis detector. Separation of phenolic compounds in samples  
260 was carried out using a Shim-pack VP-ODS C18 column (ID 5 µm × 50 mm × 4.6 mm)  
261 with a GVP-ODS Cartridge guard column (2 pieces) (ID 10 mm × 4.6 mm). Analytical  
262 conditions: The mobile phase used consisted of (A) 0.5 % acetic acid in  
263 water and (B) absolute methanol. Analysis was carried out using a gradient system in  
264 the following order: initial conditions of 10 % B in A to 50 % B in A were maintained for  
265 40 minutes; then 100 % B was maintained for 20 minutes. Next the column was re-  
266 equilibrated with 10 % B in A maintained for 10 minutes before analysis of the next  
267 sample. The sample flow rate was set at 1.0 ml/min with a controlled temperature at 40  
268 °C. Detection was used at a wavelength of 280 nm. The reference standard used were  
269 gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,5-dicaffeoylquinic acid, 3,4-  
270 dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid. All of the reference standard was

271 dissolved in distilled water and prepared similar to the samples before injected in  
272 HPLC.

273

## 274 EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

275 The research design used a randomized block design with two factors, i.e., the  
276 steeping temperature (T) and the storage ~~time~~period (B). *Pluchea* leaf blades were  
277 subjected to 4 steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and  
278 95 °C (T4), and the storage ~~time~~period of 0 year /~~fresh~~un-stored (B1), and 5  
279 year/stored (B2). ~~The research resulted~~resulting in 8 treatment combinations (T1B1,  
280 T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic was  
281 repeated two ~~time~~periods. The data of samples were analyzed by ANOVA at  $\alpha \leq 0.05$ ,  
282 and continued analysis using a paired T test at  $\alpha \leq 0.05$ . treatment means of specific  
283 phenolic compounds that were identified were expressed as the mean  $\pm$  SD. The  
284 analysis used SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

285

## 286 RESULTS AND DISCUSSIONS

287 *Pluchea* leaf infusion is produced by young *Pluchea* leaf from 1-6 level on each  
288 branch the shoot (Widyawati et al., 2022), that is steeped at 95 °C for 5 min, has many  
289 biological activities, such as antioxidant activity (Widyawati et al., 2016), antidiabetic  
290 activity (Werdani and Widyawati, 2018), anti-inflammatory (Srisook et al., 2015). The  
291 chemical constituents in *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols,  
292 cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2  
293 g/100 mL steeping *Pluchea* tea has total phenolic content 9.3 mg gallic acid

Corresponding Author: paini@ukwms.ac.id

**Commented [A9]:** Were all analyses replicated 2 periods only? What do you mean by 'continued analysis using a paired t-test at  $\alpha \leq 0.05$ '? Rewrite this part of the paragraph.

294 equivalents (GAE)/g samples, total flavonoid content 22.0 mg catechin equivalents  
295 (CE)/g samples, DPPH free radical scavenging activity 27.2 mg gallic acid equivalents  
296 (GAE)/g samples, and ferric reducing power 10.2 mg gallic acid equivalents (GAE)/g  
297 samples (Widyawati et al., 2016). Previous research has informed related to the  
298 composition of phytochemical compounds in *Pluchea* leaves, such as phenolic acids  
299 such as chlorogenic acids, caffeic acids, 3-*O*-caffeoylquinic acids, 4-*O*-caffeoylquinic  
300 acids, 5-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic  
301 acids, and 4,5-di-*O*-caffeoylquinic acids; total flavonoids which cover quercetin,  
302 kaempferol, myricetin, anthocyanin;  $\beta$ -carotene; and total carotenoids (Suriyaphan,  
303 2014; Vongsak et al., 2018; Ruan et al., 2019; Chan et al., 2022; Widyawati et al.,  
304 2022). Presence of phytochemical compounds in herbal product were influenced by  
305 environmental factors, i.e., temperature, light exposure, oxygen level, pH and moisture.  
306 The structure of phytochemical compounds in herbal tea is very sensitive of the  
307 surrounding changes. The effect arising from these changes causes the structure of the  
308 phytochemical molecule to be degraded to produce smaller size molecules or to  
309 combine to produce larger size molecules (Ali et al., 2018; Jayani et al. 2022,  
310 Ramphinwa et al., 2023). Therefore, this study was conducted to determine the effect of  
311 steeping temperature and storage ~~time~~period of *Pluchea* tea on levels of the bioactive  
312 compounds, antioxidant and antidiabetic properties and phenolic compound profile.

**Commented [A10]:** Delete this part. Information in here are already found in the Introduction section.

313

## 314 BIOACTIVE COMPOUNDS

315

### Phenolics Compounds

**Formatted:** Centered

316 The bioactive compounds are active compounds in plants that are essential to  
317 protect a body health (Nguyen and Chuyen, 2020). These compounds usually have  
318 many biological activities, such as antioxidant, antidiabetic, anti-inflammatory,  
319 anticancer, antimicrobial, antibacterial, anti-cholesterol and so on (Suriyaphan, 2014;  
320 Acar et al., 2022). Phenolic compounds have potential redox properties that can  
321 scavenge free radicals that can cause a number of chronic diseases (Noreen et al.,  
322 2017; Aryal et al., 2019; Acar et al., 2022).

323 The total phenolic content (TPC) of *Pluchea* infusion at different steeping  
324 temperature and storage period generally significantly increased with increasing  
325 steeping temperature and storage period based on paired  $T$ -test at  $\alpha \leq 0.05$  (Figure  
326 1a). Steeped and stored infusion had significantly higher amounts of phenolic  
327 compounds than the samples that were steeped and un-stored. Further, the highest  
328 total phenolic content was observed in samples infused at 95 °C and stored for 5 years  
329 (at 71.38±4.14 mg GAE/g samples) while the lowest was measured in the un-stored  
330 samples and infused at 60 °C (at mg GAE/g sample). Phenolic content of stored  
331 samples that were infused at different temperatures that then stored were steeped only  
332 at 60 and 95 °C also showed a significant increase in their phenolic content. This  
333 implies that the steeping temperature and the storage periods significantly resulted in  
334 the high amounts of the phenolic compounds of the infusions. Results also indicated  
335 that phenolic compounds were generally greater in the infusion at high steeping  
336 temperatures and long storage period. (Figure 1a). This could have been due to that  
337 fact that during steeping fresh *Pluchea* tea had a lower total phenolic content than  
338 stored *Pluchea* tea for 5 years, besides that the higher the steeping temperature also

339 caused the greater the extracted total phenolic content. The temperature of infusion  
340 influenced total phenolic content, it could relate to. This could have been due to the fact  
341 that the steeping temperature and storage period can cause the process of degradation,  
342 oxidation, and leaching/release of phenolic compounds. Phenolic compounds are water  
343 soluble and thus soaking in hot water for a certain period of period as in steeping  
344 causes the migration process of more phenolic compounds to the water because of  
345 longer increasing contact exposure between of phenolic compounds to water (Castiglioni  
346 et al. (2015); Kilic et al. (2017), and Acar et al. (2022)). Su et al. (2019) reported that  
347 temperature treatment can stimulate the release of phenolic compounds of lychee juice  
348 stored at different temperatures of 4 and 45 °C and different long storage (fresh and 72  
349 hours).

350 . this compounds and water. The same phenomena also occurred in Castiglioni  
351 et al. (2015); Kilic et al. (2017), and Acar et al. (2022).

352 This occurrence showed that steeping temperature and storage period caused  
353 the process of degradation and oxidation of phenolic compounds. Su et al. (2019)  
354 reported that temperature treatment can stimulate the release of phenolic compounds  
355 and increase antioxidant activity of lychee juice stored at different temperatures of 4 and  
356 45 °C and different long storage (fresh and 72 hours). Hydrogen bonding is affected by  
357 T temperature treatment because the degrades (or hydrolyzes) the hydrogen bond  
358 between phenolic compounds and proteins can be degraded that the measured levels  
359 resulting in an increase of phenolic compounds when exposed to a higher  
360 temperatures. The phenomena were supported by (Ali et al. (2018); Jayani et al. (2022),  
361 and Ramphinwa et al. (2023). Zhang et al. (2021) reported that phenolic compounds



362 present in plants are not completely stable, but are easily degraded during storage after  
363 harvest. Reblova (2012) claimed that antioxidant compounds can be slowly degraded  
364 with increasing temperature. Besides that, Fibrianto et al. (2021) also stated that the  
365 brewing temperature has an effect on the extracted antioxidant compounds, such as  
366 alkaloids, catechins and tannins. Thus, there is an assumption that temperature and  
367 storage caused the degradation, oxidation and hydrolysis of the phenolic compounds  
368 that the phenolic compounds in *Pluchea* infusion are degraded due to oxidation and  
369 hydrolysis because of temperature and storage time period and can be easily extracted  
370 during steeping, thus resulting in the increased amount of the phenolic content  
371 compounds as the at higher steeping temperature and longer storage increase period.

372 Based on using of a reference standard could be informed that Simple phenolic  
373 compounds identified in steeped and stored in *Pluchea* leaf infusion, including gallic  
374 acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-O-caffeoylquinic acids,  
375 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic acids was showed in Table 1.

376 The treatment effects results of statistical analysis using a paired T test at  $\alpha \leq 0.05$   
377 showed that gallic acid and kaempferol contents of *Pluchea* infusion were insignificantly  
378 different at various steeping temperature and long storage periods. Nevertheless, the  
379 The concentration of quercetin and 3,5-di-O-caffeoylquinic acid of the un-stored and  
380 stored *Pluchea* infusion was significantly different from the rest of the samples between  
381 of two treatments except at 70 °C. The while (+)-catechin concentration of *Pluchea*  
382 infusion was only significantly different at 95 °C, but the myricetin content was  
383 significantly different concentration at 80 and 95 °C. The 3,4-di-O-  
384 caffeoylquinic acid content showed significace difference at 60, 80 and 95 °C and while

385 4,5-O-dicaffeoylquinic acid compounds content from *Pluchea* infusion were only  
386 significantly different at 60 °C; however the concentration of 3,4-dicaffeoylquinic acid  
387 was also significantly different at 80 and 95 °C.

388 Based on the analysis of concentration of Results further showed simple phenolic  
389 compounds showed that gallic acids and kaempferol were relatively stable phenolic acid  
390 because of as reflected by the insignificant changes when exposed no changes at to the  
391 different steeping temperature and storage time period, with concentration about 0.21 ±  
392 0.00 to 0.24 ± 0.02 µg/g samples and 0.14 ± 0.02 to 0.95 ± 0.03 µg/g samples, respectively.

393 However, myricetin Myricetin, (+)-catechin and 3,4-di-O-caffeoylquinic acid showed a  
394 drastic increasing increase at higher steeping temperature and longer storage period  
395 implying It's meant that these compounds tended to be relatively labile. Quercetin, 3,5-  
396 di-O-caffeoylquinic acid and 4,5-di-O-caffeoylquinic acid underwent moderate changes  
397 compared to the other two groups of phenolic acids. Therefore, myricetin, (+)-catechin  
398 and 3,4-di-O-caffeoylquinic acid were easier to dissolve or degraded to form simple  
399 phenolic compounds at higher steeping temperature and storage time period. can cause  
400 macromolecules of three phenolic acids in herbal tea convenient degradable to form  
401 simple phenolic compounds for storage, as explained by (Su et al. (2019), Ali et al.  
402 (2018); Jayani et al. (2022); Ramphinwa et al. (2023), and Zhang et al. (2021).  
403 Degradable polyphenol compounds have a simple structure and free hydroxyl groups  
404 that can react with Folin-Ciocalteu's Phenol reagent, resulting complex blue solution  
405 that can detected as total phenolic content.

406 **Flavonoid Content (TFC)**

**Commented [A11]:** Does the negative sign mean an increase or decrease

**Commented [A12]:** What does the negative (-) sign implies? What is your basis of classifying the simple phenolic compounds as relatively labile, moderate?

**Formatted:** Centered

407 Flavonoids are the major phenolic compounds that have potential chemical and  
408 biological activities, such as radical scavenging and antimicrobial activities (Ayele et al.,  
409 2022; Chandra et al., 2014) that can protect the human body from the oxidative stress  
410 caused many degenerative diseases, especially cancer, cardiovascular problems and  
411 ageing (Mathur and Vijayvergia, 2017). The total flavonoid content of steeped *Pluchea*  
412 infusion decreased with longer storage period. Un-stored samples exhibited higher  
413 flavonoid content than the stored samples. The statistical analysis using a paired T test  
414 at  $\alpha= 0.05$  showed that total flavonoid content of *Pluchea* infusion was significantly  
415 different between ~~two treatments~~the steeped un-stored and steeped stored samples  
416 (Figure 1b). The highest total flavonoid content was exhibited by ~~fresh~~the un-stored  
417 samples steeped at 95 °C at about 147.42±14.03 mg CE/g samples. Total flavonoid  
418 content was significantly lower in the stored ~~samples regardless of steeping~~  
419 ~~temperature~~ than those of the un-stored ~~around 24.75±2.47 to 33.71±3.06 mg CE/g~~  
420 samples implying that the increase in the flavonoid content of the infusion was affected  
421 primarily by the steeping temperature.

#### 422 Tannin Content (TTC)

423 Tannins are bioactive compounds that provide properties, such as astringent,  
424 anti-diarrheal, antibacterial and antioxidant (Malangngi et al., 2012). Generally, results  
425 indicated that the total tannin content of *Pluchea* infusion significantly increased with  
426 increasing steeping temperature and storage period (Figure 1c). Among, the un-stored  
427 steeped samples, the tannin content was significantly lowest in the samples infused at  
428 60 °C at about 4.81±0.58 to 17.42±1.04 mg TAE/g samples, ~~which is~~was significantly  
429 different ~~lower~~ from ~~that of~~ the lowest tannin content of the stored samples. Among the

Commented [A13]: cite similar studies to support your findings

Formatted: Centered

430 stored and steeped samples, the highest tannin content was observed at samples  
431 steeped at 95 °C about  $17.42 \pm 1.04$  mg TAE/g samples and was significantly different  
432 from that of the highest tannin content of the un-stored steeped samples at 95 °C about  
433  $9.22 \pm 1.48$  mg TAE/g samples. ~~Indicating that the tannin content was primarily affected~~  
434 ~~by both high steeping temperature and long storage period than high steeping~~  
435 ~~temperature and that the presence of high tannin content was primarily brought about by~~  
436 ~~long storage period.~~ ~~Kowalska et al. (2021) informed that~~ The condensation of  
437 catechins to tannins ~~of polyphenolic compounds~~ is a dominant process ~~occurred~~  
438 ~~occurring~~ in tea leaves that is accelerated during maceration of raw ~~material~~ tea leaves  
439 ~~(Kowalska et al. (2021) could have had contributed to the observed increase in the~~  
440 ~~tannin content in the treated samples. However, the high temperature can degrade~~  
441 ~~polyphenolic compounds to form simple phenolic compounds that is essential to body~~  
442 ~~health. The results showed, that the higher the brewing temperature and the longer the~~  
443 ~~storage time caused the tannin compound to degrade to result catechin compounds.~~  
444 ~~This phenomenon is in line with the increase in total phenol levels and the concentration~~  
445 ~~of (+) catechin compounds. Ali et al. (2018) said that pH, storage temperature, chemical~~  
446 ~~structure and concentration, light, oxygen, enzymes and metal ions affect the presence~~  
447 ~~of bioactive compounds in the material. Nevertheless,~~  
448 ~~Although, high temperature and long storage period can cause the degradation~~  
449 ~~of tannins to catechins, Rusita et al. (2019) emphasized that tannins are a polar~~  
450 ~~thermostable complex compounds, that is-are resistant to heating, indicating that even~~  
451 ~~with the exposure to high temperature, the tannins still remained high in the treated~~  
452 ~~samples. as a result the tannin content in *Pluchea* tea increases with increasing steeping~~

453 ~~temperature and storage timeperiod, this is caused tannins are thermostable complex~~  
454 ~~compounds.~~

#### 456 ANTIOXIDANT ACTIVITY

457 Antioxidant activity is capability of compounds to inhibit the oxidation of  
458 macromolecules from biological target that involve in oxidative chain reactions (Ali et al.,  
459 2005; Oh et al., 2013). The antioxidant activity assay was done in this research using  
460 DPPH Free Radical Scavenging Activity (DPPH) and ferric reducing power (FRAP)  
461 methods. The phenolic compounds are an active antioxidant that have antioxidant  
462 capability that depends on their redox properties. The structure of phenolic compounds  
463 determine the effectivity to ~~doner donate~~ hydrogen atom which is negatively correlated  
464 with the O-H phenolic bond strength. The higher antioxidant power of phenolic  
465 compounds is caused by the weaker O-H phenolic bond (Kruk et al., 2022). The  
466 mechanism of phenolic compounds is involved as antioxidants through depends on their  
467 the ability to donate hydrogen atom ands, transfer electrons, and as reducing agents  
468 and singlet oxygen quenchers (Ali et al., 2005; Huang et al. 2005).

#### 470 DPPH Free Radical Scavenging Activity

471 DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to  
472 evaluate antioxidant activity because this method is simple that is suitable to measure  
473 the donating hydrogen atoms capability of herbal infusion. This reaction can cause the  
474 purple color of DPPH to change to yellow color (Munteanu and Apetrei, 2021; Baliyan et  
475 al., 2022). Figure 2a shows that the free radical scavenging property of the stored and

Formatted: Centered

Formatted: Not Highlight

Commented [A14]: what do you mean? rewrite

Formatted: Centered

476 steeped samples were significantly higher than the un-stored steeped samples. The  
477 result of DPPH assay. It can also be observed indicates that the free radical scavenging  
478 property DPPH values accrued was significantly different among the stored and steeped  
479 samples but insignificant among the un-stored and steeped samples at higher steeping  
480 temperature and longer storage time period. Statistical analysis by ANOVA using a  
481 paired T test at  $\alpha \leq 0.05$  proven that the higher the steeping temperature of fresh  
482 *Pluchea* infusion (T1B1, T2B1, T3B1, and T4B1) was consistent the ability to DPPH free  
483 radicals scavenging activity, whereas the stored *Pluchea* infusion resulted in the higher  
484 activity and the values went up as rising of the infusion temperature. *Pluchea* infusion  
485 stored at room temperature for 5 years resulted in the high DPPH free radical  
486 scavenging activity by more than 100 %. Steeping at higher temperatures  
487 significantly increased the DPPH free radical scavenging activity in stored *Pluchea*  
488 infusion by around 15 to 25 %. Steeping at 80-95 °C in stored *Pluchea* infusion  
489 insignificantly affected the free radical scavenging property of the bioactive compounds  
490 (Figure 2a). This implies that that the higher free radical scavenging property was  
491 primarily affected by the storage period than steeping temperature. During the storage  
492 process it is possible to form complex phenolic compounds which provide a high ability  
493 to scavenge DPPH-free radicals (Thanajiruschaya et al., 2010).

494 Scavenging The scavenging activity of DPPH free radicals of the the samples  
495 was strongly and positively correlated with total phenolic and tannin  
496 contents levels, but inversely to with total flavonoid levels. Based on Pearson correlation  
497 at Table 2, the correlated coefficient values (r) between DPPH and TPC, TTC and TFC  
498 were 0.993, 0.942, and -0.940, respectively. During the storage process it is possible to

Commented [A15]:

Commented [A16]: Clarify on how you were able to come up with free radical scavenging activity by more than 100 %. Steeping temperatures significantly increased the free radical scavenging activity in stored *Pluchea* infusion by around 15 to 25 %

Commented [A17]: Explain/interpret this observation based on the data that you were able to obtain.

499 ~~form complex phenolic compounds which provide a high ability to scavenge DPPH free~~  
500 ~~radicals (Thanajiruchaya et al., 2010).~~ This ~~research study~~ also demonstrated that  
501 longer storage ~~timeperiod~~ and higher infusion temperature produced many simple  
502 phenolic compounds with free hydroxyl groups capable to donor hydrogen atom to  
503 DPPH free radical. Many phenolic acids, such as gallic acids, (+)-catechins, myricetins,  
504 kaempferols, quercetins, 3,5-di-O-caffeoylquinic acids, 3,4-di-O-caffeoylquinic acids,  
505 4,5-di-O-caffeoylquinic acids have established potential antioxidant activity (Kumar and  
506 Goel, 2019) (Table 1). Kruk et al (2022) informed that the capability of phenolic  
507 compounds to donor hydrogen atom depends on chemical structure, number and  
508 position of hydroxyl groups attached to a benzene ring, a double bond between C2 and  
509 C3 rings and a carbonyl group (C=O) on the C ring at C4. The effectivity of antioxidant  
510 compounds donor hydrogen atom is determined by O-H bond dissociation energy.

511 The DPPH-free radical scavenging property observed in the study was not in  
512 consistent with the results of the study by Moraes-de-Souza et al. (2008). The research  
513 shows that total phenolic content of herbal infusion is low correlated with DPPH-free  
514 radical scavenging activity. However, Dobrinas et al. (2021) informed that total phenolic  
515 content of tea infusion is positively and significantly correlated with the free radical  
516 scavenging property. ~~inhibitor activity of DPPH. of tea infusion.~~

517

#### 518 Ferric Reducing Antioxidant Power (FRAP)

519 FRAP is an analysis of antioxidant power of the phytochemical compounds  
520 based on the reaction among antioxidant compounds, potassium ferricyanide,  
521 trichloroacetic acid, and ferric chloride to produce a color complex, that can be

Formatted: Centered

522 measured at  $\lambda$  700 nm (Fu et al., 2011; Al-Temimi and Choudhary, 2013). The principle  
523 of the assay measures that is based of the ability of antioxidant compounds to reduce  
524 iron ions of potassium ferrocyanide ( $\text{Fe}^{3+}$ ) to be potassium ferrocyanide ( $\text{Fe}^{2+}$ ).  
525 Potassium ferrocyanide reacts with ferric chloride to form a ferric-ferrous complex and  
526 results green color solution (Widyawati et al., 2017; Raharjo and Haryoto, 2019).

527 The results showed that the ferric reducing antioxidant power (FRAP) increased  
528 with at higher steeping temperature and longer storage timeperiod. The lowest FRAP  
529 was observed in the un-stored samples which was steeped at 60 °C at  $3.95 \pm 0.17$  mg  
530 gallic acid equivalents (GAE)/g samples, and the highest was owned exhibited by in  
531 *Pluchea* infusion which was stored for 5 years at 95 °C at  $48.63 \pm 10.83$  mg gallic acid  
532 equivalents (GAE)/g samples (Figure 2b). FRAP increased significantly as steeping  
533 temperature was increased. FRAP of the samples stored for 5 years was also  
534 significantly higher than the un-stored samples at  $\alpha \leq 0.05$ . Based on Pearson  
535 correlation, the FRAP of *Pluchea* infusion was strongly and positively significant  
536 correlated with the DPPH, TPC and TTC, but inversely to TFC. The correlated  
537 coefficient values (r) between FRAP and DPPH, TPC, TTC and TFC were 0.956, 0.953,  
538 0.948 and -0.826, respectively.

539 This case was is in contrast to with the study on the antioxidant activity of DPPH  
540 and FRAP on of matcha, because The the longer storage timeperiod reduces the levels  
541 of catechin content due to the catechins, such as epigallocatechin gallat (EGCG),  
542 epicatechin gallat (ECG), epigallocatechin (EGC), and epicatechin (EC) which are  
543 bioactive compounds that have high antioxidant activity (Kim et al. 2020), and also the  
544 case of the effect of temperature and storage time in betel (*Piper bettle* L.) extract. Light



545 and temperature influence degradation of phenolic compounds of betel that determine  
546 antioxidant activity. Different structure of phenolic compounds determines their stability  
547 to degrade accelerating of light and temperature. Hydroxychavicol is the best stability of  
548 phenolic compounds of betel compared with eugenol, isoeugenol and allyl pyrocatechol  
549 (Ali et al., 2018). Thanajiruschaya et al. (2010) revealed that the antioxidant activity of  
550 rice stored at high temperatures is greater than that stored at low temperatures. The  
551 ferric reducing capability of *Pluchea* could have due infusion corresponded to the  
552 presence of simple phenolic acid values that have the ability to transfer electron from  
553 their free hydroxyl groups of, presence of them in samples could accrue antioxidant  
554 activity because of ability of the electron transfer from free hydroxyl groups of phenolic  
555 acids. The FRAP of *Pluchea* infusion was strongly and positively significant correlated  
556 with the DPPH, TPC and TTC, but inversely to TFC.

Commented [A18]: Relate these with Figure 2b. Rewrite

## 557 ANTIDIABETIC ACTIVITY

558  $\alpha$ -Amylase enzyme inhibition activity (AA)

Formatted: Centered

559 Antidiabetic activity is a measure of the potency of phenolic compounds to  
560 regulate the uptake of glucose by the cells from the blood through the mediation of 2-  
561 digestive enzymes i.e.,  $\alpha$ -amylase and  $\alpha$ -glucosidase, which are involved the control of  
562 dietary carbohydrate digestion and release in the postprandial blood glucose in human  
563 body (Fu et al., 2017). The phenolic compounds have the capability to bind with the  
564 protein component of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Martinez-Solis et al.,  
565 2022) resulting in the reduced activity of the enzymes. The results showed, that the  
566 lower steeping *Pluchea* leaf infusion was able to inhibit the action of the  $\alpha$ -amylase  
567 enzymes (Figure 3a). The *Pluchea* infusion had very good activity, exhibited a good  $\alpha$ -

568 ~~mylase enzyme inhibition activity of more than 50 % and even almost 100 % for fresh in~~  
569 ~~the un-stored *Pluchea* infusion which steeped was brewed at 60, 70 and 80 °C with~~  
570 ~~highest at 60 °C, and in stored *Pluchea* leaf infusion which was steeped at 60 °C.~~  
571 ~~Whereas The stored fresh *Pluchea* leaf infusion steeped at 70, 80 and 95 °C for 5~~  
572 ~~minutes had lower enzyme inhibition activity an activity of of less than 50 % with lowest~~  
573 ~~at 95 °C inhibiting the  $\alpha$ -amylase enzyme of less than 50 %, which was equal to~~  
574 ~~40.08 $\pm$ 1.12 %. Widyawati et al. (2017) detected found that the ability to inhibit the  $\alpha$ -~~  
575 ~~amylase enzyme from in fresh un-stored *Pluchea* infusion steeped at 95 °C for 5~~  
576 ~~minutes by was also low at 28.79 %. Increasing the steeping temperature and storage~~  
577 ~~timeperiod reduced the ability to of the phytochemicals in the *Pluchea* infusions to inhibit~~  
578 ~~the  $\alpha$ -amylase enzyme activity. The results of the analysis based on a paired T test at  $\alpha$~~   
579  ~~$\leq$  0.05 showed, that the steeping temperature and storage timeperiod had a significant~~  
580 ~~effect on the ability to inhibit the  $\alpha$ -amylase enzyme. Based on Pearson correlation,~~  
581 ~~the Table 2 further shows that the AA of *Pluchea* infusion was strongly and negatively~~  
582 ~~significant correlated with TPC, TTC, DPPH and FRAP, but it was moderately and~~  
583 ~~negatively significant correlated with TFC. The correlated coefficient values (r) between~~  
584 ~~AA and TPC, TTC, DPPH, FRAP and TFC were -0.708, -0.857, -0.696, -0.806 and~~  
585 ~~0.429, respectively.~~

586 This inhibitory activity was thought to be contributed by other bioactive  
587 compounds, besides phenolics which are sensitive to steeping temperature and storage  
588 timeperiod. Li et al. (2018) stated that there are flavonoid compounds that contribute to  
589 the ability to inhibit the  $\alpha$ -amylase enzyme. Flavonoid compounds with a hydroxyl  
590 structure at C-4' in ring B are more effective than C-6 in ring A. Akah et al. (2011)

Corresponding Author: paini@ukwms.ac.id

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Formatted: Pattern: Clear (Yellow)

Commented [A19]: Why?/Explain

Commented [A20]: Implications?Explain

591 ~~informed-reported~~ that the phytochemical compounds, such as terpenoids, saponins,  
592 flavonoids, glycosides and carbohydrate, and alkaloids are good antidiabetic  
593 metabolites ~~or  $\alpha$ -amylase enzyme activity inhibitor~~. Sangeetha and Vedaşree (2012)  
594 explained, that the ability to inhibit the  $\alpha$ -amylase enzyme was determined by the  
595 content of the phenolic compound and protein. The  $\alpha$ -amylase inhibitor enzyme present  
596 in *Pluchea* infusion may be proteinaceous in nature. Aleixandre et al. (2022) informed  
597 that phenolic acids have inhibition activity to  $\alpha$ -amylase enzyme depending their  
598 structures. Besides that, capability of phenolic acids to inhibit  $\alpha$ -amylase enzyme was  
599 determined by low half-maximum inhibitory concentration ( $IC_{50}$ ). There are C=C double  
600 bond conjugated with a carbonyl group of phenolic structures that stabilizes the binding  
601 forces to the active site of the  $\alpha$ -amylase. The hydroxyl groups of them are able to bind  
602 by non-covalent interaction, such as hydrogen binding, cation- $\pi$  interactions, salt bridge  
603 interactions, ionic interactions or electrostatic forces with amino acid residue at the  
604 active site in  $\alpha$ -amylase enzyme. ~~Elevated steeping temperature and longer storage~~  
605 ~~period. The steeping temperature and storage time can easily cause the removal of the~~  
606 ~~e~~ hydroxyl groups of phenolic compounds that can reduce their ability of enzyme  
607 inhibition. The phenolic acids with a greater number of hydroxyl groups ~~are-exhibits~~  
608 stronger capability to obstruct the  $\alpha$ -amylase enzyme.

#### $\alpha$ -Glucosidase enzyme inhibition activity (GA)

610 ~~Alpha~~-glucosidase is an important enzyme in carbohydrates digestion, that  
611 catalysis the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and  
612 converts them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et  
613 al., 2014; Proenca et al., 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -

**Commented [A21]:** What content or what is in the content the influenced the ability to inhibit the enzyme?

**Commented [A22]:** How will this affect the ability to inhibit the enzyme?

**Commented [A23]:** Lines 585 to 595, Either delete or rewrite for better readability and understanding referring to enzyme activity inhibition

**Commented [A24]:** This part is disorganized. Avoid duplicating statements, observation facts etc

**Formatted:** Centered

614 glucosidase enzyme is used to determine ~~their~~ antidiabetics activity. ~~This is supported~~  
615 ~~by~~ Werdani and Widyawati (2018) ~~stated~~; that **Pluchea infusion** has the potential as an  
616 antidiabetic agent. Widyawati et al. (2020) found that brewing fresh **Pluchea infusion** at  
617 95 °C for 5 minutes has an inhibitory effect on the α-glucosidase enzyme of 67.857 %.

618 ~~The results showed~~; **Figure 3b shows** that the ability of the **Pluchea leaf infusion**  
619 to inhibit the α-glucosidase enzyme decreased with increasing steeping temperature  
620 and storage ~~timeperiod~~. Steeping at 95 °C ~~for fresh~~ of the ~~un-stored~~ **Pluchea leaf**  
621 ~~infusion (un-stored)~~ obtained the lowest inhibitory ability, i.e.,  $48.32 \pm 1.27$  %, and the  
622 highest inhibitory activity was found at 70 °C ~~steeping temperature for fresh~~ **Pluchea**

623 ~~infusion, which was at~~  $95.11 \pm 0.70\%$ . ~~(Figure 3b)~~. The results of **a paired T test showed**  
624 **that GA of Pluchea infusion** was significantly different ~~at both~~ ~~between~~ steeping  
625 temperature and long storage. ~~The antidiabetic activity of Pluchea infusion~~ **Figure 3**

626 ~~further showed~~ ~~shows~~ that the ability of ~~Pulchea leaf infusion~~ to inhibit the **α-**  
627 **glucosidase enzyme** tended to be higher than the ability to inhibit the **α-amylase**  
628 **enzyme**. Li et al. (2018) informed that flavonoid compounds have the ability to inhibit the

629 action of the α-amylase and α-glucosidase enzymes. This is due to the total flavonoids  
630 in steeped **Pluchea** infusion which tended to have the same pattern as the ability to  
631 inhibit the activity of the α-amylase and α-glucosidase enzymes. The statistical analysis

632 using Pearson correlation showed that GA of **Pluchea** infusion was strongly and  
633 negatively correlated with TPC, TTC, DPPH and FRAP

634 ~~, with r was -0.555, -0.715, -0.527 and -0.560, respectively~~. However, GA was  
635 moderately and positively correlated to TFC, ~~with r was 0.350 and strongly and~~  
636 positively correlated to AA, ~~with r was 0.725~~. Flavonoid compounds, such as rutin,

Formatted: Font: Not Italic

Commented [A25]: Explain

Formatted: Font: Italic

Commented [A26]: This does not explain explain lines 597-599 with the manner it is written. Include statements that connect the explanation with the observation. Having 'same pattern' is not observed in the figure/graph

Commented [A27]: Interpret/Implications

637 myricetin, kaempferol, and quercetin ~~which~~ have antioxidant and antihyperglycemic  
638 activities. The ability to inhibit the action of enzymes from flavonoid compounds is  
639 determined by the position and number of hydroxyl groups and the number of double  
640 bonds in rings A and B and the heterocyclic ring in ring C. The ability to inhibit the  $\alpha$ -  
641 glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping  
642 temperature and long storage. The capability of *Pluchea* infusion to obstruct the  $\alpha$ -  
643 glucosidase enzyme was greater than the  $\alpha$ -amylase enzyme because the mechanism  
644 of two enzymes was different, according to the opinion of McCue et al. (2005).  
645 Widyawati et al. (2017) informed that phenolic and non-phenolic compounds determine  
646 the inhibitory activity of the  $\alpha$ -glucosidase enzyme. The ability of bound phenolic  
647 compounds to inhibit  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds.  
648 The presence of polymerization and degradation reactions, that may be occurred in  
649 *Pluchea* infusion during storage, affects the structure and profile of phenolic and non-  
650 phenolic compounds. Asriningtyas et al. (2014) claimed that *Pluchea* leaves contain  
651 3,5-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid methyl ester, 3,4,5-tri-O-  
652 caffeoylquinic acid methyl ester, 3,4,5-tri-O-caffeoylquinic acid, and 1,3,4,5-tetra-O-  
653 caffeoylquinic acid. Quinic acid is methyl esterified with the number of caffeic groups in  
654 the molecule that determines the activity of inhibiting the  $\alpha$ -glucosidase enzyme.  
655 Analysis of caffeoylquinic acids in *Pluchea* infusion was obtained that the higher  
656 steeping temperature and long storage caused increased concentration of them, but  
657 the  $\alpha$ -glucosidase inhibition activity of them was reduced. Aleixandre et al. (2022)  
658 reported that the simple phenolic acids forming a dipole-dipole interaction of active site  
659 from  $\alpha$ -glucosidase enzyme are effectively inhibiting the enzyme.

**Commented [A28]:** Delete literature citations that are unnecessary to explain the findings

**Commented [A29]:** Unnecessary because this is not included as one of the derived simple phenolic acids

660 This study ~~was obtained information~~ showed that the increasing of steeping  
661 temperature and storage ~~time~~ period caused a degradation reaction of polyphenol  
662 compounds to produce simple phenolic compounds, such as gallic acid, (+)-catechin,  
663 myricetin, quercetin, kaempferol, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic  
664 acid, and 4,5-di-O-caffeoylquinic acid, supported the results of total phenolic content  
665 and total tannin content assays. Increased concentration of simple phenolic compounds  
666 determined the ability of these compounds as antioxidant agents, but reduced their  
667 capability as antidiabetic agents.

## 669 CONCLUSION

670 The steeping temperature and storage ~~time~~ period of *Pluchea* infusion  
671 significantly influenced bioactive contents, antioxidant and antidiabetic activities. TPC,  
672 TTC, and TFC were significantly different at various steeping temperature and storage  
673 period based on statistical analysis using a paired ~~T~~ t-test at  $\alpha \leq 0.05$ . ~~There was the~~  
674 ~~difference of t~~ The phenolic compound profile in ~~fresh~~ the un-stored and stored of  
675 *Pluchea* infusion ~~and at~~ various steeping temperature. ~~The included~~ simple phenolic  
676 compounds ~~were detected in Pluchea infusion including~~ such as gallic acid, (+)-catechin,  
677 quercetin, myricetin, kaempferol, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic  
678 acid, and 4,5-di-O-caffeoylquinic acid. The results of statistical analysis using a paired ~~T~~  
679 ~~t~~-test at  $\alpha \leq 0.05$  showed that gallic acid and kaempferol of *Pluchea* infusion were  
680 insignificantly different at various steeping temperature and long storage. ~~Nevertheless,~~  
681 ~~The~~ concentration of quercetin and 3,5-dicaffeoylquinic acid of *Pluchea* infusion was  
682 significantly different of two treatments except at 70 °C. The (+)-catechin concentration

Corresponding Author: paini@ukwms.ac.id

Commented [A30]: Not clear, re-write

Commented [A31]: Organize the discussion to explain the observation one at a period. ex:

1) 'Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme decreased with increasing steeping temperature and storage period.' (This must be followed by an explanation or support.)

2) However, GA was moderately and positively correlated to TFC and positively correlated to AA..(This must be followed by implications/support/explanation.)

The ability to inhibit the  $\alpha$ -glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping temperature and long storage.(This can be integrated in 1)

4) The capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater than the  $\alpha$ -amylase enzyme because the mechanism of two enzymes was different, according to the opinion of McCue et al. (2005). (This can be integrated in 1)

The mechanism must be explained -:the mechanism of two enzymes was different,

5) Flavonoid compounds, such as rutin, myricetin, kaempferol, and quercetin which have antioxidant and antihyperglycemic activities

6) . Li et al. (2018) informed that flavonoid compounds have the ability to inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. ( May also be integrated in 1)

7) Widyawati et al. (2017) informed that phenolic and non-phenolic compounds determine the inhibitory activity of the  $\alpha$ -glucosidase enzyme. The ability of bound phenolic compounds to inhibit  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. (May also be incorporated in 1).

8) Lines 618 to 629 into 1)

Commented [A32]: Reconcile with your discussion

Commented [A33]: Suggested conclusion

## CONCLUSION

The total phenolic content (TPC) of *Pluchea* infusion at different steeping temperature and storage period generally significantly increased with increasing steeping temperature and storage period. Steeped and stored infusion had significantly higher amounts of phenolic compounds than the samples that were steeped and un-stored. TPC was highest in the stored and steeped at 95 °C and lowest in the un-stored and steeped at 60 °C. Un-stored steeped samples exhibited significantly higher flavonoid content than tr

683 of *Pluchea* infusion was significantly different at 95 °C, but the myricetin was different  
684 concentration at 80 and 95 °C. The 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic  
685 acid compounds from *Pluchea* infusion were significantly different at 60 °C, however the  
686 concentration of 3,4-dicaffeoylquinic acid was also significantly different at 80 and 95  
687 °C. TPC, TTC and TFC of *Pluchea* infusion were significantly different at various  
688 steeping temperature and storage period. TPC and TTC significantly increased with  
689 increasing steeping temperature and long storage, but TFC significantly increased at  
690 various steeping temperature and significantly decreased at long storage. The bioactive  
691 compounds of *Pluchea* infusion influenced antioxidant activities (DPPH and FRAP) and  
692 antidiabetic activity (AA and GA). The DPPH was strongly and positively correlated with  
693 TPC and TTC, but it was strongly and negatively correlated with TFC, with coefficient  $r$   
694 0.993, 0.942, and -0.940, respectively. The correlated pattern between FRAP and  
695 bioactive contents of *Pluchea* infusion was similar to it between DPPH and bioactive  
696 contents. The correlated coefficient values ( $r$ ) between FRAP and TPC, TTC and TFC  
697 were 0.953, 0.948 and -0.826, respectively. The AA and GA were strongly and  
698 negatively correlated with TPC, TTC, DPPH and FRAP, but it was moderately and  
699 negatively significant correlated with TFC. Between the antioxidant activity of DPPH and  
700 FRAP and the antidiabetic activity of AA and GA of *Pluchea* infusion were strongly and  
701 positively correlated with correlation coefficient ( $r$ ) values of 0.956 and 0.725,  
702 respectively.

703

#### 704 DATA AVAILABILITY

705 Table and figure used to support of this study were included in the article.

Corresponding Author: paini@ukwms.ac.id

706

#### 707 CONFLICT OF INTEREST

708 The authors declare no conflict of interest.

709

#### 710 ACKNOWLEDGEMENTS

711 The authors would like to thank the he Ministry of Education and Culture of the Republic  
712 of Indonesia for fundamental research grant to higher education institutions in 2022

713

#### 714 REFERENCES

715 ACAR A, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with  
716 food wastes:evaluation of temperature and time effects on quality parameters. Applied  
717 Food Res. 2(100087):1-8.

718 ALEIXANDRE A et al. 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  
719  $\alpha$ -glucosidase and influence of reaction conditions. Food Chem. 372(131231):1-9.

720 ALI et al. 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile.  
721 Food Chem. 89(2005):27-36.

722 ALI A. et al. 2018. Impact of storage conditions on the stability of predominant phenolic  
723 constituents and antioxidant activity of dried *piper bettle* extracts. Molecules.23(24):1-  
724 13.

725 AL-TEMIMI A, CHOUDHARY R. 2013. Determination of antioxidant activity in different  
726 kinds of plants *in vivo* and *in vitro* by using diverse technical methods. J Nut. Food Sci.  
727 3(1):1-6.

**Commented [A34]:** Check for authors that may had been deleted in the paper due to revisions done.



728 AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and  
729 methanol extract and fractions of *Gongronema latifolium* (Asclepidaceae) leaves in  
730 alloxan diabetic rats. J Applied Pharm. Sci. 01(09): 99-102.

731 ARSININGTYAS IS. et al. 2014. Identification of  $\alpha$ -glucosidase inhibitors from the  
732 leaves of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural  
733 product use. Natural Product Res. 28(17):1350-1353.

734 ARYAL S et al. 2019. Total phenolic content, flavonoid content and antioxidant of wild  
735 vegetables from Western Nepal. Plants. 8(96): 1-12.

736 AYELE DT, AKELE ML, MELESE A.T. 2022. Analysis of total phenolic contents,  
737 flavonoids, antioxidant and antibacterial activities of *Croton macrostachyus* root extracts.  
738 BMC Chem. 16(30): 1-9.

739 BALIYAN S et al. 2022. Determination of antioxidants by DPPH radical scavenging  
740 activity and quantitative phytochemical analysis of *Ficus religiosa*. Molecules.  
741 27(1326): 1-19.

742 CASTIGLIONI S et al. 2015. Influence of steeping conditions (time, temperature, and  
743 particle size) on antioxidant properties and sensory attributes of some white and green  
744 teas. Intern. J. Food Sci. Nut. Online 1-7.

745 Chan EWC et al. 2022. *Pluchea indica*: An updated review of its botany, uses, bioactive  
746 compounds and pharmacological properties. Pharmaceutical Sciences Asia. 49(1): 77-  
747 85.

748 CHANDRA S et al. 2014. Assessment of total phenolic and flavonoid content,  
749 antioxidant properties, and yield of aeroponically and conventionally grown leafy

Corresponding Author: paini@ukwms.ac.id

750 vegetables and fruit crops: A Comparative study. Hindawi Publishing Corporation  
751 Evidence-Based Complementary Alter. Med. 2014:1-9 (2014)

752 CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic,  
753 flavonoids, tannin and chlorophyll content of leaves of *Strobilanthes Kunthiana*  
754 (*Neelakurinji*). J.Med. Plants Studies. 4(4):282-286.

755 DOBRINAS S, SOCEANU A, POPESCU V, POPOVICI IC, JITARIU D. 2021.  
756 Relationship between total phenolic content, antioxidant capacity, Fe and Cu content  
757 from tea plant samples at different brewing times. Processes. 9(1311):1-11.c

758 FERRARIS Z. 2023. *Pluchea indica* Less.iNaturalist.org.  
759 <https://www.gbif.org/occurrence/4022273660>.

760 FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional  
761 properties and visual appearance of Dampit Robusta coffee leaves tea. Advances in  
762 Food Sci. Sustain. Agric. Agro-industrial Engineering. 4(1):25-32.

763 FU L et al. 2017. Total phenolic contents and antioxidant capacities of herbal and tea  
764 infusions. Intern. J. Molecular Sci. 12:2112-2124.

765 GAO MR et al. 2019. A theoretical and experimental study: the influence of different  
766 standards on the determination of total phenol content in the Folin–Ciocalteu assay. J.  
767 Food Measurement and Characteristic. 13:1349–1356.

768 HUANG D, OU B, PRIOR RL. 2005. The Chemistry behind antioxidant capacity assays.  
769 J. Agric. Food Chem. 53:1841-1856.

770 ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties  
771 of rosehip tea beverage: study by response surface methodology. J. Food Sci. Tech.  
772 54(11):3737–3743.

Corresponding Author: paini@ukwms.ac.id

773 JAYANI NIE et al. 2022. Evaluation of stability and quality characteristics of moringa  
774 (*Moringa oleifera*) herbal tea during storage. Food Res. 6(4):399-406.

775 KIM JM et al. 2020. Effect of storage temperature on the antioxidant activity and  
776 catechins stability of Matcha (*Camellia sinensis*). Food Science and Biotechnology.  
777 29(9):1261–1271.

778 KILIC et al. 2017. Antioxidant properties of some herbal teas (green tea, senna, corn  
779 silk, rosmery) brewed at different temperatures. Intern. J. Secondary Metabolite.  
780 4(3):142-248 Special Issue 1.

781 KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of  
782 extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica*  
783 leaves from different provenances in Thailand. Revista Brasileira de Farmacognosia.  
784 28(2018): 145–150.

785 KOWALSKA J et al. 2021. Influence of tea brewing parameters on the antioxidant  
786 potential of infusions and extracts depending on the degree of processing of the leaves  
787 of *Camellia sinensis*. Molecules.26(4773):1-17.

788 KRUK J, ABOUL-ENAIN BH, DUCHNIK E, MARCHLEWICZ M. 2022. Antioxidative  
789 properties of phenolic compounds and their effect on oxidative stress induced by severe  
790 physical exercise. J. Physiol. Sci. 72(19):1-24.

791 KUMAR N, GOEL N. 2019. Phenolic acids: natural versatile molecules with promising  
792 therapeutic applications. Biotechnology Reports. 24(e00370):1-10.

793 LI K et al. 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the  
794 flavonoids rich extract from *Scutellaria baicalensis* shoots and interpretation of structure

Corresponding Author: paini@ukwms.ac.id

795 activity relationship of its eight flavonoids by a refined assign score method. Chem.  
796 Central J. 12(82):1-11.

797 LIN YS. et al. 2020. Effects of storage time and temperature on antioxidants in juice  
798 from *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser.  
799 *Molecules*. 25(3614): 1-12.

800 MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content  
801 and antioxidant activity test of avocado seed extract (*Persea Americana* Mill). Jurnal  
802 MIPA Unsrat Online. 1(1): 5-10. [https://media.neliti.com/media/publications/116214-ID-](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf)  
803 [penentuan-kandungan-tanin-dan-uji-aktivi.pdf](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf) [In Bahasa Indonesia]

804 MARTINEZ-SOLIS J et al. 2022. Antidiabetic and toxicological effect of the tea infusion  
805 of summer collection from *Annona cherimola* Miller leaves. *Plants*. 11(3224):1-17.

806 MATHUR R, VIJAYVERGIA R. 2017. Determination of total flavonoid and phenol  
807 content in *Mimusops Elengi* Linn. *Intern. J. Pharm. Sci. Res.* 8(12): 5282-5285.

808 MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of  
809 sprouted and solid-state bioprocessed soybean. *Asia Pacific J. Clinical Nut.* 14(2): 145-  
810 152.

811 MORAES-DE-SOUZA RA, OLDONI TLC, REGITANO-D'ARCE MAB, ALENCAR SM.  
812 2008. Antioxidant activity and phenolic composition of herbal infusions consumed in  
813 Brazil. *Cienc Tecnol. Aliment.* 6(1):41-47.

814 MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining  
815 antioxidant activity: A Review. *Intern. J. Molecular Sci.* 22(3380):1-30.

Corresponding Author: paini@ukwms.ac.id

816 NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus*  
817 *sabdariffa* L.): Effect of dying temperature and brewing conditions on total soluble solid,  
818 phenolic content, antioxidant capacity and sensory quality. *Beverages*.6(2):1-11 (2020).  
819 NOREEN H. et al. 2017. Measurement of total phenolic content and antioxidant activity  
820 of aerial parts of medicinal plant *Coronopus didymus*. *Asian Pacific J. Tropical Med.*  
821 10(8): 792–801.  
822 NURCHOLIS GW et al. 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity  
823 in-vitro and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum*  
824 *pictum* (L.). *Current Biochem.* 1(2): 58-65.  
825 OH J et al. 2013. Antioxidant and antimicrobial activities of various leafy herbal teas.  
826 *Food Control.* 31(2013):403-409.  
827 PROENCA C et al. 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an in vitro and in silico  
828 structure–activity relationship study. *J. Enzyme Inhibition Med. Chem.* 32(1): 1216–  
829 1228.  
830 PUREWAL SS et al. 2022. Unraveling the effect of storage duration on antioxidant  
831 properties, physicochemical and sensorial parameters of ready to serve Kinnow-aml  
832 beverages. *Applied Food Res.* 2(1): 1-8.  
833 RAHARJO D, HARYOTO. 2019. Antioxidant activity of mangrove *Sonneratia caseolaris*  
834 L. using the FRAP method. *ISETH.* 2019: 623-629.  
835 RAMPHINWA ML et al. 2023. Eco-physiological response of secondary metabolites of  
836 teas: review of quality attributes of herbal tea. *Frontiers in Sustain. Food Systems.*  
837 7(990334):1-14.

Corresponding Author: paini@ukwms.ac.id

838 REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids.  
839 Czech J. Food Sci. 30(2):171-177.

840 RUAN J et al. 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea*  
841 *indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel  
842 column chromatography fractions. Molecules. 24(2784):1-20.

843 RUSITA YD et al. 2019. Comparison of tannin concentration on fresh and dried tea leaf  
844 (*Camelia sinensis* L.) extract using UV-visible spectrophotometric method. 1st  
845 International Conference of Health, Science & Technology (ICOHETECH) 2019 E-ISBN:  
846 978-623-92207-0-9. November 16<sup>th</sup>, 2019.LPPM Universitas Duta Bangsa Surakarta,  
847 Surakarta.

848 SANGEETHA R, VEDASREE N. 2012. In Vitro  $\alpha$ -Amylase inhibitory activity of the  
849 leaves of *Thespesia populnea*. Intern. Scholarly Res.Network. 2012: 1-4 (2012)

850 SHRAIM AM et al. 2021. Determination of total flavonoid content by aluminum chloride  
851 assay: A critical evaluation. LWT Food Sci. Tech. 150(111932):1-11.

852 SILVA-RAMIREZ KA et al. 2020. Effect of drying and steeping temperature on the  
853 phenolic content, antioxidant activity, aromatic compounds and sensory properties of  
854 *Culila polyantha* Benth. Infusions. Processes. 8(1378):2-20.

855 SRISOOK K et al. 2012. Antioxidant and anti-inflammatory activities of hot water extract  
856 from *Pluchea indica* Less. herbal tea. J. Med. Plants Res. 6(23):4077-4081.

857 SURIYAPHAN O. 2014. Nutrition, health benefits and applications of *Pluchea indica* (L)  
858 Less leaves. Pharm. Sci. Asia. 41:1–10.

Corresponding Author: paini@ukwms.ac.id

859 SU D et al. 2019. Impact of thermal processing and storage temperature on phenolic  
860 profile and antioxidant activity of different varieties of lychee juice. LWT-Food Tech.  
861 116(108578):1-9.

862 THANAJIRUSCHAYA P et al. 2010. Effect of storage ~~time~~ period and temperature on  
863 antioxidant components and properties of milled rice. Khon Kaen Univ. Res.  
864 J.15(9):843-851.

865 VONGSAK B et al. 2018. Comparison of active constituents, antioxidant capacity, and  
866  $\alpha$ glucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. Food  
867 Biosci. 25: 68–73.

868 WANG SC. et al. 2022. Effect of brewing conditions on polyphenols in the dark tea  
869 (*Camellia sinensis* L.) infusions: content, composition and antioxidant activities. Food  
870 Sci.Tech. 42(e36322):1-9.

871 WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica*  
872 Less as functional beverage in diabetic patients. ASSEHR. 98:164-167.

873 WIDYAWATI PS et al. 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract  
874 as antioxidant and antiwarmed over flavor (WOF) of duck meat. International  
875 Congress on Challenges of Biotechnological Research in Food and Health. November  
876 15<sup>th</sup> 2014. Agricultural Technology Faculty, Slamet Riyadi University, Surakarta.

877 WIDYAWATI PS et al. 2016. The physicochemical and antioxidant properties of  
878 *Pluchea indica* Less drink in tea bag packaging. IJFNS. 5: 13–120.

879 WIDYAWATI PS et al. 2017. In vitro antioxidant capacities and antidiabetic properties of  
880 pluchea leaves. IJPPS. 9(8): 203-208.

Corresponding Author: paini@ukwms.ac.id

881 WIDYAWATI PS et al. 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica*  
882 Less beverage. CJFST.12(4): 125-139.

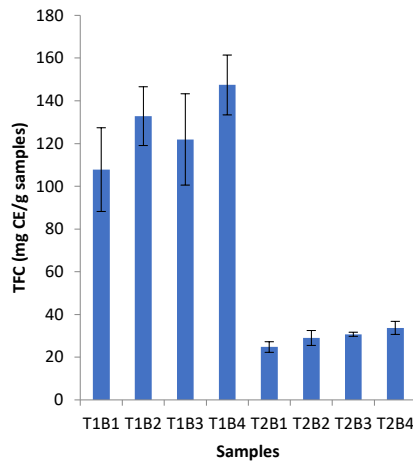
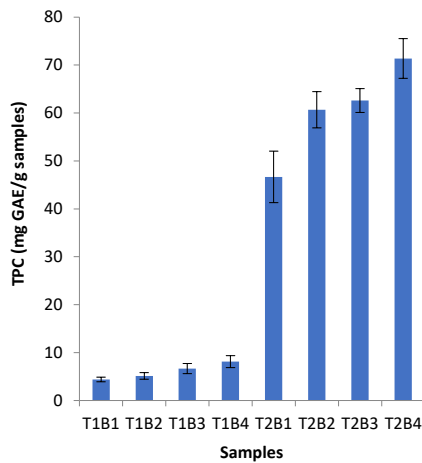
883 WIDYAWATI PS ET AL. 2022. The effect of κ-carrageenan proportion and hot water  
884 extract of the *P luche* *indica* Less leaf tea on the quality and sensory properties of  
885 stink lily (*Amorphophallus muelleri*) wet noodles. Molecules. 27(5062):1-16.

886 XU P, CHEN L, WANG Y. 2019. Effect of storage ~~time~~period on antioxidant activity and  
887 inhibition on α-amylase and α-glucosidase of white tea. Food Sci. Nut. 7(2): 636-644.

888 ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction ~~time~~period on  
889 caffeine in coffee. Lantanida J. 6(2):103-202. [https://jurnal.ar-](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811)  
890 [raniry.ac.id/index.php/lantanida/article/view/3811](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811). [In Bahasa Indonesia]

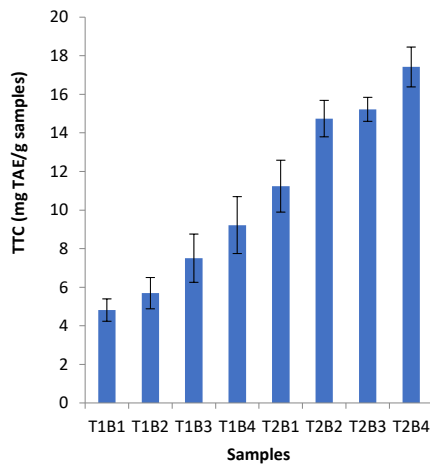
891 ZHANG Y et al. 2021. Effect of storage conditions and ~~time~~period on the polyphenol  
892 content of wheat flours. Processes. 9(248):1-11.





(a)

(b)



(c)

Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping temperature and storage time period (a) Total phenolic content (b) Total flavonoid content (c) Total tannin content. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-

Formatted: Left, Indent: Left: 0.5", Hanging: 0.69", Line spacing: single

902  
903  
904  
905  
906  
907  
908

stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$ .

Commented [A35]:

Table 1. Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperature and storage time period

Phenolic Compounds	Steeping Temperature (°C)	Mean±SD Un-stored	Mean±SD Stored	Mean difference ±SD	Sig (2-tailed)
Gallic Acid (µg/g samples)	60	0.2132±0.0027	0.2364±0.0015	0.0375±0.0175	0.2030
	70	0.2157±0.0013	0.2324±0.0214	0.0167±0.0227	0.4870
	80	0.2234±0.0122	0.2347±0.0078	0.0386±0.0264	0.2870
	95	0.2316±0.0104	0.2402±0.0169	0.0086±0.1990	0.8500
(+) -Catechin (µg/g samples)	60	0.3425±0.0110	0.5085±0.0111	-0.1576±0.0885	0.241
	70	0.3260±0.0265	0.5448±0.0006	-0.2188±0.0259	0.053
	80	0.3240±0.0222	0.5023±0.0773	-0.1451±0.0248	0.077
	95	0.4039±0.0320	0.5995±0.0372	-0.2049±0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756±0.1234	1.4762±0.0271	-1.2887±0.3222	0.111
	70	0.2587±0.0160	1.4245±0.2526	-1.1657±0.2695	0.103
	80	0.4175±0.0104	1.4570±0.0925	-1.0391±0.0841	0.036*
	95	0.8786±0.0434	2.6138±0.0695	-1.735±0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220±0.0268	0.6220±0.0706	-0.5999±0.9733	0.544
	70	0.1530±0.0511	1.0708±0.0289	-0.9177±0.0222	0.011*
	80	0.3666±0.0103	0.8629±0.0815	-0.1082±0.4462	0.790
	95	0.6559±0.0570	2.0230±0.0573	-1.4123±0.3203	0.101
Kaempferol (µg/g samples)	60	0.1394±0.0202	0.3675±0.0183	-0.3207±0.1122	0.154
	70	0.0514±0.0037	0.3726±0.0944	0.3213±0.0907	0.125
	80	0.3699±0.0924	0.7966±0.0366	-0.4267±0.2727	0.271
	95	0.5913±0.0239	0.9478±0.0287	-0.3565±0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103±0.0628	2.4863±0.0270	-1.8760±0.2074	0.050*
	70	0.6271±0.0099	2.3403±0.0325	-1.7131±0.3152	0.082
	80	0.7967±0.03060	2.6278±0.0211	-1.8311±0.0095	0.002*
	95	1.5386±0.0668	4.0211±0.0851	-2.4825±0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635±0.0628	0.9449±0.0501	-0.2814±0.4458	0.536
	70	0.6162±0.0099	0.9485±0.0794	-0.3323±0.0301	0.041*
	80	0.6601±0.0306	0.9099±0.0387	-0.2498±0.3127	0.461
	95	0.6642±0.0668	1.3156±0.0166	-0.6514±0.2666	0.179

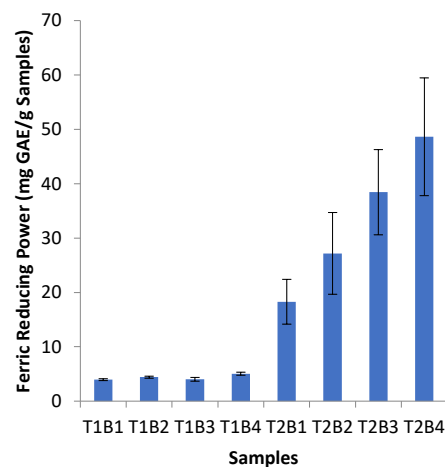
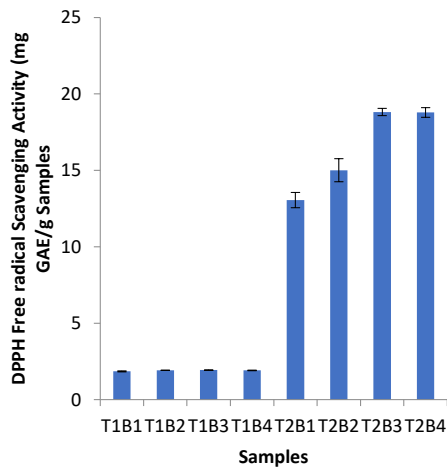
4,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.4906±0.0060	1.1842±0.0120	-0.6886±0.2723	0.018*
	70	0.4807±0.0034	1.0089±0.0736	-0.5281±0.0702	0.060
	80	0.5299±0.0053	1.2382±0.1435	-0.7082±0.1489	0.094
	95	1.0018±0.0526	1.3797±0.2170	-0.3086±0.3086	0.333

910 Note : Data were expressed as mean ±standard deviation (n=2). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-  
 911 steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60  
 912 °C, stored for 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-  
 913 steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping  
 914 temperature, calculated using a paired T test at  $\alpha \leq 0.05$ . \*  $\alpha \leq 0.05$ .  
 915

Formatted: Line spacing: single

Commented [A36]: Improve

916



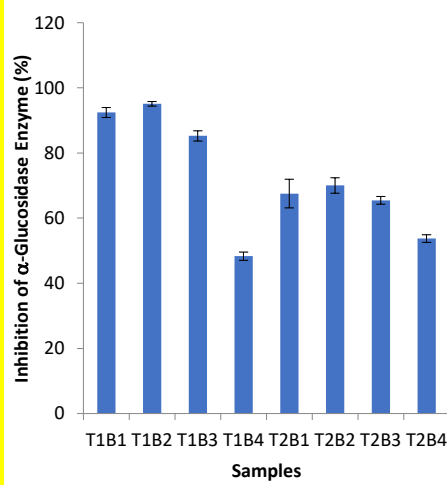
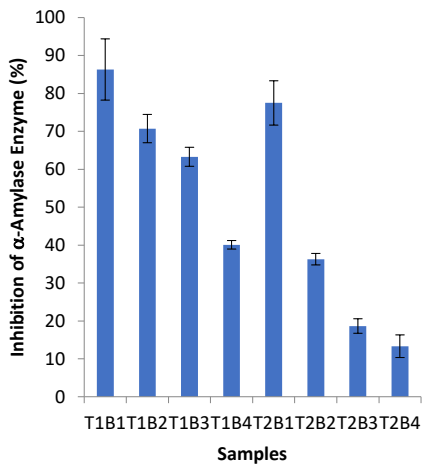
(a)

(b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage ~~time period~~ (a) DPPH (b) FRAP. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T2B2-steeped at 70 °C, stored for 5 years; T2B3-steeped at 80 °C, stored for 5 years; T2B4-steeped at 95 °C, stored for 5 years. ~~Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$~~

Formatted: Justified, Indent: Left: 0.5", Hanging: 0.69", Line spacing: single

Commented [A37]: Improve



(a)

(b)

**Figure 3.** Antidiabetic activity of pluchea tea at different steeping temperature and storage ~~time period~~ (a)  $\alpha$ -amylase (b)  $\alpha$ -glucosidase. Data were expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T2B2-steeped at 70 °C, stored for 5 years; T2B3-steeped at 80 °C, stored for 5 years; T2B4-steeped at 95 °C, stored for 5 years. Within group differences at unstored vs stored for 5 years at certain steeping temperature, calculated using a paired T test at  $\alpha \leq 0.05$

**Formatted:** Indent: Left: 0.44", Hanging: 0.75"

**Formatted:** Justified, Indent: Left: 0.44", Hanging: 0.75", Line spacing: single, Tab stops: 1.19", Left

**Commented [A38]:** Improve

942  
943

Table 2. Pearson correlation coefficients between bioactive contents (TPC, TFC and TAC), antioxidant activity (DPPH and FRAP) and antidiabetic activity (AA and GA)\*

Formatted: Space After: 0 pt, Line spacing: single

	TPC	TFC	TTC	DPPH	FRAP	Alpha Glucosidase	Alpha Amylase
TPC	1						
TFC	-0.93589	1					
TTC	0.960028	-0.81321	1				
DPPH	0.992776	-0.93992	0.942273	1			
FRAP	0.953366	-0.82636	0.947778	0.956242	1		
Alpha Glucosidase	-0.55512	0.349873	-0.71534	-0.5272	-0.55947	1	
Alpha Amylase	-0.70842	0.429393	-0.8569	-0.69579	-0.80548	0.725161631	1

944  
945

Note: \*Correlation Ssignificant at the 0.05 level (2-tailed)

## Response of reviewer's comments

PJS paper Ms 23-158

No	Line	Reviewer's Comments	Response
1	Line 1	Delete time and insert period	Time has been deleted and period has been inserted
2	Line 10	Delete time and insert period	Time has been deleted and period has been inserted
3	Line 23	Delete time and insert period	Time has been deleted and period has been inserted
4	Line 24	Delete . (dot)	. (dot) has been deleted
5	Line 25	Delete variety of the and insert <i>Pluchea</i> leaf blades were exposed to 4	Variety of the has been deleted and <i>Pluchea</i> leaf blades were exposed to 4 has been inserted
6	Line 25	Delete time and insert period	Time has been deleted and period has been inserted
7	Line 26	Delete includes, insert of and give space of °C	Includes has been deleted, of inserted and °C has been spaced
8	Line 26	Delete (	( has been deleted
9	Line 26	Delete )	) has been deleted
11	Line 27	Delete time and insert period	Time has been deleted and period has been inserted
12	Line 27	Delete (	( has been deleted
13	Line 27	Delete )	) has been deleted
14	Line 27	Delete the research resulted and insert resulting in	The research resulted has been deleted and inserted resulting in
15	Line 28	Delete T	T has been deleted and t has been inserted
16	Line 29	Delete influenced and insert affected	Influenced has been deleted and affected has been inserted
17	Line 29	Delete ) and (	) has been deleted and inserted [ and ]
18	Line 31		Potential has been inserted
19	Line 32	Delete inhibitors, activities and samples	Inhibitors, activities, and samples ...have been deleted
20	Line 32-40	Insert properties, the <i>pluchea</i> leaf infusion, TPC decreased during	Properties, the <i>pluchea</i> leaf infusion, TPC decreased during storage period but significantly



		storage period but significantly increased at higher steeping temperature	increased at higher steeping temperature ....have been inserted
21	Line 34-36	Move the AA and GA of pluchea infusion increased until 70°C of the steeping temperature, but decreased until 95°C.	The statement was changed to be ..... TPC, TTC, DPPH, and FRAP significantly increased for the storage period and the steeping temperatures. Then, TFC decreased during the storage period but significantly increased at higher steeping temperatures. The AA and GA of pluchea leaf infusion increased until 70°C of the steeping temperature, but decreased until 95°C. The DPPH and FRAP of the pluchea leaf infusion were strongly and positively correlated with TPC and TTC. The GA and AA of pluchea leaf infusion were not influenced by the TPC and TTC but were weakly and positively correlated with TFC. The antioxidant activity of the pluchea leaf infusion was inversely proportional to the antidiabetic activity.
22	Line 33-34	Comment : describe treatment effects on total phenolics, tannins, antioxidant, antidiabetic in one brief sentence each and indicate statistical significance	
21	Line 39	Delete between and change t to be T Comment state briefly results of the correlation analysis.	
22	Line 40-42	Delete the treatments gave different effect of and Insert derived from pluchea leaf infusion at different sleeping temperatures and storage included	The treatments gave different effect of ... has been deleted and derived from pluchea leaf infusion at different sleeping temperatures and storage included has been inserted
23	Line 49	Delete n	n has been deleted
24	Line 54		Change is to be are
25	Line 60	Delete infusion by and insert in	Infusion by has been deleted and in has been inserted
26	Line 61	Delete n area	n area has been deleted
27	Line 62	Delete with brewing of and insert by steeping 2 g of	Brewing of has been deleted and by steeping 2 g of ... has been inserted
28	Line 62	Delete by and insert in 100 mL of	By has been deleted and in has been inserted

29	Line 63	Delete water and each tea bag contained 2 g of pluchea leaf powder is steeped with 100 mL hot water or boiling water.	Water and each tea bag contained 2 g of pluchea leaf powder is steeped with 100 mL hot water or boiling water. ....has been deleted
30	Line 64	Delete results and content and insert rexhibits.	Results and content have deleted and rexhibits
31	Line 64	Insert s at content	S has been inserted at content to be contents
32	Line 65	Delete of and insert to and at	Of has been deleted and to and at have been inserted
32	Line 69-71	Delete s at samples	S at samples has been deleted to be sample
33	Line 72	Delete , (comma)	, (comma) has been deleted
34	Line 73	Delete , (comma)	, (comma) has been deleted
35	Line 74	Delete ir	Ir has been deleted
36	Line 77	Delete time and insert storage	Time has been deleted and storage has been inserted
37	Line 78	Insert, (comma)	, (comma) has been inserted
38	Line 79	Delete time and insert periods	Time has been deleted and periods has been inserted
39	Line 80	Delete to and insert on the	To has been deleted and on the has been inserted
40	Line 82	Insert on	On has been inserted
41	Line 83	Delete ly and time, and insert period	Ly and time have been delete and period has been inserted
42	Line 84	Insert on the caffeine content extracted at the brewing temperature of coffee Delete the coffee	On the caffeine content extracted at the brewing temperature of coffee ....has been inserted The coffee has been deleted
43	Line 85	Delete influences the caffeine content extracted ...., the steeping...	Influences the caffeine content extracted ... and ....the steeping ....has been deleted
44	Line 85	Insert and the high total phenol content and antioxidant activity	And the high total phenol content and antioxidant activity ... has been inserted
45	line 86	Delete results the highest total phenol content and antioxidant activity	....results the highest total phenol content and antioxidant activity... has been deleted
46	Line 87	Insert the most efficient	The most efficient ...has been inserted
47	Line 89	Insert and	... and... has been inserted

48	Line 90	Delete on the other hand, storage time Insert period tea usually for several months until years	on the other hand, storage time... has been deleted ...period tea usually for several months until years... has been inserted
49	Line 91	Delete ...because this herbal tea usually is stored for a several months until years	...because this herbal tea usually is stored for a several months until years... has been deleted
50	Line 93	Replace alu foil	Aluminum foil has been replaced
51	Line 93	Replace standing proud	Standing Pouch has been replaced
52	Line 93	Delete informed and insert reported	Informed has been deleted and reported has been inserted
53	Line 94	Delete time and insert the	Time has been deleted and the has been inserted
54	Line 98-103	Delete time and the study was done to determine total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC), DPPH free radical scavenging activity (DPPH), ferric reducing power (FRAP), $\alpha$ -amylase (AA) and $\alpha$ -glucosidase (GA) inhibition activities	Therefore, this research studied the effect of steeping temperature and storage period on the bioactive compounds [total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP)], and antidiabetic activities [( $\alpha$ -amylase (AA) and $\alpha$ -glycosidase (GA) inhibition)] of the infusion from powdered <i>Pluchea</i> leaves and on the phenolic compound profile.
55	Line 108	Insert the	The has been inserted
56	Line 109	Insert specifications	Specifications has been inserted
57	Line 110	Delete of, insert s at level and insert from	Of ... has been deleted, s at level and from has been inserted
58	Line 111	Delete e and get, and insert a and of	e and get have been deleted and a and of ... have been inserted
59	Line 112-113	Insert is, pulverized, powder, T, dried in an Delete powdering of, done, get, and then, the heating of , t, and done using a drying	Is, pulverized, powder, T, dried in an... have been inserted powdering of, done, get, and then, the heating of , t, and done using a drying... have been inserted
60	Line 114-116	Delete and , using, that made from paper filter around 2g/bag, and then	and , using, that made from paper filter around 2g/bag, and then all of samples called, pluchea herbal

		<p>all of samples called, pluchea herbal tea was</p> <p>Insert then 2 g of the powder were, into a paper filter, packed samples were , (un-stored), (stored)</p>	<p>tea was...have been deleted</p> <p>and , using, that made from paper filter around 2g/bag, and then all of samples called, pluchea herbal tea was... have been inserted</p>
61	Line 117-122	<p>Delete Therefore, this research studied the effect of steeping temperature and storage period on the bioactive compounds [(total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP)] and antidiabetic activities [(<math>\alpha</math>-amylase (AA) and <math>\alpha</math>-glycosidase (GA) inhibition]] of the infusion from powdered <i>Pluchea</i> leaves, and on the phenolic compound profile.</p>	<p>Replace to be ...</p> <p>.In the research, the one tea bag of <i>Pluchea</i> herbal tea that was stored 0 (B1) and 5 (B2) year, was steeped with 100 mL hot water at various temperatures, including 60 (T1), 70 (T2), 80 (T3), 95 (T4) °C for 5 min with infusion method obtaining 8 treatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2. After the temperature of <i>Pluchea</i> infusion similar to ambient temperature was analyzed further.</p>
62	Line 125	<p>Delete compounds, to analysis, including</p> <p>Insert reagents, in the analysis include</p>	<p>compounds, to analysis, including ... has been deleted</p> <p>reagents, in the analysis include ... has been inserted</p>
63	Line 137	<p>Change center ... analysis of the bioactive compounds</p>	<p>Analysis of the bioactive compounds ... has been changed to center</p>
64	Line 142-143	<p>Delete entered, and distilled water was added until 10 mL volume</p> <p>Add color specific</p> <p>Insert ...added and filled up to 10 mL volume with distilled water, ....the blue color intensity of</p>	<p>Entered, and distilled water was added until 10 mL volume .... Have been deleted</p> <p>Blue color ...has been added ...added and filled up to 10 mL volume with distilled water, ....the blue color intensity of the solution was measured...have been inserted</p>

		the solution was measured	
65	Line 151-156	Delete ...an..., distilled Insert ...the..., ...resulted in..., ...distilled...	...the..., ...resulted in..., ..distilled .. has been inserted
66	Line 165-167	Delete ....was added until 10 mL volume,....that... Insert ...with..., ...with.., filled up to 10 mL volume with distilled ..., ..was..., ...in...	...was added until 10 mL volume,....that...has been deleted  ...with..., ...with.., filled up to 10 mL volume with distilled ..., ..was..., ...in... has been inserted
67	Line 172	Change center... analysis of the antioxidant potential	Analysis of the antioxidant potential ... has been changed to center
68	Line 175-180	Delete ...antioxidant activity of..., ...donor..., ...with..., ...entered..., ..and..., ...and then the solution was..., ...ed..., ...and... Insert ... the ability of the phytochemicals in..., ...donate..., ...the..., ...in the formation of..., exhibiting ..., ...poured into..., ...into which was added..., after incubation..., ...the	...antioxidant activity of..., ...donor..., ...with..., ...entered..., ..and..., ...and then the solution was..., ...ed..., ...and.. has been deleted  ... the ability of the phytochemicals in..., ...donate..., ...the..., ...in the formation of..., exhibiting ..., ...poured into..., ...into which was added..., after incubation..., ...the. has been inserted
69	Line 188-194	Delete ...1%..., ...incubation..., ...signed...distillated  Insert... of 1%..., ...the...incubated., into the..., .. distilled, ...indicated..., ...the..., ...was...,	...1%..., ...incubation..., ...signed...distillated...has been deleted  ...1%..., ...incubation..., ...signed...distillated... has been inserted
70	Line 198		Analysis of the antidiabetic properties ... has been added and centered
71	Line 201-208	Delete..were mixed..., then..., each..., and..., ), and., d,...that could be analyzed based on absorbance	...were mixed..., then..., each..., and..., ), and., d. , that could be analyzed based on absorbance has been deleted  ... was mixed with..., into a., the.,

		Insert... was mixed with..., into a., the., was added an., then., mixture..., into which was., the., to., was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1900, Shimadzu, Japan)	was added an., then., mixture..., into which was., the., to., was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1900, Shimadzu, Japan)... has been inserted
72	Line 216-223	Delete... contained..., finally, the..., or.. steeping...tea,...to enzyme..  Insert ...containing., ..the., ..the., inhibition,..the.. infusion..  Rewrite the residue of this enzyme hydrolyzed p-nitrophenyl- $\alpha$ -glucopyranoside (pNPG) as a substrate to result p-nitrophenol.	contained..., finally, the..., or.. steeping...tea,...to enzyme...has been deleted  ...containing., ..the., ..the., inhibition,..the.. infusion..has been inserted  Rewrite to be...The amount of these enzymes that didn't react with bioactive compounds of <i>Pluchea</i> infusion hydrolyzed p-nitrophenyl- $\alpha$ -D-glucopyranoside (pNPG) as a substrate to result in p-nitrophenol.
73	Line 230	Delete HPLC	HPLC has been deleted
74	Line 239	Delete analytical conditions..., ...t... Insert T	analytical conditions..., ...t...has been deleted ...T... has been inserted
75	Line 248	Delete distilled and insert distilled	Distilled has been deleted and distilled has been inserted
76	Line 253	Delete time and insert period	Time has been deleted and period has been inserted
77	Line 255-261	Delete time, fresh, the research resulted, time Insert period, un-stored, resulting in, period  Comment : Were all analyses replicated 2 periods only? What do you mean by 'continued analysis using a paired t-	time, fresh, the research resulted, time ... have been deleted  period., un-stored, resulting in, period ... have been inserted  Statement changes to be... The HPLC analysis of phenolic was repeated six periods. The data analysis of samples was repeated for six periods. The data were analyzed using a paired t-test at $\alpha$ .

		test at $\alpha \leq 0.05$ ? Rewrite this part of the paragraph.	$\leq 0.05$ , treatment means of specific phenolic compounds that were identified were expressed as the mean $\pm$ SD. The analysis used SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).
78	Line 264	Delete <i>Pluchea</i> leaf infusion is produced by young <i>Pluchea</i> leaf from 1-6 levels on each branch of the shoot (Widyawati et al., 2022), that is steeped at 95 °C for 5 min, has many biological activities, such as antioxidant activity (Widyawati et al., 2016), antidiabetic activity (Werdani and Widyawati, 2018), anti-inflammatory (Srisook et al., 2015). The chemical constituents in <i>Pluchea</i> tea involve alkaloids, flavonoids, phenolics, sterols, cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2 g/100 mL steeping <i>Pluchea</i> tea has total phenolic content 9.3 mg gallic acid equivalents (GAE)/g samples, total flavonoid content 22.0 mg catechin equivalents (CE)/g samples, DPPH free radical scavenging activity 27.2 mg gallic acid equivalents (GAE)/g samples, and ferric reducing power 10.2 mg gallic acid equivalents (GAE)/g samples (Widyawati et al., 2016). Previous research has informed the	..... has been done

		<p>composition of phytochemical compounds in <i>Pluchea</i> leaves, such as phenolic acids such as chlorogenic acids, caffeic acids, 3-<i>O</i>-caffeoylquinic acids, 4-<i>O</i>-caffeoylquinic acids, 5-<i>O</i>-caffeoylquinic acids, 3,4-di-<i>O</i>-caffeoylquinic acids, 3,5-di-<i>O</i>-caffeoylquinic acids, and 4,5-di-<i>O</i>-caffeoylquinic acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin; <math>\beta</math>-carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Chan et al., 2022; Widyawati et al., 2022).</p> <p>The presence of phytochemical compounds in herbal products was influenced by environmental factors, i.e., temperature, light exposure, oxygen level, pH, and moisture. The structure of phytochemical compounds in herbal tea is very sensitive to the surrounding changes. The effect arising from these changes causes the structure of the phytochemical molecule to be degraded to produce smaller size molecules or to combine to produce larger size molecules (Ali et al., 2018; Jayani et al. 2022, Ramphinwa et al., 2023).</p>	
--	--	--	--



79	Line 266	Change center position of phenolic compounds	Phenolic compounds... has been centered
80	Line 276	Delete T and insert t and Figure 1a	T ...has been deleted and t and Figure 1a ... have been inserted
81	Line 278	Delete t and insert that	t.. has been delete and that...has been inserted
82	Line 280	Delete s	S ..has been deleted
83	Line 281-282	Insert phenolic content value ... and stored  Delete that were infused at different temperatures..., ..then stored..., ...also	Phenolic content value ..and ...stored....have been inserted  ....that were infused at different temperatures.... ,....then stored... and ...also..have been deleted
84	Line 283	Insert content	Content ...has been inserted
85	Line 286 -295	Delete (figure 1a). this could have been due to fact that during steeping fresh Pluchea tea had a lower total phenolic content than stored pluchea tea for 5 years, besides that the higher the steeping temperature also caused the greater the extracted total phenolic content. The temperature of insusion influenced total phenolic content, it could relate to...., increasing..., contact...., between,....this compounds and water. The same phenomena also occurred in...  Move water (Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al. (2022).	Change to be ...period. This could have been due to the fact that the steeping temperature and storage period could cause the process of degradation, oxidation, and leaching/release of phenolic compounds. Phenolic compounds are water soluble and thus soaking in hot water for a certain period of period as in steeping causes the migration process of more phenolic compounds to the water because of longer exposure of phenolic compounds to water (Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al. (2022). Su et al. (2019) reported that temperature treatment can stimulate the release of phenolic compounds and increase antioxidant activity of lychee juice stored at different temperatures of 4 and 45 oC and different long storage (fresh and 72 hours).
86	Line 296-299	Delete this occurrence showed that steeping temperature and storage period caused the process of degradation and oxidation of phenolic	Change to be..... Temperature treatment degrades (or hydrolyzes) the hydrogen bond between phenolic compounds and proteins resulting in an

		<p>compounds. Su et al. (2019) reported that temperature treatment can stimulate the release of phenolic compounds and increase antioxidant activity of lychee juice stored at different temperatures of 4 and 45°C and different long store (fresh and 72 hours)</p> <p>Delete hydrogen bonding is affected by</p> <p>Delete t Delete because the...</p> <p>Delete can be degraded that the measured levels</p> <p>Delete are the phenomena were supported by Delete and Delete besides that,</p>	<p>increase of phenolic compounds when exposed to higher temperatures (Ali et al. (2018); Jayani et al. (2022) and Ramphinwa et al. (2023)).</p>
87	Line 302	Delete besides that	Besides that...has been deleted
88	Line 304-307	<p>Delete that the phenolic compounds in pluchea infusion are degraded due to ..</p> <p>Delete ...and can be easily extracted...</p> <p>Delete ing..</p> <p>Delete the</p> <p>Delete the..</p> <p>Delete content</p> <p>Delete as the</p> <p>Delete increase</p>	<p>Change to be ... that temperature and storage caused the degradation, oxidation, and hydrolysis of the phenolic compounds period resulting in the increased amount of the phenolic compounds at higher steeping temperature and longer storage period.</p>
89	Line 308-319	<p>Delete based on using of a reference</p> <p>Delete ing</p> <p>Delete . (dot)</p> <p>Delete ing</p> <p>Delete results of statistical analysis</p>	<p>Change to be... Simple phenolic compounds are identified in steeped and stored. <i>Pluchea</i> leaf infusion included gallic acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-O-</p>

		<p>Delete of pluchea infusion  Delete long  Delete nevertheless, the  Delete of two treatments expect  Delete the  Delete but  Delete t  Delete different concentration  Delete and  Delete compounds  Delete from pluchea infusion were  Delete however the concentration of 3,4-dicaffeoylquinic acid was also significantly different at 80and 90°C.</p>	<p>caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic acids was showed in Table 1. The treatment effects using a t-test at <math>\alpha \leq 0.05</math> showed that gallic acid and kaempferol content were insignificantly different at various steeping temperatures and storage periods. The concentration of quercetin and 3,5-di-O-caffeoylquinic acid of the un-stored and stored <i>Pluchea</i> infusion was significantly different from the rest of the samples between 70 °C while (+)-catechin concentration of <i>Pluchea</i> infusion was only significantly different at 95 °C. The myricetin content was significantly different at 80 and 95 °C. The 3,4-di-O-caffeoylquinic acid content showed significant difference at 60, 80, and 95 °C while 4,5-di-O-caffeoylquinic acid content was only significantly different at 60 °C.</p>
90	Line 320-331	<p>Delete based on the analysis of concentration of .., simple phenolic compounds showed..., phenolic acid because of...no changes at..., time..., with concentration about 0.21 ± 0.00 to 0.24±0.02 µg/g samples and 0.14±0.02 to 0.95±0.03 µg/g samples respectively..., however, myricetin..., increasing..., it's meant..., compared to the other two groups of phenolic acids..., T, ...time.., can cause macromolecules of three phenolic acids in herbal tea, (, )</p>	<p>Change sentence to be...  Results further showed that gallic acids and kaempferol were relatively stable as reflected by the insignificant changes when exposed to the different steeping temperature and storage period. Myricetin, (+)-catechin, and 3,4-di-O-caffeoylquinic acid showed a drastic increase at higher steeping temperatures and longer storage period implying that these compounds tended to be relatively labile. Quercetin, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid underwent moderate changes. Therefore, myricetin, (+)-catechin, and 3,4-di-O-caffeoylquinic acid were easier to dissolve or degrade to form simple phenolic acids at higher temperatures and storage period</p>

			(Su et al. (2019, Ali et al. (2018); Jayani et al. (2022); Ramphinwa et al. (2023), and Zhang et al. (2021). Degradable polyphenol compounds have a simple structure and free hydroxyl groups that can react with Folin-Ciocalteu's Phenol reagent, resulting complex blue solution that can detected as total phenolic content.
91	Line 332	Change flavonoid content (TFC) to be center position	Has been done
92	Line 337		Change ageing to be aging
93	Line 340		Change T to be t
94	Line 341-344	Insert the steeped un-stored and steeped stored samples..., the un-stored , at..., samples	The steeped un-stored and steeped stored samples..., the un-stored , at..., samples... have been inserted
95	Line 347	Center Tannin content (TTC)	Tannin content (TTC) has been centered
96	Line 352-362	Delete is., lower., l., both high steeping temperature and ..., and that the presence of high tannin content was primarily brought about by long storage period., informed that., t, of polyphenolic compounds., occurred., material., l (, ., however the high temperature can degree polyphenolic compounds that is essential to body health, the results showed that the higher the brewing temperature and the longer the storage time caused the tannin compound to degrade to result catechin compounds. This phenomenon is in line with the increase in total	Is., lower., l., both high steeping temperature and ..., and that the presence of high tannin content was primarily brought about by long storage period., informed that., t, of polyphenolic compounds., occurred., material., l (, ., however the high temperature can degree polyphenolic compounds that is essential to body health, the results showed that the higher the brewing temperature and the longer the storage time caused the tannin compound to degrade to result catechin compounds. This phenomenon is in line with the increase in total phenol levels and the concentrations of (+)-catechin compounds. Ali et al. (2018) said that pH, storage temperature, chemical structure and concentration, light, oxygen, enzymes and metal ions affect the presence of bioactive compounds

		<p>phenol levels and the concentrations of (+)-catechin compounds. Ali et al. (2018) said that pH, storage temperature, chemical structure and concentration, light, oxygen, enzymes and metal ions affect the presence of bioactive compounds in the material,.. nevertheless.., s., s., as a result the tannin content in pluchea tea increases with increasing steeping temperature and storage time</p> <p>Insert ...the.., at.., ., was, that of, I, primarily, than high steeping temperature, T, occurring, tea leaves (, could have had contributed to the observed increase in the tannin content in the treated samples.., although high temperature and long storage period can cause the degradation of tannins to catechins.., thermostable complex,..s., .are.., indicating that even with the exposure to high temperature, the tannins still remained high in the treated samples.</p> <p>Move ..Kowalska et al. (2021)</p>	<p>in the material,.. nevertheless... s., s., as a result the tannin content in pluchea tea increases with increasing steeping temperature and storage time have ben deleted.</p> <p>...the.., at.., ., was, that of, I, primarily, than high steeping temperature, T, occurring, tea leaves (, could have had contributed to the observed increase in the tannin content in the treated samples, although high temperature and long storage period can cause the degradation of tannins to catechins.., thermostable complex,..s., .are.., indicating that even with the exposure to high temperature, the tannins still remained high in the treated samples.... have been inserted</p> <p>..Kowalska et al. (2021)... has been moved</p>
97	Line 368	Center antioxidant activity	Antioxidant activity ...has been entered
98	Line 374-380	Delete donor, involved, through, s	donor, involved, through, s... have been deleted

		<p>Insert that, s, the, donate, depends on their, and, and as</p> <p>Rewrite: the higher antioxidant power of phenolic compounds is caused the weaker O-H phenolic bond</p>	<p>That, s, the, donate, depends on their, and, and as ... has been inserted</p> <p>Rewrite to be... The antioxidant power of phenolic compounds is due to the weak hydrogen bonds in the OH group of the phenolic compound so that it is easier to donate hydrogen atoms</p>
99	Line 382	Center DPPH free radical scavenging activity	DPPH free radical scavenging activity has been centered
100	Line 387-398	<p>Delete the result of DPPH assay., indicates, DPPH values accrued, at higher steeping temperature and longer storage time, statistical analysis by Anova using a paired T test at <math>\alpha \leq 0.05</math> proven that the higher the steeping temperature of fresh Pluchea infusion (T1B1, T2B1, T3B1 and T4B1) was consistent the ability to DPPH free radicals scavenging activity whereas the stored pluchea infusion resulted in the higher activity and the values went up as rising of the infusion temperature, DPPH, s, at higher, DPPH, steeping at 80-95°C in stored pluchea infusion insignificantly affected the free radial, scavenging property of the, DPPH</p> <p>Insert figure 2a shows that the free radical scavenging property of the stored and steeped samples were</p>	<p>Delete the result of DPPH assay., indicates, DPPH values accrued, at higher steeping temperature and longer storage time, statistical analysis by Anova using a paired T test at <math>\alpha \leq 0.05</math> proven that the higher the steeping temperature of fresh Pluchea infusion (T1B1, T2B1, T3B1 and T4B1) was consistent the ability to DPPH free radicals scavenging activity whereas the stored pluchea infusion resulted in the higher activity and the values went up as rising of the infusion temperature, DPPH, s, at higher, DPPH, steeping at 80-95°C in stored pluchea infusion insignificantly affected the free radial, scavenging property of the, DPPH</p> <p>Figure 2a shows that the free radical scavenging property of the stored and steeped samples were significantly higher than the un-stored steeped samples, it can also be observed, free radical</p>

		<p>significantly higher than the un-stored steeped samples, it can also be observed, free radical scavenging property, was significantly different among the stored and steeped samples but insignificant among the un-stored and steeped samples, period, high, by, during the store process it is possible to form omplex phenolic compounds which provide a high ability to scavenge DPPH free radicals (Thanajirushaya et al., 2010)</p> <p>Move during the store process it is possible to form omplex phenolic compounds which provide a high ability to scavenge DPPH free radicals (Thanajirushaya et al., 2010)</p> <p>Clarify on how you were able to come up with free radical scavenging activity by more than 100%. Steeping temperatures significantly increased the free radical scavenging activity in stored pluchea infusion by around 15 to 25%</p>	<p>scavenging property, was significantly different among the stored and steeped samples but insignificant among the un-stored and steeped samples, period, high, by, during the store process it is possible to form omplex phenolic compounds which provide a high ability to scavenge DPPH free radicals (Thanajirushaya et al., 2010) ... have been inserted</p> <p>during the store process it is possible to form omplex phenolic compounds which provide a high ability to scavenge DPPH free radicals (Thanajirushaya et al., 2010 during the store process it is possible to form omplex phenolic compounds which provide a high ability to scavenge DPPH free radicals (Thanajirushaya et al., 2010 ...has been moved</p> <p>Change to be...  <i>Pluchea</i> infusion stored at room temperature for 5 years resulted in high free radical scavenging activity by more than 10%. Steeping at higher temperatures significantly increased the DPPH free radical scavenging activity in stored <u>Pluchea</u> infusion by around 15 to 25 %.</p>
101	Line 399-411	Delete scavenging, of DPPH free radicals, with total, levels, to, based on pearson correlated, research, time, donor	Scavenging, of DPPH free radicals, with total, levels, to, based on pearson correlated, research, time ... have been deleted

		<p>Insert the scavenging, of the samples, with total, content, with, study, period, donate, table 1.</p> <p>Comment: explain this observation based on the data that you were able to obtain</p>	<p>the scavenging, of the samples, with total, content, with, study, period, donate, table 1... have been inserted</p> <p>explain to be ...</p> <p>The scavenging activity of the samples was strongly and positively correlated with total phenol and tannin contents, but inversely with total flavonoid levels (Table 2). The antioxidant activity was strongly and negatively correlated with flavonoid content. The storage period could be reduced flavonoid content.</p>
102	Line 414	<p>Delete DPPH, DPPH, of tea infusion, inhibitor activity of DPPH</p> <p>Insert ...In, the free radical scavenging property, of tea infusion</p>	<p>DPPH, DPPH, of tea infusion, inhibitor activity of DPPH have been deleted</p> <p>In, the free radical scavenging property, of tea infusion... have been inserted</p>
103	Line 419	center ferric reducing antioxidant power (FRAP)	ferric reducing antioxidant power (FRAP) has been centered
104	Line 420-424	<p>Delete based on the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a color complex, that can be measured at <math>\lambda</math> 700 nm (Fu et al., 2011; Al-Temi8ni and Choudhary, 2013). Principle of the assay measures, ...</p> <p>Insert that is based</p>	<p>...based on the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a color complex, that can be measured at <math>\lambda</math> 700 nm (Fu et al., 2011; Al-Temi8ni and Choudhary, 2013). Principle of the assay measures, ...have been deleted</p> <p>that is based... has been inserted</p>
105	Line 425-432	<p>Delete with,...time, ... owned, .. by.., based on pearson correlation, the FRAP of Pluchea infusion was strongly and positively significant correlated with the</p>	<p>with,...time, ... owned, .. by.., based on pearson correlation, the FRAP of Pluchea infusion was strongly and positively significant correlated with the DPPH, TPC and TTC, but inversely to TFC. the correlated coefficient values ( r</p>



		<p>DPPH, TPC and TTC, but inversely to TFC. the correlated coefficient values ( r) between FRAP and DPPH, TPC, TTC and TFC were 0.956, 0.948, and -0.826, respectively</p> <p>Insert at, er, period, exhibited, in, un-</p>	<p>between FRAP and DPPH, TPC, TTC and TFC were 0.956, 0.948, and -0.826, respectively... have been deleted</p> <p>at, er, period, exhibited, in, un-... have been inserted</p>
106	Line 433-440	<p>Delete case was, to, on, ,, because, the, time, due to catechins, ,, and also the case of the effect of temperature and storage time in betel (piler betle L.) extract. Light and temperature influence degradation of phenolic compounds of betel that determine antioxidant activity. Different structure of phenolic compounds determines their stability to degrade accelerating of light and temperature. Hydroxychavicol is the best stability of phenolic compounds of betel compared with eugenol, isoeugenol and allyl pyrocatechol (Ali et al., 2018), Infusion corresponded, to, values, presence of them sample</p> <p>Insert .. is in, with, study on the, of, ,, the, period, which, ,, could have due, to the presence, of, that have the ability to transfer electron from their free hydroxyl groups of.., The FRAP of pluchea infusion was strongly and positively significant correlated</p>	<p>Case was, to, on, ,, because, the, time, due to catechins, ,, and also the case of the effect of temperature and storage time in betel (piler betle L.) extract. Light and temperature influence degradation of phenolic compounds of betel that determine antioxidant activity. Different structure of phenolic compounds determines their stability to degrade accelerating of light and temperature. Hydroxychavicol is the best stability of phenolic compounds of betel compared with eugenol, isoeugenol and allyl pyrocatechol (Ali et al., 2018), Infusion corresponded, to, values, presence of them sample ... have been deleted</p> <p>.. is in, with, study on the, of, ,, the, period, which, ,, could have due, to the presence, of, that have the ability to transfer electron from their free hydroxyl groups of sample, The FRAP of pluchea infusion was strongly and positively significant correlated with the DPPH, TPC, and TTC but inversely to TFC. ... have been inserted</p>

		with the DPPH, TPC, and TTC but inversely to TFC.	
107	Line 443	Center $\alpha$ -amylase enzyme inhibition activity (AA)	$\alpha$ -amylase enzyme inhibition activity (AA) has been centered
108	Line 445-463	<p>Delete,, the, had very good activity, for fresh, which, was brewed, whereas, fresh, an activity of, inhibiting the <math>\alpha</math>-alylase enzyme of less than 50%, which was equal to <math>40.08\pm 1.12</math>, detected, from, fresh, by, time, to, the results of the analysis based on a paired Ttest at <math>\alpha\leq 0.05</math> showed, that the steeping temperature and storage time, had a significant effect on the ability to inhibit the <math>\alpha</math>-amylse enzyme, besed on pearson correlation, the, the correlated coefficient..</p> <p>Pattern: clear (yellow), not highlight</p> <p>Insert lower, leaf, exhibited a good <math>\alpha</math>-amylase, in the un-stored, steeped, with highest at 60 °C, in, leaf, was, the stored, leaf, 70, 80 and, lower enzyme inhibition activity, of, less than 50% with lowest at 95 oC, found that, in, un-stored, was also low at, period, of the phytochemicals in the,</p>	<p>digestive... has been deleted</p> <p>digestive ... has been inserted</p> <p>,, the, had very good activity, for fresh, which, was brewed, whereas, fresh, an activity of, inhibiting the <math>\alpha</math>-alylase enzyme of less than 50%, which was equal to <math>40.08\pm 1.12</math>, detected, from, fresh, by, time, to, the results of the analysis based on a paired Ttest at <math>\alpha\leq 0.05</math> showed, that the steeping temperature and storage time, had a significant effect on the ability to inhibit the <math>\alpha</math>-amylse enzyme, besed on pearson correlation, the, the correlated coefficient.. have been deleted</p> <p>... has been done</p> <p>lower, leaf, exhibited a good <math>\alpha</math>-amylase, in the un-stored, steeped, with highest at 60 °C, in, leaf, was, the stored, leaf, 70, 80 and, lower enzyme inhibition activity, of, less than 50% with lowest at 95 oC, found that, in, un-stored, was also low at, period, of the phytochemicals in the, activity, period, Table 2 further shows that the, formatted ... have been inserted and formatted</p>

		activity, period, Table 2 further shows that the, formatted	
109	Line 466-485	<p>Insert period, reported, <math>\alpha</math>-amylase enzyme activity inhibition, elevated steeping temperature and longer storage period, easily cause the, al of the, ir, exhibits, ility,</p> <p>Delete time, flavonoid compounds with..., informed, the steeping temperature and storage time, e,</p> <p>Comment : rewrite</p>	<p>period, reported, <math>\alpha</math>-amylase enzyme activity inhibition, elevated steeping temperature and longer storage period, easily cause the, al of the, ir, exhibits, ility, ... have been inserted</p> <p>time, flavonoid compounds with..., informed, the steeping temperature and storage time, e,... have been deleted</p> <p>Delete by content and insert content</p> <p>ability of <i>Threspesia populnea</i> extract to inhibit the <math>\alpha</math>-amylase enzyme was determined of their phenolic compound content and protein. Moreover, the presence of <math>\alpha</math>-amylase enzyme inhibitor in this extract may be proteinaceous or nonproteinaceous in nature. It was assumed that this enzyme inhibitory activity in <i>Pluchea</i> infusion also was determined with their protein and polyphenolic content. Aleixandre et al. (2022) also stated that phenolic acids have inhibition activity to <math>\alpha</math>-amylase enzyme depending on their structures. There are C=C double bonds conjugated with a carbonyl group of phenolic structures that stabilize the binding forces to the active site of the</p>

			<p><math>\alpha</math>-amylase. The hydroxyl groups can bind by non-covalent interaction (hydrogen bonding, cation-<math>\pi</math> interactions, salt bridge interactions, ionic interactions, or electrostatic forces) with amino acid residue at the active site in <math>\alpha</math>-amylase enzyme. have been inserted</p>
110	Line 485	center $\alpha$ -glucosidase enzyme inhibition activity (GA)	$\alpha$ -glucosidase enzyme inhibition activity (GA)... has been centered
111	Line 486-512	<p>Delete <math>\alpha</math>, s, this supported by, the results showed, time, for fresh, un-stored, found, steeping temperature for fresh pluchea infusion, which was, T, Figure 3b, at both, the antidiabetic activity of pluchea infusion, showed</p> <p>Insert alpha, their, stated, Figure 3b shows, of the Pluchea leaf infusion, period, of the un-stored, leaf, at, between, Figure 3 further, shows, of pluchea leaf infusion</p> <p>Comment : rewrite and delete literature because unnecessary</p>	<p><math>\alpha</math>, s, this supported by, the results showed, time, for fresh, un-stored, found, steeping temperature for fresh pluchea infusion, which was, T, Figure 3b, at both, the antidiabetic activity of pluchea infusion, showed... have been deleted</p> <p>alpha, their, stated, Figure 3b shows, of the Pluchea leaf infusion, period, of the un-stored, leaf, at, between, Figure 3 further, shows, of pluchea leaf infusion... have been inserted</p> <p>Data analysis in Table 2. showed that the TFC of the <i>Pluchea</i> leaf infusion was influenced weakly and positively by GA and AA, but the GA and AA were not affected by TPC, TTC, DPPH, and FRAP. Li et al. (2018) stated that flavonoid compounds can inhibit the action</p>

			<p>of the <math>\alpha</math>-amylase and <math>\alpha</math>-glucosidase enzymes. Dias et al. (2021) stated that flavonoid compounds, such as rutin, myricetin, kaempferol, and quercetin have antioxidant and antihyperglycemic activities. The ability to inhibit the action of enzymes from flavonoid compounds is determined by the position and number of hydroxyl groups, the number of double bonds in rings A and B, and the heterocyclic ring in ring C. Tadera et al. (2006) and Zhang et al. (2014) also explained that flavonoid compounds of samples significantly inhibit the <math>\alpha</math>-glucosidase enzyme activity.</p>
112	Line 513-548	Rewrite the statement and delete literature not support	<p>Rewrite to be ...</p> <p>The ability to inhibit the <math>\alpha</math>-glucosidase enzyme from <i>Pluchea</i> infusion was significantly affected by the steeping temperature and long storage. Figure 3 also showed that the capability of <i>Pluchea</i> infusion to obstruct the <math>\alpha</math>-glucosidase enzyme was greater than the <math>\alpha</math>-amylase enzyme because the mechanism of the two enzymes was different, according to the opinion of McCue et al. (2005). The mechanism of the <math>\alpha</math>-glucosidase enzyme inhibitor includes making the sugar mimic structure, binding using ionic bonds with nucleophilic, making the transition state-like structure, binding hydrogen with catalytic acid residue, interacting ionic and hydrophobic with site other than the active site, and binding covalent with enzymes through an epoxy or aziridine group (Moorthy et al., 2012). Then, the mechanism of the <math>\alpha</math>-amylase enzyme inhibitor includes blocking carbohydrates,</p>

		<p>limiting the digestibility and absorption of carbohydrates, and blocking the active centers of several subsites of the enzyme (Gong et al., 2020).</p> <p>Widyawati et al. (2017) stated that phenolic and non-phenolic compounds can inhibit of the <math>\alpha</math>-glucosidase enzyme activity. The ability of bound phenolic compounds to inhibit <math>\alpha</math>-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, that may be occurred in <i>Pluchea</i> infusion during storage, affects the structure and profile of phenolic and non-phenolic compounds. Asriningtyas et al. (2014) explained that the methyl-esterified quinic acid with the caffeic groups, such as 3,5-di-<i>O</i>-caffeoylquinic acid, 4,5-di-<i>O</i>-caffeoylquinic acid methyl ester, 3,4,5-tri-<i>O</i>-caffeoylquinic acid methyl ester, 3,4,5-tri-<i>O</i>-caffeoylquinic acid, and 1,3,4,5-tetra-<i>O</i>-caffeoylquinic acid of <i>Pluchea</i> leaves inhibits the <math>\alpha</math>-glucosidase enzyme activity. The resulting analysis of caffeoylquinic acids (3,4-di-<i>O</i>-caffeoylquinic acid, 3,5-di-<i>O</i>-caffeoylquinic acid, and 4,5-di-<i>O</i>-caffeoylquinic acid in stored <i>Pluchea</i> leaf infusion higher concentration than in un-stored <i>Pluchea</i> infusion, and the concentrations of the simple phenolic compounds were increased at higher steeping temperature, but the <math>\alpha</math>-glucosidase inhibition activity of them was reduced. It means that the methyl-esterified quinic acid with the caffeic groups had more potential to inhibit <math>\alpha</math>-glucosidase enzyme than free caffeoylquinic acid.</p>
--	--	--

			<p>This study showed that the increasing steeping temperature and storage period caused degradation of polyphenol compounds to produce simple phenolic compounds, such as gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid that increased the total phenolic content and total tannin content. The increase in the simple phenolic concentration of the <i>Pluchea</i> leaf infusion caused higher antioxidant activity and lower antidiabetic activity.</p>
113	Line 549-590	Rewrite conclusion	Rewrite conclusion has been done
114	Line 600-784	Delete, add, and revise reference	Delete, add, and revise reference have been done
115	Line 789-830	<p>Delete time, T, within group differences at unstored vs stored for 5 years at certain steeping temperatures, calculated using a paired T test at <math>\alpha \leq 0.05</math></p> <p>Insert period, t,</p> <p>Formatted figure and table, description of figures and tables</p>	<p>time, T, within group differences at unstored vs stored for 5 years at certain steeping temperatures, calculated using a paired T test at <math>\alpha \leq 0.05</math>... have been deleted</p> <p>period, t, ... have been inserted</p> <p>figure and table, description of figures and tables... have been formatted</p>

1 **Effect of Steeping Temperature and Storage **Period** on the Bioactive Compounds,**  
2 **Antioxidant and Antidiabetic Activities of Infusion from Powdered Pluchea Indica**  
3 **Less**

4 Painsi Sri Widyawati<sup>1\*)</sup>, Yufita Ratnasari Wilianto<sup>2)</sup>

5 <sup>1)</sup>Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala  
6 Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

7 <sup>2)</sup>Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic  
8 University, Kalisari Street Number 1, Surabaya 60272, Indonesia

9 Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature,  
10 Pluchea indica Less, storage **period**

11

12

13

14

15

16

17

18

19

20



21 ABSTRACT

22 This study was done to determine the effects of steeping temperature and storage  
23 **period** on the bioactive contents, antioxidant and antidiabetic activities of *Pluchea* leaf  
24 infusion. The research used a randomized block design with two factors, i.e., steeping  
25 temperature (T) and storage **period** (B). **The *Pluchea* leaf blades were exposed to 4**  
26 **steeping temperatures of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) °C** with the storage  
27 **period** of 0 (B1) and 5 (B2) years **resulting in** 8 treatment combinations (T1B1, T1B2,  
28 T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). Statistical analysis using a paired **t-test** at  $\alpha \leq$   
29 0.05 showed that treatments significantly **affected** the bioactive contents [(total phenol  
30 (TPC), total tannin (TTC), total flavonoid (TFC)], antioxidant [(DPPH scavenging activity  
31 (DPPH) and ferric reducing antioxidant power (FRAP)] **potential** and antidiabetic [( $\alpha$ -  
32 amylase (AA) and  $\alpha$ -glucosidase (GA) **inhibition)] properties of the *Pluchea* leaf infusion.**  
33 **TPC, TTC, DPPH, and FRAP significantly increased for the storage period and the**  
34 **steeping temperatures.** Then, TFC decreased during the storage period but significantly  
35 **increased at higher steeping temperatures.** The AA and GA of *Pluchea* leaf infusion  
36 **increased until 70 °C of the steeping temperature, but decreased until 95 °C.** The DPPH  
37 **and FRAP of the *Pluchea* leaf infusion were strongly and positively correlated with TPC**  
38 **and TTC.** The GA and AA of *Pluchea* leaf infusion were not influenced by the TPC  
39 **and TTC but were weakly and positively correlated with TFC.** The antioxidant activity of  
40 **the *Pluchea* leaf infusion was inversely proportional to the antidiabetic activity.** The  
41 **simple phenolic compounds derived from *Pluchea* leaf infusion at different steeping**  
42 **temperatures and storage included** gallic acid, kaempferol, myricetin, (+)-catechin,

43 quercetin, 3,4-di-O-caffeoylquinic acid, 3,5- di-O-caffeoylquinic acid, and 4,5-di-O-  
44 caffeoylquinic acid.

45

## 46 INTRODUCTION

47 Pluchea herbal tea is a product of dried Pluchea leaf processing introduced by  
48 world people (Srisook et al., 2012; Widyawati et al., 2016) because of the efficacy of the  
49 active components in Pluchea leaves, as a herbal plant that has been widely used for  
50 traditional medicine and food (Chan et al., 2022). Pluchea leaves are composed of  
51 many nutrients and bioactive compounds useful to body health. The nutrient  
52 compositions in the Pluchea leaves include protein, fat, ash, insoluble fiber, soluble  
53 fiber, carbohydrates, calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive  
54 compounds are comprised, i.e., chlorogenic acid, caffeic acid, 3-O-caffeoylquinic acid,  
55 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-  
56 caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, quercetin, myricetin, kaempferol, total  
57 anthocyanin,  $\beta$ -carotene, and total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018;  
58 Ruan et al., 2019; Widyawati et al., 2022, Chan et al., 2022).

59 The steeping process of Pluchea leaves can be performed with fresh or dry  
60 leaves in hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et al.,  
61 2020; Jayani et al., 2022). In Asia, especially in Indonesia, people usually consume the  
62 Pluchea infusion by steeping 2 g of powdered Pluchea leaves in a tea bag in 100 mL of  
63 hot or boiling water. Widyawati et al. (2016) claimed that steeping of 2 g of Pluchea leaf  
64 powder at 95 °C for 5 minutes exhibits total phenolic and total flavonoid contents, the  
65 ability to scavenge DPPH free radicals, and the capability to reduce ferric ions at 9.3 mg

66 gallic acid equivalent (GAE)/g sample, 22.0 mg gallic acid equivalent (GAE)/g sample,  
67 27.2 mg gallic acid equivalent (GAE)/g sample, and 10.2 mg gallic acid equivalent  
68 (GAE)/g sample, respectively. Werdani and Widyawati (2018) reported that drinking  
69 *Pluchea* leaf powder infusion in the morning and evening regularly (2 g/100 mL) can  
70 decline blood sugar levels.

71 The steeping of *Pluchea* herbal tea with hot water at 95 °C for 5 minutes certainly  
72 determines the stability and amount of extracted bioactive compounds that  
73 influence the biological activity especially antioxidant and antidiabetic activities.  
74 Silva-Ramirez et al. (2020) reported that the infusion process can influence the content  
75 and composition of the bioactive compounds and antioxidant activity of tea. Acar et al.  
76 (2022) informed that the infusion quality of herbal tea extract depends on several  
77 factors, i.e., storage and temperature. The polyphenol profile and antioxidant properties  
78 of herbal tea infusion decline with an increase in steeping/brewing and storage  
79 temperatures, and longer exposure periods.

80 Several studies have mentioned the effect of steeping temperature on the  
81 bioactive compound contents and antioxidant activity, such as some white and green  
82 teas are effective with hot water at 90 °C for 7 min (Castiglioni et al., 2015), on  
83 rosehip tea is effective at infusion period around 6-8 min at temperatures of 84-86 °C (Ilyasoglu  
84 and Arpa, 2017), on the caffeine content extracted at the brewing temperature of coffee  
85 (Zarwinda and Sartika, 2018), and the high total phenol content and antioxidant activity  
86 of dark tea at 92 °C for 27 min (Wang et al., 2022). The study of the effect of steeping  
87 temperature on *Pluchea* infusion was carried out to afford information about the most

88 efficient preparation of powdered *Pluchea* leaves to get higher bioactive compounds,  
89 antioxidant, and antidiabetic activities.

90 Storage period tea usually for several months to years *Pluchea* herbal tea also  
91 affects the levels of the bioactive compounds and biological activity (Jayani et al., 2022).  
92 Tea or herbal tea is generally stored at ambient temperature and packed in a tea bag or  
93 aluminum foil standing pouch or a combination of both. Many researchers reported that  
94 the storage period decreases the bioactive compounds, antioxidant and antidiabetic  
95 activities, i.e., juice from *Momordica charantia* L. (Lin et al., 2020), dried *Piper bettle*  
96 extracts (Ali et al., 2018), white tea (Xu et al., 2019), kinnow-amlam beverages (Purewal  
97 et al., 2022), whole wheat flour (Zhang et al., 2021).

98 Therefore, this research studied the effect of steeping temperature and storage  
99 period on the bioactive compounds [total phenolic content (TPC), total flavonoid content  
100 (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging activity  
101 (DPPH), ferric reducing antioxidant power (FRAP)], and antidiabetic activities [( $\alpha$ -  
102 amylase (AA) and  $\alpha$ -glycosidase (GA) inhibition] of the infusion from powdered  
103 *Pluchea* leaves and on the phenolic compound profile.

104

## 105 MATERIALS AND METHODS

### 106 RAW MATERIALS AND PREPARATION

107 The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya,  
108 East Java, Indonesia. The *Pluchea* plants were included in the *Asteraceae* family with  
109 specifications according to the GBIF taxon ID number database:3132728 (Ferraris,  
110 2023). *Pluchea* leaves at 1-6 levels of each branch from the shoot were collected,

111 sorted, washed, and dried to get a moisture content of around  $11.16 \pm 0.09$  % dry  
112 basis (Widyawati et al., 2022). The dried *Pluchea* leaves was pulverized to a 45-mesh  
113 size powder. The *Pluchea* leaf powder was dried in an oven (Binder, Merck KGaA,  
114 Darmstadt, Germany) at 120 °C for 10 min to reduce microbial organisms. Then, 2 g of  
115 the powder were packed into a paper filter infusion bag. Packed samples were stored  
116 for 0 (un-stored) and 5 (stored) years in standing pouch before analysis.

117 In the research, the one tea bag of *Pluchea* herbal tea that was stored 0 (B1)  
118 and 5 (B2) year, was steeped with 100 mL hot water at various temperatures, including  
119 60 (T1), 70 (T2), 80 (T3), 95 (T4) °C for 5 min with infusion method obtaining 8  
120 treatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2.  
121 After the temperature of *Pluchea* infusion similar to ambient temperature was analyzed  
122 further.

123

## 124 REAGENTS

125 The reagents used in the analysis include 2,2-diphenyl-1-picrylhydrazyl (DPPH),  
126 sodium carbonate, gallic acid,  $\alpha$ -amylase,  $\alpha$ -glucosidase, pNPG (p-nitrophenyl- $\alpha$ -  
127 glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-O-caffeoylquinic  
128 acid, 4,5-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and (+)-catechin were  
129 purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, Folin–Ciocalteu's  
130 Phenol, sodium nitric, aluminum chloride, ferric chloride, sodium dihydrogen phosphate,  
131 sodium phosphate, potassium ferricyanide, starch, acetic acid, and sodium hydroxide  
132 were purchased from Merck (Kenilworth, NJ, USA). All reagents used were of analytical

133 grade except for distilled water which was purchased from PT Aqua Industry  
134 Surabaya.

135

## 136 METHODOLOGY

### 137 ANALYSIS OF THE BIOACTIVE COMPOUNDS

#### 138 TOTAL PHENOLIC CONTENT ANALYSIS

139 Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using  
140 the technique by Gao et al. (2019). About 10  $\mu$ L *Pluchea* infusion and 1 mL Folin-  
141 Ciocalteu's phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated  
142 for 5 min. And then 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5 % was added and filled up to 10 mL volume with  
143 distilled water. The blue color intensity of the solution was measured in the  
144 spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at  $\lambda$  760 nm with gallic acid as the  
145 reference standard. The total phenolic content was calculated using the formula:  
146  $y=0.00009x+0.008$  with  $R^2=0.9941$ . The results were expressed as mg gallic acid  
147 equivalent (GAE)/g samples.

148

#### 149 TOTAL FLAVONOID CONTENT ASSAY

150 Total flavonoid content (TFC) of the samples was measured based on the  
151 reaction between AlCl<sub>3</sub> and NaNO<sub>2</sub> with the aromatic ring of flavonoid compounds,  
152 especially flavonol and flavon (Shraim et al., 2021). The reaction between AlCl<sub>3</sub> and  
153 flavonoid compounds resulted in a yellow solution. About 30  $\mu$ L *Pluchea* infusion was  
154 mixed with 0.3 mL NaNO<sub>2</sub> 5 % in 10 mL volumetric flash and incubated for 5 min. The  
155 mixture was added with 0.3 mL AlCl<sub>3</sub> 10 % for 5 min. Then, 2 mL NaOH 1 M and

156 distilled water were added until 10 mL volume. Then, the red solution was produced  
157 after NaOH solution addition that was measured by a spectrophotometer  
158 (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  510 nm with (+)-catechin as  
159 the reference standard compound, and the results were expressed as mg catechin  
160 equivalents (CE)/g samples using the formula:  $y=0.00008x-0.0023$  with  $R^2= 0.9980$ .

161

## 162 TOTAL TANNIN CONTENT ANALYSIS

163 Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu  
164 method (Chandran and Indira, 2016). Approximately 10  $\mu$ L *Pluchea* infusion was added  
165 with 1 mL Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flask and  
166 incubated for 5 min. Then, the mixture was added with 2 mL  $\text{Na}_2\text{CO}_3$  7.5 % and filled up  
167 to 10 mL volume with distilled water. The blue dark color solution was measured in UV-  
168 Vis spectrophotometer 1800 (Shimadzu, Japan) at  $\lambda$  760 nm with tannic acid as the  
169 reference standard. Calculation of TTC was expressed as mg tannic acid equivalents  
170 (TAE)/g samples used the formula:  $y=0.00009x+0.0021$  with  $R^2=0.9993$

171

## 172 ANALYSIS OF THE ANTIOXIDANT POTENTIAL

### 173 DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

174 The DPPH free radical scavenging activity (DPPH) was measured by the  
175 spectrophotometric method (Widyawati et al., 2017) to determine the ability of the  
176 phytochemicals in the *Pluchea* leaf infusion to donate hydrogen atom to the nitrogen  
177 atom in DPPH resulting in the formation of DPPH-H compound exhibiting a yellow-  
178 colored solution. About 25  $\mu$ L *Pluchea* leaf infusion was poured into reaction tube into

179 which was added 3 mL DPPH solution (4 mg/100 mL). After incubation for 15 min in a  
180 dark room, the absorbance was measured by a spectrophotometer (Spectrophotometer  
181 UV-Vis 1800, Shimadzu, Japan) at  $\lambda$ . 517 nm. The reference standard compound was  
182 gallic acid and the results of analysis were expressed as mg gallic acid equivalents  
183 (GAE)/g samples that calculated using formula:  $y=0.146x+1.7896$  with  $R^2=0.9975$ .

184

#### 185 FERRIC REDUCING POWER ANALYSIS

186 Ferric-reducing power (FRAP) was determined following the method used by  
187 Widyawati et al. (2014) method. Approximately 10  $\mu$ L of samples were added to 2.5 mL  
188 phosphate buffer pH 6.6 and 2.5 mL of 1% potassium ferricyanide in the reaction tube.  
189 And then mixture was shaken and incubated for 20 min at 50 °C. Finally, 2.5 mL  
190 chloroacetic acid 10% (w/v) was added. Into the 2.5 mL supernatant was added 2.5 mL  
191 distilled water, 0.5 mL ferric chloride 0.1% w/v, and incubated for 10 min. Potency of the  
192 samples reducing iron (III) to iron (II) ion was indicated by the intensity of blue color  
193 formed that was measured using UV-Vis spectrophotometer (Spectrophotometer UV-  
194 Vis 1800, Shimadzu, Japan) at  $\lambda$  700 nm. Intensity of the blue color indicated higher  
195 reducing capacity. The reducing power expressed as mg gallic acid equivalent (GAE)/g  
196 samples was calculated using the formula:  $y=0.0002x+0,0256$  with  $R^2=0,9906$ .

197

#### 198 ANALYSIS OF THE ANTIDIABETIC PROPERTIES

##### 199 $\alpha$ -AMYLASE ENZYME INHIBITION CAPACITY ASSAY

200 In vitro inhibition of  $\alpha$ -amylase enzyme (AA) followed the procedure as described  
201 by Widyawati et al. (2020). Each 500  $\mu$ L of samples, was mixed with starch 1 % (w/v)



202 and sodium acetate buffer pH 5. Into a 250  $\mu$ L of the mixture was added an  $\alpha$ -amylase  
203 solution (0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in 50 mL of 0.2 M  
204 sodium acetate pH 5. Mixture was shaken into which was and added 2 mL sodium  
205 hydroxide 1M. Before the analysis, this mixture was incubated at 37  $^{\circ}$ C for 10 min.  
206 Then, the capacity of the  $\alpha$ -amylase enzyme to hydrolyze the starch to release glucose  
207 was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800,  
208 Shimadzu, Japan) at  $\lambda$  540 nm. The inhibition percentage of  $\alpha$ -amylase was assessed  
209 using the formula:  $(AC_b - AC_a) - (A_s - A_b) / (AC_b - AC_a) \times 100 \%$ . Where,  $AC_b$  is the  
210 absorbance of 100 % enzyme activity (solvent with the enzyme),  $AC_a$  is the absorbance  
211 of 0 % enzyme activity (solvent without the enzyme),  $A_s$  is the absorbance of test  
212 sample with enzyme,  $A_b$  is absorbance of test sample without enzyme.

213

#### 214 $\alpha$ -GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

215 The analysis of the  $\alpha$ -glycosidase inhibitor activity (GA) was done by Widyawati  
216 et al. (2020) method with slight modification. About 150  $\mu$ L samples containing 100  $\mu$ L  
217 *Pluchea* infusion and 50  $\mu$ L pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH  
218 7) were reacted with 50  $\mu$ L  $\alpha$ -glycosidase 2 mM (0.0833 unit/mL), and then the mixture  
219 was incubated at 37  $^{\circ}$ C for 15 min. The reaction was stopped with the addition of 1000  
220  $\mu$ L sodium carbonate 0.2 M. The amount of these enzymes that didn't react with  
221 bioactive compounds of *Pluchea* infusion hydrolyzed p-nitrophenyl- $\alpha$ -D-glucopyranoside  
222 (pNPG) as a substrate to result in p-nitrophenol. The inhibition activity of the *Pluchea*  
223 infusion was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800,  
224 Shimadzu, Japan) at  $\lambda$  405 nm. The inhibition percentage of  $\alpha$ -glycosidase was

225 calculated using formula:  $(AC_b - AC_a) - (A_s - A_b) (AC_b - AC_a) \times 100 \%$ . Where,  $AC_b$   
226 is the absorbance of 100 % enzyme activity (solvent with enzyme),  $AC_a$  is the  
227 absorbance of 0 % enzyme activity (solvent without enzyme),  $A_s$  is the absorbance of  
228 test sample with enzyme,  $A_b$  is the absorbance of test sample without enzyme.

229

## 230 ANALYSIS OF PHENOLICS

231 The phenolic compounds of the samples were analyzed by HPLC based on  
232 Kongkiatpaiboon et al. (2018) method with modifications. Each *Pluchea* infusion was  
233 sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter  
234 syringe (Whatmann, 0.2  $\mu$ m, NYL). About 20  $\mu$ L of sample was injected in an HPLC  
235 (LC20AD series, Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence  
236 UFLC LC-20AD pump, CTO-30A column oven, CBM-20A/20 Alite system controller,  
237 and SPD-20A/20 AV UV-Vis detector. Separation of phenolic compounds in samples  
238 was carried out using a Shim-pack VP-ODS C18 column (ID 5  $\mu$ m  $\times$  50 mm  $\times$  4.6 mm)  
239 with a GVP-ODS Cartridge guard column (2 pieces) (ID 10 mm  $\times$  4.6 mm). The mobile  
240 phase used consisted of a solution of (A) 0.5 % acetic acid in water and (B) absolute  
241 methanol. Analysis was carried out using a gradient system in the following order: initial  
242 conditions of 10 % B in A to 50 % B in A were maintained for 40 minutes; then 100 % B  
243 was maintained for 20 minutes. Next the column was re-equilibrated with 10 % B in A  
244 maintained for 10 minutes before analysis of the next sample. The sample flow rate was  
245 set at 1.0 ml/min with a controlled temperature at 40 °C. Detection was used at a  
246 wavelength of 280 nm. The reference standard used were gallic acid, (+)-catechin,  
247 myricetin, quercetin, kaempferol, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and

248 4,5-dicaffeoylquinic acid. All of reference standard was dissolved in distilled water and  
249 prepared similar to the samples before injected in HPLC.

250

## 251 EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

252 The research design used a randomized block design with two factors, i.e., the  
253 steeping temperature (T) and the storage period. *Pluchea* leaf blades were subjected to  
254 4 steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95 °C (T4),  
255 and the storage period of 0 year /un-stored (B1), and 5 year/stored (B2) resulting in 8  
256 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The  
257 HPLC analysis of phenolic was repeated six periods. The data analysis of samples was  
258 repeated for six periods. The data were analyzed using a paired t-test at  $\alpha \leq 0.05$ ,  
259 treatment means of specific phenolic compounds that were identified were expressed  
260 as the mean  $\pm$  SD. The analysis used SPSS 23.0 software (SPSS Inc., Chicago, IL,  
261 USA).

262

## 263 RESULTS AND DISCUSSIONS

264

### 265 BIOACTIVE COMPOUNDS

266

#### Phenolic Compounds

267 The bioactive compounds are active compounds in plants that are essential to  
268 protect a body health (Nguyen and Chuyen, 2020). These compounds usually have  
269 many biological activities, such as antioxidant, antidiabetic, anti-inflammatory,  
270 anticancer, antimicrobial, antibacterial, anti-cholesterol, and so on (Suriyaphan, 2014;

271 Acar et al., 2022). Phenolic compounds have potential redox properties that can  
272 scavenge free radicals that can cause a number of chronic diseases (Noreen et al.,  
273 2017; Aryal et al., 2019; Acar et al., 2022).

274 The total phenolic content (TPC) of *Pluchea* infusion at different steeping  
275 temperature and storage period generally significantly increased with increasing  
276 steeping temperature and storage period based on paired t-test at  $\alpha \leq 0.05$  (Figure 1a).  
277 Steeped and stored infusion had significantly higher amounts of phenolic compounds  
278 than the samples that were steeped and un-stored. Further, the highest total phenolic  
279 content was observed in samples infused at 95 °C and stored for 5 years (at 71.38±4.14  
280 mg GAE/g sample) while the lowest was measured in the un-stored samples and  
281 infused at 60 °C (at 4.39±0.49 mg GAE/g sample). The phenolic content of stored  
282 samples that were steeped only at 60 and 95 °C showed a significant increase in their  
283 phenolic content. This implies that the steeping temperature and the storage periods  
284 significantly resulted in the high amounts of phenolic compounds in the infusions.  
285 Results also indicated that phenolic compounds were generally greater in the infusion at  
286 high steeping temperatures and long storage period. This could have been due to the  
287 fact that the steeping temperature and storage period could cause the process of  
288 degradation, oxidation, and leaching/release of phenolic compounds. Phenolic  
289 compounds are water soluble and thus soaking in hot water for a certain period of  
290 period as in steeping causes the migration process of more phenolic compounds to the  
291 water because of longer exposure of phenolic compounds to water (Castiglioni et al.  
292 (2015); Kilic et al. (2017), and Acar et al. (2022). Su et al. (2019) reported that  
293 temperature treatment can stimulate the release of phenolic compounds and increase

294 antioxidant activity of lychee juice stored at different temperatures of 4 and 45 °C and  
295 different long storage (fresh and 72 hours).

296 Temperature treatment degrades (or hydrolyzes) the hydrogen bond between  
297 phenolic compounds and proteins resulting in an increase of phenolic compounds when  
298 exposed to higher temperatures (Ali et al. (2018); Jayani et al. (2022) and Ramphinwa  
299 et al. (2023)). Zhang et al. (2021) reported that phenolic compounds present in plants  
300 are not completely stable, but are easily degraded during storage after harvest. Reblova  
301 (2012) claimed that antioxidant compounds can be slowly degraded with increasing  
302 temperature. Fibrianto et al. (2021) also stated that the brewing temperature has an  
303 effect on the extracted antioxidant compounds, such as alkaloids, catechins, and  
304 tannins. Thus, there is an assumption that temperature and storage caused the  
305 degradation, oxidation, and hydrolysis of the phenolic compounds period resulting in the  
306 increased amount of the phenolic compounds at higher steeping temperature and  
307 longer storage period.

308 Simple phenolic compounds are identified in steeped and stored. *Pluchea leaf*  
309 infusion included gallic acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-  
310 O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic acids  
311 was showed in Table 1. The treatment effects using a t-test at  $\alpha \leq 0.05$  showed that  
312 gallic acid and kaempferol content were insignificantly different at various steeping  
313 temperatures and storage periods. The concentration of quercetin and 3,5-di-O-  
314 caffeoylquinic acid of the un-stored and stored *Pluchea* infusion was significantly  
315 different from the rest of the samples between 70 °C while (+)-catechin concentration  
316 of *Pluchea* infusion was only significantly different at 95 °C. The myricetin content was

317 significantly different at 80 and 95 °C. The 3,4-di-O-caffeoylquinic acid content showed  
318 significant difference at 60, 80, and 95 °C while 4,5-di-O-caffeoylquinic acid content was  
319 only significantly different at 60 °C.

320 Results further showed that gallic acids and kaempferol were relatively stable as  
321 reflected by the insignificant changes when exposed to the different steeping  
322 temperature and storage period. Myricetin, (+)-catechin, and 3,4-di-O-caffeoylquinic  
323 acid showed a drastic increase at higher steeping temperatures and longer storage  
324 period implying that these compounds tended to be relatively labile. Quercetin, 3,5-di-O-  
325 caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid underwent moderate changes.  
326 Therefore, myricetin, (+)-catechin, and 3,4-di-O-caffeoylquinic acid were easier to  
327 dissolve or degrade to form simple phenolic acids at higher temperatures and storage  
328 period (Su et al. (2019, Ali et al. (2018); Jayani et al. (2022); Ramphinwa et al. (2023),  
329 and Zhang et al. (2021). Degradable polyphenol compounds have a simple structure  
330 and free hydroxyl groups that can react with Folin-Ciocalteu's Phenol reagent, resulting  
331 complex blue solution that can detected as total phenolic content.

### 332 Flavonoid Content (TFC)

333 Flavonoids are the major phenolic compounds that have potential chemical and  
334 biological activities, such as radical scavenging and antimicrobial activities (Ayele et al.,  
335 2022; Chandra et al., 2014) that can protect the human body from the oxidative stress  
336 caused by many degenerative diseases, especially cancer, cardiovascular problems  
337 and aging (Mathur and Vijayvergia, 2017). The total flavonoid content of steeped  
338 *Pluchea* infusion decreased with longer storage period. Un-stored samples exhibited  
339 higher flavonoid content than the stored samples. The statistical analysis using a paired

340 t-test at  $\alpha \leq 0.05$  showed that the total flavonoid content of *Pluchea* infusion was  
341 significantly different between the steeped un-stored and steeped stored samples  
342 (Figure 1b). The highest total flavonoid content was exhibited by the un-stored samples  
343 steeped at 95°C at about 147.42±14.03 mg CE/g sample. Total flavonoid content was  
344 significantly lower in the stored samples than those of the un-stored samples implying  
345 that the increase in the flavonoid content of the infusion was affected primarily by the  
346 steeping temperature.

### 347 Tannin Content (TTC)

348 Tannins are bioactive compounds that provide properties, such as astringent,  
349 anti-diarrheal, antibacterial and antioxidant (Malangngi et al., 2012). Generally, results  
350 indicated that the total tannin content of *Pluchea* infusion significantly increased with  
351 increasing steeping temperature and storage period (Figure 1c). Among, the un-stored  
352 steeped samples, the tannin content was significantly lowest in the samples infused at  
353 60 °C at about 4.81±0.58 to 17.42±1.04 mg TAE/g samples which was significantly  
354 different lower from that of the lowest tannin content of the stored samples. Among the  
355 stored and steeped samples, the highest tannin content was observed at samples  
356 steeped at 95 °C about 17.42 ± 1.04 mg TAE/g samples, and was significantly different  
357 from that of the highest tannin content of the un-stored steeped samples at 95 °C about  
358 9.22 ± 1.48 mg TAE/g samples. Indicating that the tannin content was primarily affected  
359 by a longer storage period than high steeping temperature. The condensation of  
360 catechins to tannins is a dominant process occurring in tea leaves that is accelerated  
361 during the maceration of raw tea leaves (Kowalska et al., 2021) and could have had  
362 contributed to the observed increase in the tannin content in the treated samples.

363 Although, high temperature and long storage period can cause the degradation  
364 of tannins to catechins. Rusita et al. (2019) emphasized that tannins are polar  
365 thermostable complex compounds, that are resistant to heating, indicating that even  
366 with the exposure to high temperature, the tannins still remained high in the treated  
367 samples period.

### 368 Antioxidant Activity

369 Antioxidant activity is capability of compounds to inhibit the oxidation of  
370 macromolecules from biological target that involve in oxidative chain reactions (Ali et al.,  
371 2005; Oh et al., 2013). The antioxidant activity assay was done in this research using  
372 DPPH Free Radical Scavenging Activity (DPPH) and ferric reducing power (FRAP)  
373 methods. The phenolic compounds are an active antioxidant that have antioxidant  
374 capability that depends on their redox properties. The structure of phenolic compounds  
375 determines the effectivity to donate hydrogen atom which is negatively correlated with  
376 the O-H phenolic bond strength. The antioxidant power of phenolic compounds is due to  
377 the weak hydrogen bonds in the OH group of the phenolic compound so that it is easier  
378 to donate hydrogen atoms (Kruk et al., 2022). The mechanism of phenolic compounds  
379 as antioxidants depends on their ability to donate hydrogen atoms and transfer  
380 electrons, and as reducing agents and singlet oxygen quenchers (Ali et al., 2005;  
381 Huang et al. 2005).

### 382 DPPH Free Radical Scavenging Activity

383 DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to  
384 evaluate antioxidant activity because this method is simple that is suitable to measure  
385 the donating hydrogen atoms capability of herbal infusion. This reaction can cause the



386 purple color of DPPH to change to yellow color (Munteanu and Apetrei, 2021; Baliyan et  
387 al., 2022). Figure 2a. shows that the free radical scavenging properties of the stored  
388 and steeped samples were significantly higher than the un-stored steeped samples. It  
389 can also be observed that the free radical scavenging property was significantly  
390 different among the stored and steeped samples but insignificant among the un-stored  
391 and steeped sample period. *Pluchea* infusion stored at room temperature for 5 years  
392 resulted in high free radical scavenging activity by more than 10%. Steeping at higher  
393 temperatures significantly increased the DPPH free radical scavenging activity in stored  
394 *Pluchea* infusion by around 15 to 25 %. This implies that the higher free radical  
395 scavenging property was primarily affected by the storage period than the steeping  
396 temperature. During the storage process, it is possible to form complex phenolic  
397 compounds which provide a high ability to scavenge free radicals (Thanajiruschaya et  
398 al., 2010).

399 The scavenging activity of the samples was strongly and positively correlated  
400 with total phenolic and tannin contents, but inversely with total flavonoid levels (Table  
401 2). The antioxidant activity was strongly and negatively correlated with flavonoid  
402 content. The storage period could be reduced flavonoid content. The study also  
403 demonstrated that longer storage period and higher infusion temperatures produced  
404 many simple phenolic compounds with free hydroxyl groups capable to donate  
405 hydrogen atoms to DPPH free radicals. Many phenolic acids, such as gallic acids, (+)-  
406 catechins, myricetins, kaempferols, quercetins, 3,5-di-O-caffeoylquinic acids, 3,4-di-O-  
407 caffeoylquinic acids, 4,5-di-O-caffeoylquinic acids have established potential antioxidant  
408 activity (Kumar and Goel, 2019) (Table 1). Kruk et al (2022) informed that the

409 capability of phenolic compounds to donate hydrogen atom depends on chemical  
410 structure, number and position of hydroxyl groups attached to a benzene ring, a double  
411 bond between C2 and C3 rings, and a carbonyl group (C=O) on the C ring at C4. The  
412 effectivity of antioxidant compounds to donate hydrogen atom is determined by O-H  
413 bond dissociation energy.

414 **The free radical** scavenging property observed in the study was not **in** consistent  
415 with the results of the study by Moraes-de-Souza et al. (2008). The research shows that  
416 total phenolic content of herbal infusion is low correlated with **free radical scavenging**  
417 activity. However, Dobrinas et al. (2021) informed that total phenolic content **is positively**  
418 and significantly correlated with **the free radical scavenging property of tea infusion.**

#### 419 **Ferric Reducing Antioxidant Power (FRAP)**

420 FRAP is an analysis of the antioxidant power of the phytochemical compounds  
421 **that is based of** the ability of antioxidant compounds to reduce iron ions of potassium  
422 ferricyanide ( $\text{Fe}^{3+}$ ) to potassium ferrocyanide ( $\text{Fe}^{2+}$ ). Potassium ferrocyanide reacts with  
423 ferric chloride to form a ferric-ferrous complex and results green color solution  
424 (Widyawati et al., 2017; Raharjo and Haryoto, 2019).

425 The results showed that the ferric reducing antioxidant power (FRAP) increased  
426 **at** higher steeping temperature and longer **er** storage **period.** The lowest FRAP was  
427 observed in the un-stored samples which were steeped at 60 °C at  $3.95 \pm 0.17$  mg gallic  
428 acid equivalents (GAE)/g samples, and the highest was **exhibited in** *Pluchea* infusion  
429 which was stored for 5 years at 95 °C at  $48.63 \pm 10.83$  mg gallic acid equivalents  
430 (GAE)/g samples (Figure 2b). FRAP increased significantly as the steeping temperature

431 was increased. FRAP of the samples stored for 5 years was also significantly higher  
432 than the un-stored samples at  $\alpha \leq 0.05$ .

433 This is in contrast with the study on the antioxidant activity of DPPH and FRAP of  
434 matcha. The longer storage period reduces the levels of catechin content due to the  
435 catechins, such as epigallocatechin gallat (EGCG), epicatechin gallat (ECG),  
436 epigallocatechin (EGC), and epicatechin (EC) which are bioactive compounds that have  
437 high antioxidant activity (Kim et al. 2020). The ferric-reducing capability of *Pluchea*  
438 could have been due to the presence of simple phenolic acid that can transfer electrons  
439 from their free hydroxyl groups of sample. The FRAP of *Pluchea* infusion was strongly  
440 and positively significantly correlated with the DPPH, TPC, and TTC, but inversely to  
441 TFC.

## 442 ANTIDIABETIC ACTIVITY

### 443 Alpha amylase enzyme inhibition activity (AA)

444 Antidiabetic activity is a measure of the potency of phenolic compounds to  
445 regulate the uptake of glucose by the cells from the blood through the mediation of 2-  
446 digestive enzymes i.e.,  $\alpha$ -amylase and  $\alpha$ -glucosidase, which are involved the control of  
447 dietary carbohydrate digestion and release in the postprandial blood glucose in human  
448 body (Fu et al., 2017). The phenolic compounds have the capability to bind with the  
449 protein component of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Martinez-Solis et al.,  
450 2022) resulting in the reduced activity of the enzymes. The results showed that lower  
451 steeping *Pluchea* leaf infusion was able to inhibit the action of the  $\alpha$ -amylase enzymes  
452 (Figure 3a). The *Pluchea* infusion exhibited a good  $\alpha$ -amylase enzyme inhibition activity  
453 of more than 50 % and even almost 100 % in un-stored *Pluchea* infusion steeped at 60,

454 70, and 80 °C with the highest at 60 °C, and in stored *Pluchea* leaf infusion which was  
455 steeped at 60 °C. The stored *Pluchea* leaf infusion steeped at 70, 80, and 95 °C for 5  
456 minutes had lower enzyme inhibition activity of less than 50 % with the lowest at 95 °C  
457 around 13 %. Widyawati et al. (2017) found that the ability to inhibit the  $\alpha$ -amylase  
458 enzyme in un-stored *Pluchea* infusion steeped at 95 °C for 5 minutes was also low at  
459 28.79 %. Increasing the steeping temperature and storage period reduced the ability of  
460 the phytochemicals in the *Pluchea* infusion to inhibit the  $\alpha$ -amylase enzyme activity  
461 period. Table 2 further shows that the AA of *Pluchea* infusion was strongly and  
462 negatively significantly correlated with TPC, TTC, DPPH, and FRAP, but it was weakly  
463 and positively significantly correlated with TFC.

464 This inhibitory activity was thought to be contributed by other bioactive  
465 compounds, besides phenolics which are sensitive to steeping temperature and storage  
466 period. Li et al. (2018) stated that there are flavonoid compounds that contribute to the  
467 ability to inhibit the  $\alpha$ -amylase enzyme. Akah et al. (2011) reported that phytochemical  
468 compounds, such as terpenoids, saponins, flavonoids, glycosides/carbohydrates, and  
469 alkaloids are good antidiabetic metabolites or  $\alpha$ -amylase enzyme activity inhibitors.  
470 Sangeetha and Vedesree (2012) explained that the ability of *Threspesia populnea*  
471 extract to inhibit the  $\alpha$ -amylase enzyme was determined of their phenolic compound  
472 content and protein. Moreover, the presence of  $\alpha$ -amylase enzyme inhibitor in this  
473 extract may be proteinaceous or nonproteinaceous in nature. It was assumed that this  
474 enzyme inhibitory activity in *Pluchea* infusion also was determined with their protein and  
475 polyphenolic content. Aleixandre et al. (2022) also stated that phenolic acids have  
476 inhibition activity to  $\alpha$ -amylase enzyme depending on their structures. There are C=C

477 double bonds conjugated with a carbonyl group of phenolic structures that stabilize the  
478 binding forces to the active site of the  $\alpha$ -amylase. The hydroxyl groups can bind by non-  
479 covalent interaction (hydrogen bonding, cation- $\pi$  interactions, salt bridge interactions,  
480 ionic interactions, or electrostatic forces) with amino acid residue at the active site in  $\alpha$ -  
481 amylase enzyme. Elevated steeping temperature and longer storage period can easily  
482 cause the removal of the hydroxyl groups of phenolic compounds that can reduce their  
483 ability of enzyme inhibition. The phenolic acids with a greater number of hydroxyl  
484 groups exhibits stronger capability to obstruct the  $\alpha$ -amylase enzyme.

#### 485 Alpha glucosidase enzyme inhibition activity (GA)

486 Alpha glucosidase is an important enzyme in carbohydrate digestion, that  
487 catalysis the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and  
488 converts them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et  
489 al., 2014; Proenca et al., 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -  
490 glucosidase enzyme is used to determine their antidiabetic activity. This is supported by  
491 Werdani and Widyawati (2018) stated that *Pluchea* infusion has the potential as an  
492 antidiabetic agent. Widyawati et al. (2020) found that the steeping of un-stored *Pluchea*  
493 infusion at 95 °C for 5 minutes has an inhibitory effect on the  $\alpha$ -glucosidase enzyme of  
494 67.857 %.

495 Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -  
496 glucosidase enzyme decreased with increasing steeping temperature and storage  
497 period. Steeping at 95 °C of the un-stored *Pluchea* leaf infusion obtained the lowest  
498 inhibitory ability, i.e.,  $48.32 \pm 1.27$  %, and the highest inhibitory activity was at 70 °C at  
499  $95.11 \pm 0.70\%$ . The results of a paired t-test showed that GA of *Pluchea* infusion was

500 significantly different between steeping temperature and long storage. Figure 3 further  
501 shows that the ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme  
502 tended to be higher than the ability to inhibit the  $\alpha$ -amylase enzyme. Data analysis in  
503 Table 2. showed that the TFC of the *Pluchea* leaf infusion was influenced weakly and  
504 positively by GA and AA, but the GA and AA were not affected by TPC, TTC, DPPH,  
505 and FRAP. Li et al. (2018) stated that flavonoid compounds can inhibit the action of the  
506  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. Dias et al. (2021) stated that flavonoid  
507 compounds, such as rutin, myricetin, kaempferol, and quercetin have antioxidant and  
508 antihyperglycemic activities. The ability to inhibit the action of enzymes from flavonoid  
509 compounds is determined by the position and number of hydroxyl groups, the number of  
510 double bonds in rings A and B, and the heterocyclic ring in ring C. Tadera et al. (2006)  
511 and Zhang et al. (2014) also explained that flavonoid compounds of samples  
512 significantly inhibit the  $\alpha$ -glucosidase enzyme activity.

513 The ability to inhibit the  $\alpha$ -glucosidase enzyme from *Pluchea* infusion was  
514 significantly affected by the steeping temperature and long storage. Figure 3 also  
515 showed that the capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme  
516 was greater than the  $\alpha$ -amylase enzyme because the mechanism of the two enzymes  
517 was different, according to the opinion of McCue et al. (2005). The mechanism of the  $\alpha$ -  
518 glucosidase enzyme inhibitor includes making the sugar mimic structure, binding using  
519 ionic bonds with nucleophilic, making the transition state-like structure, binding  
520 hydrogen with catalytic acid residue, interacting ionic and hydrophobic with site other  
521 than the active site, and binding covalent with enzymes through an epoxy or aziridine  
522 group (Moorthy et al., 2012). Then, the mechanism of the  $\alpha$ -amylase enzyme inhibitor

523 includes blocking carbohydrates, limiting the digestibility and absorption of  
524 carbohydrates, and blocking the active centers of several subsites of the enzyme (Gong  
525 et al., 2020).

526 Widyawati et al. (2017) stated that phenolic and non-phenolic compounds can  
527 inhibit of the  $\alpha$ -glucosidase enzyme activity. The ability of bound phenolic compounds to  
528 inhibit  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. The presence  
529 of polymerization and degradation reactions, that may be occurred in *Pluchea* infusion  
530 during storage, affects the structure and profile of phenolic and non-phenolic  
531 compounds. Asriningtyas et al. (2014) explained that the methyl-esterified quinic acid  
532 with the caffeic groups, such as 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic  
533 acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic  
534 acid, and 1,3,4,5-tetra-*O*-caffeoylquinic acid of *Pluchea* leaves inhibits the  $\alpha$ -  
535 glucosidase enzyme activity. The resulting analysis of caffeoylquinic acids (3,4-di-*O*-  
536 caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid in  
537 stored *Pluchea* leaf infusion higher concentration than in un-stored *Pluchea* infusion,  
538 and the concentrations of the simple phenolic compounds were increased at higher  
539 steeping temperature, but the  $\alpha$ -glucosidase inhibition activity of them was reduced. It  
540 means that the methyl-esterified quinic acid with the caffeic groups had more potential  
541 to inhibit  $\alpha$ -glucosidase enzyme than free caffeoylquinic acid.

542 This study showed that the increasing steeping temperature and storage period  
543 caused degradation of polyphenol compounds to produce simple phenolic compounds,  
544 such as gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,4-di-*O*-  
545 caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid that

546 increased the total phenolic content and total tannin content. The increase in the simple  
547 phenolic concentration of the *Pluchea* leaf infusion caused higher antioxidant activity  
548 and lower antidiabetic activity.

## 549 CONCLUSION

550 The Total Phenol (TPC) of *Pluchea* infusion at different steeping temperatures  
551 and storage periods generally significantly increased with increasing steeping  
552 temperature and storage periods. Steeped and stored infusion had significantly higher  
553 amounts of phenolic compounds than the samples that were steeped and un-stored.  
554 TPC was highest in the stored and steeped at 95°C and lowest in the un-stored and  
555 steeped at 60°C. Un-stored steeped samples exhibited significantly higher flavonoid  
556 content than the stored steeped samples. The highest total flavonoid content was  
557 exhibited by the un-stored samples steeped at 95°C. The total tannin content of *Pluchea*  
558 leaf infusion significantly increased with increasing steeping temperature and storage  
559 period. Among the un-stored steeped samples, the tannin content was significantly  
560 lowest in the samples steeped at 60°C and highest in the samples steeped at 95°C.

561 The free radical scavenging property (DPPH) of the stored and steeped *Pluchea*  
562 leaf infusion was significantly higher than the un-stored steeped samples. The free  
563 radical scavenging property was highest in the stored samples steeped at 80 and 95°C.  
564 free radical scavenging activity of the samples was strongly and positively correlated  
565 with total phenolic and tannin contents, but inversely with total flavonoid levels. The  
566 ferric-reducing antioxidant power (FRAP) significantly increased with increasing  
567 steeping temperature and longer storage periods. The lowest FRAP was found in the  
568 un-stored samples which were steeped at 60°C and the highest was exhibited in



569 *Pluchea* stored samples which were stored for 5 years and steeped at 95°C. The FRAP  
570 of *Pluchea* leaf infusion was significantly strong and positively correlated with the free  
571 radical scavenging property, total phenolic, and total tannin content, but inversely with  
572 total flavonoid content. The inhibition of the  $\alpha$ -amylase activity was generally found to be  
573 higher at lower steeping temperatures of the un-stored *Pluchea* leaf infusion than at  
574 higher steeping temperatures of the stored sample. The  $\alpha$ -amylase enzyme inhibition  
575 capacity of the *Pluchea* leaf infusion showed a significantly strong and negative  
576 correlation with TPC, TTC, DPPH, and FRAP, but it was weakly and positively  
577 correlated significantly with TFC.

578 The ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme  
579 decreased at high steeping temperatures and long storage periods. The highest  
580 inhibitory activity was obtained in the un-stored *Pluchea* leaf infusion that was steeped  
581 at 70°C while the lowest was obtained in the un-stored sample that was steeped at  
582 95°C. The ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to  
583 be higher than the ability to inhibit the  $\alpha$ -amylase enzyme. The inhibition of the  $\alpha$ -  
584 glucosidase enzyme activity was significantly strong and negative TPC, TTC, DPPH,  
585 and FRAP, and it was weakly and positively correlated significantly with TFC.

586 The simple phenolic compounds identified in *Pluchea* leaf infusion may affect the  
587 presence of the bioactive compounds, antioxidant potential, and antidiabetic properties  
588 at different steeping temperatures and storage periods including gallic acids, (+)-  
589 catechins, myricetins, kaempferols, quercetins, 3,5-di-O-caffeoylquinic acids, 3,4-di-O-  
590 caffeoylquinic acids, 4,5-di-O-caffeoylquinic acids.

591

592 DATA AVAILABILITY

593 Table and figure used to support this study were included in the article.

594 CONFLICT OF INTEREST

595 The authors declare no conflict of interest.

596 ACKNOWLEDGEMENTS

597 The authors would like to thank the Ministry of Education and Culture of the Republic of  
598 Indonesia for a fundamental research grant to higher education institutions in 2022

599 REFERENCES

600 ACAR A, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with  
601 food wastes:evaluation of temperature and time effects on quality parameters. Applied  
602 Food Res. 2(100087):1-8.

603 ALEIXANDRE A et al. 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  
604  $\alpha$ -glucosidase and influence of reaction conditions. Food Chem. 372(131231):1-9.

605 ALI et al. 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile.  
606 Food Chem. 89(2005):27-36.

607 ALI A. et al. 2018. Impact of storage conditions on the stability of predominant phenolic  
608 constituents and antioxidant activity of dried *Piper bettle* extracts. Molecules.23(24):1-  
609 13.

610 AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and  
611 methanol extract and fractions of *Gongronema latifolium* (Asclepidaceae) leaves in  
612 alloxan diabetic rats. J Applied Pharm. Sci. 01(09): 99-102.

613 ARSININGTYAS IS. et al. 2014. Identification of  $\alpha$ -glucosidase inhibitors from the  
614 leaves of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural  
615 product use. *Natural Product Res.* 28(17):1350-1353.

616 ARYAL S et al. 2019. Total phenolic content, flavonoid content and antioxidant of wild  
617 vegetables from Western Nepal. *Plants.* 8(96): 1-12.

618 AYELE DT, AKELE ML, MELESE A.T. 2022. Analysis of total phenolic contents,  
619 favonoids, antioxidant and antibacterial activities of *Croton macrostachyus* root extracts.  
620 *BMC Chem.* 16(30): 1-9.

621 BALIYAN S et al. 2022. Determination of antioxidants by DPPH radical scavenging  
622 activity and quantitative phytochemical analysis of *Ficus religiosa*. *Molecules.*  
623 27(1326): 1-19.

624 CASTIGLIONI S et al. 2015. Influence of steeping conditions (time, temperature, and  
625 particle size) on antioxidant properties and sensory attributes of some white and green  
626 teas. *Intern. J. Food Sci. Nut.* Online 1-7.

627 Chan EWC et al. 2022. *Pluchea indica*: An updated review of its botany, uses, bioactive  
628 compounds and pharmacological properties. *Pharmaceutical Sciences Asia.* 49(1): 77-  
629 85.

630 CHANDRA S et al. 2014. Assessment of total phenolic and flavonoid content,  
631 antioxidant properties, and yield of aeroponically and conventionally grown leafy  
632 vegetables and fruit crops: A Comparative study. Hindawi Publishing Corporation  
633 Evidence-Based Complementary Alter. Med. 2014:1-9 (2014)

634 CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic,  
635 flavonoids, tannin and chlorophyll content of leaves of *Strobilanthes Kunthiana*  
636 (*Neelakurinji*). J.Med. Plants Studies. 4(4):282-286.

637 DIAS MC, PINTO DCGA, SILVA AMS. 2021. Plant flavonoids: chemical characteristics  
638 and biological activity. Molecules. 26(5377):1-16.

639 DOBRINAS S, SOCEANU A, POPESCU V, POPOVICI IC, JITARIU D. 2021.  
640 Relationship between total phenolic content, antioxidant capacity, Fe and Cu content  
641 from tea plant samples at different brewing times. Processes. 9(1311):1-11.c

642 FERRARIS Z. 2023. *Pluchea indica* Less. [iNaturalist.org.](https://www.gbif.org/occurrence/4022273660)  
643 <https://www.gbif.org/occurrence/4022273660>.

644 FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional  
645 properties and visual appearance of Dampit Robusta coffee leaves tea. Advances in  
646 Food Sci. Sustain. Agric. Agro-industrial Engineering. 4(1):25-32.

647 FU L et al. 2017. Total phenolic contents and antioxidant capacities of herbal and tea  
648 infusions. Intern. J. Molecular Sci. 12:2112-2124.

649 GAO MR et al. 2019. A theoretical and experimental study: the influence of different  
650 standards on the determination of total phenol content in the Folin–Ciocalteu assay. J.  
651 Food Measurement and Characteristic. 13:1349–1356.

652 GONG L, FENG D, WANG T, REN Y, LIU Y, WANG J. 2020. Inhibitors of  $\alpha$ -amylase  
653 and  $\alpha$ -glucosidase: Potential linkage for whole cereal foods on prevention of  
654 hyperglycemia. Food Sci. Nut. 2020(8):6320-6337.

655 HUANG D, OU B, PRIOR RL. 2005. The Chemistry behind antioxidant capacity assays.  
656 J. Agric. Food Chem. 53:1841-1856.

657 ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties  
658 of rosehip tea beverage: study by response surface methodology. J. Food Sci. Tech.  
659 54(11):3737–3743.

660 JAYANI NIE et al. 2022. Evaluation of stability and quality characteristics of moringa  
661 (*Moringa oleifera*) herbal tea during storage. Food Res. 6(4):399-406.

662 KIM JM et al. 2020. Effect of storage temperature on the antioxidant activity and  
663 catechins stability of Matcha (*Camellia sinensis*). Food Science and Biotechnology.  
664 29(9):1261–1271.

665 KILIC et al. 2017. Antioxidant properties of some herbal teas (green tea, senna, corn  
666 silk, rosmery) brewed at different temperatures. Intern. J. Secondary Metabolite.  
667 4(3):142-248 Special Issue 1.

668 KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of  
669 extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica*  
670 leaves from different provenances in Thailand. Revista Brasileira de Farmacognosia.  
671 28(2018): 145–150.

672 KOWALSKA J et al. 2021. Influence of tea brewing parameters on the antioxidant  
673 potential of infusions and extracts depending on the degree of processing of the leaves  
674 of *Camellia sinensis*. Molecules.26(4773):1-17.

675 KRUK J, ABOUL-ENAIN BH, DUCHNIK E, MARCHLEWICZ M. 2022. Antioxidative  
676 properties of phenolic compounds and their effect on oxidative stress induced by severe  
677 physical exercise. J. Physiol. Sci. 72(19):1-24.

678 LI K et al. 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the  
679 flavonoids rich extract from *Scutellaria baicalensis* shoots and interpretation of structure

680 activity relationship of its eight flavonoids by a refined assign score method. Chem.  
681 Central J. 12(82):1-11.

682 LIN YS. et al. 2020. Effects of storage time and temperature on antioxidants in juice  
683 from *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser.  
684 Molecules. 25(3614): 1-12.

685 MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content  
686 and antioxidant activity test of avocado seed extract (*Persea Americana* Mill). Jurnal  
687 MIPA Unsrat Online. 1(1): 5-10. [https://media.neliti.com/media/publications/116214-ID-  
688 penentuan-kandungan-tanin-dan-uji-aktivi.pdf](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf) [In Bahasa Indonesia]

689 MARTINEZ-SOLIS J et al. 2022. Antidiabetic and toxicological effect of the tea infusion  
690 of summer collection from *Annona cherimola* Miller leaves. Plants. 11(3224):1-17.

691 MATHUR R, VIJAYVERGIA R. 2017. Determination of total flavonoid and phenol  
692 content in *Mimusops Elengi* Linn. Intern. J. Pharm. Sci. Res. 8(12): 5282-5285.

693 MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of  
694 sprouted and solid-state bioprocessed soybean. Asia Pacific J. Clinical Nut. 14(2): 145-  
695 152.

696 MOORTHY NS, RAMOS MJ, A FERNANDES P. 2012. Studies on  $\alpha$ -glucosidase  
697 inhibitors development: Magic molecules for the treatment of carbohydrate mediated  
698 diseases. Mini Rev Med Chem. 12:713–720.

699 MORAES-DE-SOUZA RA, OLDONI TLC, REGITANO-D'ARCE MAB, ALENCAR SM.  
700 2008. Antioxidant activity and phenolic composition of herbal infusions consumed in  
701 Brazil. Cienc Tecnol. Aliment. 6(1):41-47.

702 MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining  
703 antioxidant activity: A Review. Intern. J. Molecular Sci. 22(3380):1-30.

704 NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus*  
705 *sabdariffa* L.): Effect of dying temperature and brewing conditions on total soluble solid,  
706 phenolic content, antioxidant capacity and sensory quality. Beverages.6(2):1-11 (2020).

707 NOREEN H. et al. 2017. Measurement of total phenolic content and antioxidant activity  
708 of aerial parts of medicinal plant *Coronopus didymus*. Asian Pacific J. Tropical Med.  
709 10(8): 792–801.

710 NURCHOLIS GW et al. 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity  
711 in-vitro and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum*  
712 *pictum* (L.). Current Biochem. 1(2): 58-65.

713 OH J et al. 2013. Antioxidant and antimicrobial activities of various leafy herbal teas.  
714 Food Control. 31(2013):403-409.

715 PROENCA C et al. 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an in vitro and in silico  
716 structure–activity relationship study. J. Enzyme Inhibition Med. Chem. 32(1): 1216–  
717 1228.

718 PUREWAL SS et al. 2022. Unraveling the effect of storage duration on antioxidant  
719 properties, physicochemical and sensorial parameters of ready to serve Kinnow-aml  
720 beverages. Applied Food Res. 2(1): 1-8.

721 RAHARJO D, HARYOTO. 2019. Antioxidant activity of mangrove *Sonneratia caseolaris*  
722 L. using the FRAP method. ISETH. 2019: 623-629.

723 RAMPHINWA ML et al. 2023. Eco-physiological response of secondary metabolites of  
724 teas: review of quality attributes of herbal tea. *Frontiers in Sustain. Food Systems*.  
725 7(990334):1-14.

726 REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids.  
727 *Czech J. Food Sci.* 30(2):171-177.

728 RUAN J et al. 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea*  
729 *indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel  
730 column chromatography fractions. *Molecules*. 24(2784):1-20.

731 RUSITA YD et al. 2019. Comparison of tannin concentration on fresh and dried tea leaf  
732 (*Camelia sinensis* L.) extract using UV-visible spectrophotometric method. 1st  
733 International Conference of Health, Science & Technology (ICOHETECH) 2019 E-ISBN:  
734 978-623-92207-0-9. November 16<sup>th</sup>, 2019.LPPM Universitas Duta Bangsa Surakarta,  
735 Surakarta.

736 SANGEETHA R, VEDASREE N. 2012. In Vitro  $\alpha$ -Amylase inhibitory activity of the  
737 leaves of *Thespesia populnea*. *Intern. Scholarly Res.Network*. 2012: 1-4 (2012)

738 SHRAIM AM et al. 2021. Determination of total flavonoid content by aluminum chloride  
739 assay: A critical evaluation. *LWT Food Sci. Tech.* 150(111932):1-11.

740 SILVA-RAMIREZ KA et al. 2020. Effect of drying and steeping temperature on the  
741 phenolic content, antioxidant activity, aromatic compounds and sensory properties of  
742 *Culila polyantha* Benth. *Infusions. Processes*. 8(1378):2-20.

743 SRISOOK K et al. 2012. Antioxidant and anti-inflammatory activities of hot water extract  
744 from *Pluchea indica* Less. herbal tea. *J. Med. Plants Res.* 6(23):4077-4081.



745 SURIYAPHAN O. 2014. Nutrition, health benefits and applications of *Pluchea indica* (L)  
746 Less leaves. Pharm. Sci. Asia. 41:1–10.

747 SU D et al. 2019. Impact of thermal processing and storage temperature on phenolic  
748 profile and antioxidant activity of different varieties of lychee juice. LWT-Food Tech.  
749 116(108578):1-9.

750 TADERA K, MINAMI Y, TAKAMATSU K, MATSUOKA T. 2006. Inhibition of alpha  
751 glucosidase and alpha-amylase by flavonoids. J Nutr Sci Vitaminol. 52:149–153.

752 THANAJIRUSCHAYA P et al. 2010. Effect of storage **period** and temperature on  
753 antioxidant components and properties of milled rice. Khon Kaen Univ. Res.  
754 J.15(9):843-851.

755 VONGSAK B et al. 2018. Comparison of active constituents, antioxidant capacity, and  
756 αglucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. Food  
757 Biosci. 25: 68–73.

758 WANG SC. et al. 2022. Effect of brewing conditions on polyphenols in the dark tea  
759 (*Camellia sinensis* L.) infusions: content, composition and antioxidant activities. Food  
760 Sci.Tech. 42(e36322):1-9.

761 WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica*  
762 Less as functional beverage in diabetic patients. ASSEHR. 98:164-167.

763 WIDYAWATI PS et al. 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract  
764 as antioxidant and **anti-warmed** over flavor (WOF) of duck meat. International  
765 Congress on Challenges of Biotechnological Research in Food and Health. November  
766 15<sup>th</sup> 2014. Agricultural Technology Faculty, Slamet Riyadi University, Surakarta.

767 WIDYAWATI PS et al. 2016. The physicochemical and antioxidant properties of  
768 *Pluchea indica* Less drink in tea bag packaging. IJFNS. 5: 13–120.

769 WIDYAWATI PS et al. 2017. In vitro antioxidant capacities and antidiabetic properties of  
770 *pluchea* leaves. IJPPS. 9(8): 203-208.

771 WIDYAWATI PS et al. 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica*  
772 Less beverage. CJFST.12(4): 125-139.

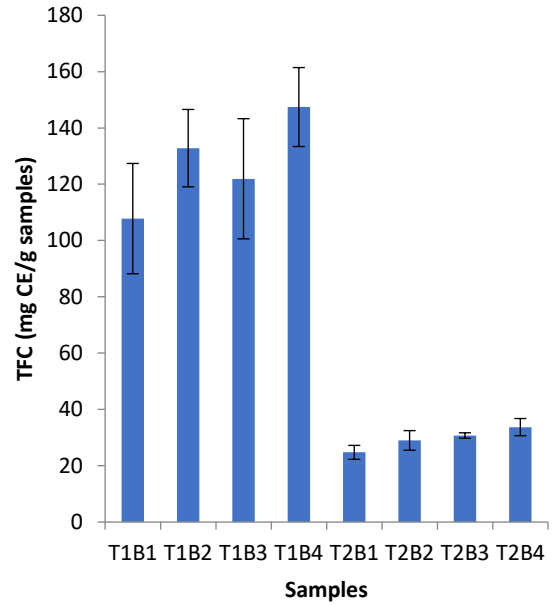
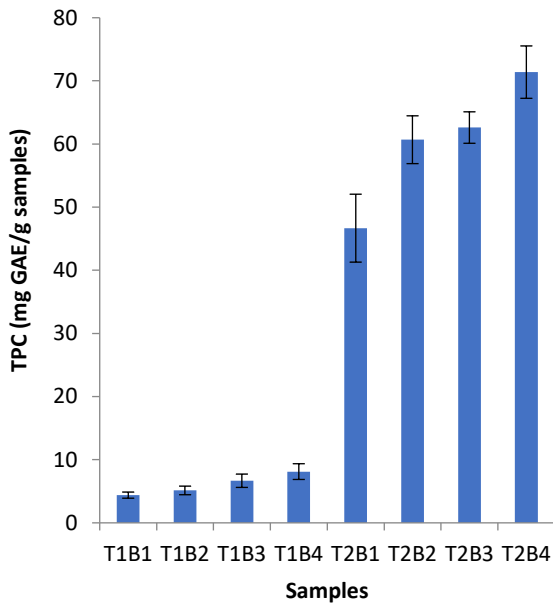
773 WIDYAWATI PS ET AL. 2022. The effect of κ-carrageenan proportion and hot water  
774 extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of stink  
775 lily (*Amorphophallus muelleri*) wet noodles. Molecules. 27(5062):1-16.

776 XU P, CHEN L, WANG Y. 2019. Effect of storage *period* on antioxidant activity and  
777 inhibition on α-amylase and α-glucosidase of white tea. Food Sci. Nut. 7(2): 636-644.

778 ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction *period* on caffeine  
779 in coffee. Lantanida J. 6(2):103-202. [https://jurnal.ar-](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811)  
780 [raniry.ac.id/index.php/lantanida/article/view/3811](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811). [In Bahasa Indonesia]

781 Zhang H, Wang G, Dong J. 2014. Inhibitory properties of aqueous ethanol extracts of  
782 propolis on alpha-glucosidase. J Evid Based Complement Alternat Med. 2015:1–8.

783 ZHANG Y et al. 2021. Effect of storage conditions and *period* on the polyphenol content  
784 of wheat flours. Processes. 9(248):1-11.

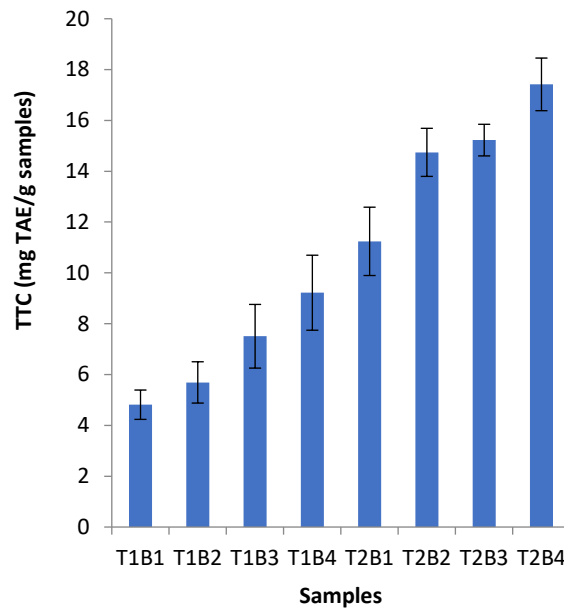


785

786

(a)

(b)



787

788

(c)

789 **Figure 1.** Bioactive compound contents of *Pluchea* infusion at different steeping  
 790 temperature and storage period (a) Total phenolic content (b) Total flavonoid  
 791 content (c) Total tannin content. Data analysis using ANOVA at  $\alpha \leq 0.05$   
 792 continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as  
 793 mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-

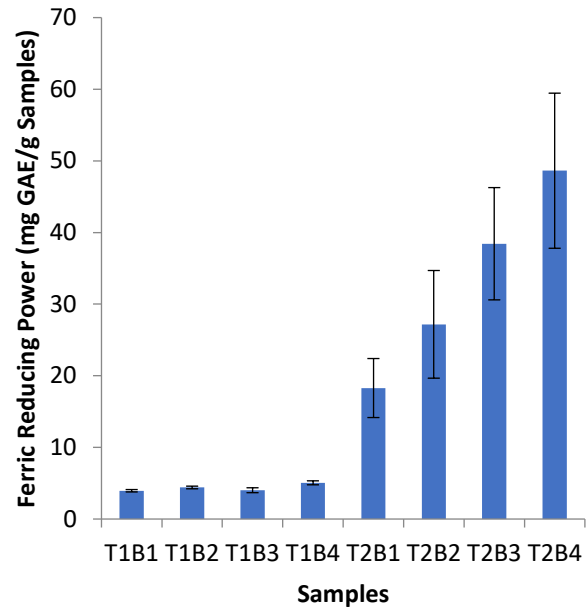
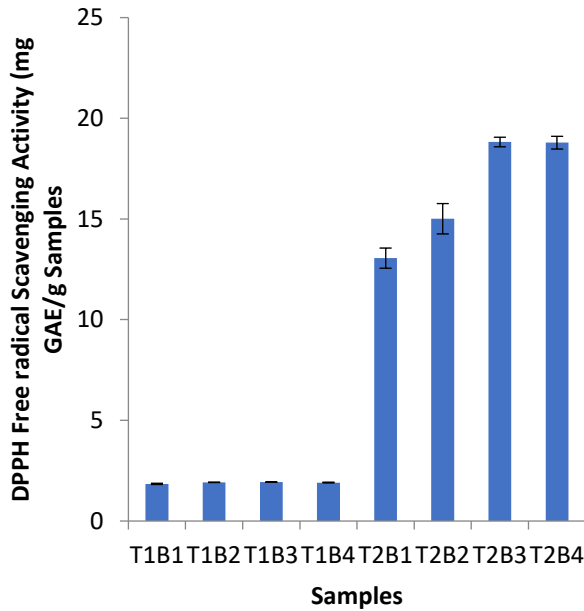
794 stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-  
795 stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for  
796 5 years; T3B2-steeped at 70 °C, stored for 5 years; T3B3-steeped at 80  
797 °C, stored for 5 years; T3B4-steeped at 95 °C, stored for 5 years.

798 Table 1. Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperature and storage period

Phenolic Compounds	Steeping Temperature (°C)	Mean±SD Un-stored	Mean±SD Stored	Mean difference ±SD	Sig (2-tailed)
Gallic Acid (µg/g samples)	60	0.2132±0.0027	0.2364±0.0015	0.0375±0.0175	0.2030
	70	0.2157±0.0013	0.2324±0.0214	0.0167±0.0227	0.4870
	80	0.2234±0.0122	0.2347±0.0078	0.0386±0.0264	0.2870
	95	0.2316±0.0104	0.2402±0.0169	0.0086±0.1990	0.8500
(+) -Catechin (µg/g samples)	60	0.3425±0.0110	0.5085±0.0111	-0.1576±0.0885	0.241
	70	0.3260±0.0265	0.5448±0.0006	-0.2188±0.0259	0.053
	80	0.3240±0.0222	0.5023±0.0773	-0.1451±0.0248	0.077
	95	0.4039±0.0320	0.5995±0.0372	-0.2049±0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756±0.1234	1.4762±0.0271	-1.2887±0.3222	0.111
	70	0.2587±0.0160	1.4245±0.2526	-1.1657±0.2695	0.103
	80	0.4175±0.0104	1.4570±0.0925	-1.0391±0.0841	0.036*
	95	0.8786±0.0434	2.6138±0.0695	-1.1735±0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220±0.0268	0.6220±0.0706	-0.5999±0.9733	0.544
	70	0.1530±0.0511	1.0708±0.0289	-0.9177±0.0222	0.011*
	80	0.3666±0.0103	0.8629±0.0815	-0.1082±0.4462	0.790
	95	0.6559±0.0570	2.0230±0.0573	-1.4123±0.3203	0.101
Kaempferol (µg/g samples)	60	0.1394±0.0202	0.3675±0.0183	-0.3207±0.1122	0.154
	70	0.0514±0.0037	0.3726±0.0944	0.3213±0.0907	0.125
	80	0.3699±0.0924	0.7966±0.0366	-0.4267±0.2727	0.271
	95	0.5913±0.0239	0.9478±0.0287	-0.3565±0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103±0.0628	2.4863±0.0270	-1.8760±0.2074	0.050*
	70	0.6271±0.0099	2.3403±0.0325	-1.7131±0.3152	0.082
	80	0.7967±0.03060	2.6278±0.0211	-1.8311±0.0095	0.002*
	95	1.5386±0.0668	4.0211±0.0851	-2.4825±0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635±0.0628	0.9449±0.0501	-0.2814±0.4458	0.536
	70	0.6162±0.0099	0.9485±0.0794	-0.3323±0.0301	0.041*
	80	0.6601±0.0306	0.9099±0.0387	-0.2498±0.3127	0.461
	95	0.6642±0.0668	1.3156±0.0166	-0.6514±0.2666	0.179

4,5-di-O-Caffeoylquinic acid ( $\mu\text{g/g}$ samples)	60	0.4906 $\pm$ 0.0060	1.1842 $\pm$ 0.0120	-0.6886 $\pm$ 0.2723	0.018*
	70	0.4807 $\pm$ 0.0034	1.0089 $\pm$ 0.0736	-0.5281 $\pm$ 0.0702	0.060
	80	0.5299 $\pm$ 0.0053	1.2382 $\pm$ 0.1435	-0.7082 $\pm$ 0.1489	0.094
	95	1.0018 $\pm$ 0.0526	1.3797 $\pm$ 0.2170	-0.3086 $\pm$ 0.3086	0.333

799 Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as  
800 mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-  
801 steeped at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped  
802 at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped at 95 °C, stored for 5 years.  
803



804

805

(a) (b)

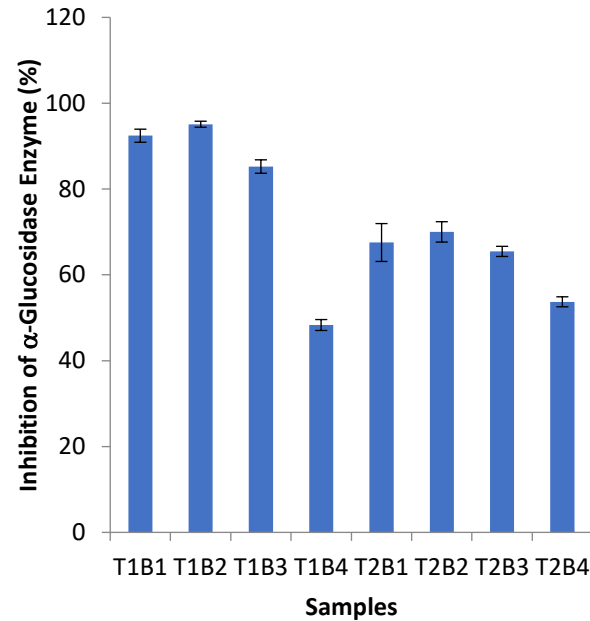
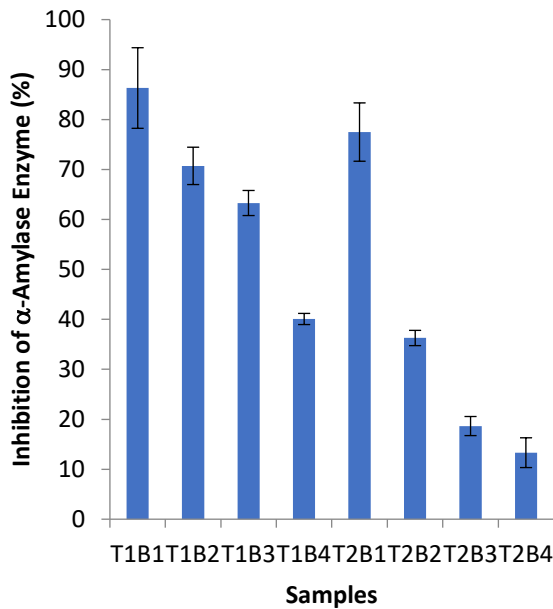
806 Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and  
 807 storage period (a) DPPH (b) FRAP. Data analysis using ANOVA at  $\alpha \leq 0.05$   
 808 continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as  
 809 mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored;  
 810 T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-  
 811 steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T2B2-  
 812 steeped at 70 °C, stored for 5 years; T2B3-steeped at 80 °C, stored for 5  
 813 years; T2B4-steeped at 95 °C, stored for 5 years.

814

815

816

817



818

819

(a)

(b)

820 **Figure 3.** Antidiabetic activity of pluchea tea at different steeping temperature and  
 821 storage period (a)  $\alpha$ -amylase (b)  $\alpha$ -glucosidase. Data analysis using ANOVA  
 822 at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were  
 823 expressed as mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60  
 824  $^{\circ}\text{C}$ , un-stored; T1B2-steeped at 70  $^{\circ}\text{C}$ , un-stored; T1B3-steeped at 80  $^{\circ}\text{C}$ , un-  
 825 stored; T1B4-steeped at 95  $^{\circ}\text{C}$ , un-stored; T2B1-steeped at 60  $^{\circ}\text{C}$ , stored for 5  
 826 years; T3B2-steeped at 70  $^{\circ}\text{C}$ , stored for 5 years; T3B3-steeped at 80  $^{\circ}\text{C}$ ,  
 827 stored for 5 years; T3B4-steeped at 95  $^{\circ}\text{C}$ , stored for 5 years.



828 Table 2. Pearson correlation coefficients between bioactive contents (TPC, TFC and TAC), antioxidant activity (DPPH  
 829 and FRAP) and antidiabetic activity (AA and GA)

	<i>TPC</i>	<i>TFC</i>	<i>TTC</i>	<i>DPPH</i>	<i>FRAP</i>	<i>Alpha Glucosidase</i>	<i>Alpha Amylase</i>
TPC	1						
TFC	-0.93589	1					
TTC	0.960028	-0.81321	1				
DPPH	0.992776	-0.93992	0.942273	1			
FRAP	0.953366	-0.82636	0.947778	0.956242	1		
Alpha Glucosidase	-0.55512	0.349873	-0.71534	-0.5272	-0.55947	1	
Alpha Amylase	-0.70842	0.429393	-0.8569	-0.69579	-0.80548	0.725161631	1

830 Significant at the 0.05 level (2-tailed)

831



Paini Sri Widyawati <paini@ukwms.ac.id>

---

## Fwd: Comments on PJS Paper Ms 23-158

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Wed, May 8, 2024 at 3:51 PM

Dear Dr. Widyawati,

We confirm the receipt of your revised Ms 23-158 paper, as well as your point-for-point response to the reviewer's comments. These will be forwarded to the PJS Editor-in-Chief for his consideration and final decision.

Thank you for your sustained contribution to PJS!

Sincerely,

Ms. CARYL MARIA MINETTE I. ULAY

Editorial Assistant

For Dr. CAESAR A. SALOMA

Editor-in-Chief

[Quoted text hidden]



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**From Caesar Saloma/12 May 2024/ Acceptance/ MS 23-158R3**

---

**Caesar Saloma** <caesar.saloma@gmail.com>

Sun, May 12, 2024 at 3:17 AM

To: paini@ukwms.ac.id

Cc: DOST STII PJS &lt;pjs@stii.dost.gov.ph&gt;

12 May 2024

DR. PAINI SRI WIDYAWATI  
Food Technology Study Program  
Agricultural Technology Faculty  
Widya Mandala Surabaya Catholic University  
Surabaya, Indonesia  
[paini@ukwms.ac.id](mailto:paini@ukwms.ac.id)

Subject: MS 23-158R3 RESEARCH NOTE

Title: Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea indica* Less Tea

Authors: PS Widyawati and YR Wilianto

Dear Dr Widyawati:

We are pleased to inform you that your third revised manuscript has been accepted for publication as a Research Note in the next available issue of the Philippine Journal of Science.

Kindly submit a final version that strictly complies with the format of a Research Note as explained in the PJS Author's Guide found in: <http://philjournalsci.dost.gov.ph/index.php/author-s-guide>.

Please send it only to the PJS Managing Editor, Mr Allyster Endozo at: [philjournalsci@gmail.com](mailto:philjournalsci@gmail.com); [pjs@stii.dost.gov.ph](mailto:pjs@stii.dost.gov.ph). It will be used to produce the galley proofs of your article.

We look forward to hearing from you soon so as not to delay the publication of your work.

Kindly direct to the PJS Managing Editor any future inquiry regarding the publication status of your article.

Thank you.

Sincerely yours,  
Caesar Saloma (signed)  
Editor-in-Chief  
The Philippine Journal of Science

## COMMENTS OF REVIEWERS

Reviewer 1

[1st evaluation] Paper secured no affirming commitment from experts

Reviewer 2

[1st evaluation] Paper as presently written is unacceptable for publication; needs extensive revision

[2nd evaluation] Reconsider only after the comments/recommendations are clarified and/or complied with. Paper should be published as a research note/short communication

Reviewer 3

[1st evaluation] Reconsider only after the comments/recommendations are clarified and/or complied with. Paper should be published as a research note/short communication

[2nd evaluation] Reconsider only after the comments/recommendations are clarified and/or complied with

Initial manuscript submitted to PJS: 05 May 2023  
Reviewers' comments sent to authors: 20 Sep 2023  
1st revised manuscript sent to PJS: 22 Nov 2023  
2nd revised manuscript sent to PJS: 17 Apr 2024  
3rd revised manuscript sent to PJS: 08 May 2024  
END.

--

Caesar Saloma, PhD NAST FSPS SMOPTICA  
Professor  
National Institute of Physics  
University of the Philippines Diliman  
Quezon City 1101, Philippines  
Voice/Fax: +632 920 9749 VOIP Trunkline: +632 981 8500 loc 3701  
E-mail: [caesar.saloma@gmail.com](mailto:caesar.saloma@gmail.com)  
[http://en.wikipedia.org/wiki/Caesar\\_Saloma](http://en.wikipedia.org/wiki/Caesar_Saloma)



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

## Manuscript has been formatted

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>

Mon, May 13, 2024 at 11:22 AM

To: Philippine Journal of Science <pjs@stii.dost.gov.ph>, Philippine Journal of Science <philjournsci@gmail.com>

Dear Mr Allyster Endozo  
Managing Editor

Greetings,

Attached I sent a manuscript with the title "Effect of Steeping Temperature and Storage Period on the Bioactive Compounds, Antioxidant and Antidiabetic Activities of Infusion from Powdered Pluchea Indica Less" with PJS paper manuscript number Ms 23-158, which I have adapted to the PJS format. Based on Mr. Caesar Saloma, this manuscript can be processed further.

Thank you for your cooperation

Regards

Paini Sri Widyawati



**PJS paper Ms 23-158-Effect of Brewing Temperature and Storage Time on Antioxidant and Antidiabetic**

**Properties of Pluchea Tea.docx**

80K

1 **Effect of Steeping Temperature and Storage Period on the Bioactive Compounds,**  
2 **Antioxidant and Antidiabetic Activities of Infusion from Powdered Pluchea Indica**  
3 **Less**

4 **Paini Sri Widyawati<sup>1\*</sup>, Yufita Ratnasari Wilianto<sup>2</sup>**

5 <sup>1</sup>Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala  
6 Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

7 <sup>2</sup>Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic  
8 University, Kalisari Street Number 1, Surabaya 60272, Indonesia

9 Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature,  
10 Pluchea indica Less, storage period

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26 ABSTRACT

27 This study was done to determine the effects of steeping temperature and storage period  
28 on the bioactive contents, antioxidant and antidiabetic activities of *Pluchea* leaf infusion.

29 The research used a randomized block design with two factors, i.e., steeping temperature  
30 (T) and storage period (B). The *Pluchea* leaf blades were exposed to 4 steeping

31 temperatures of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) °C with the storage period of 0 (B1)

32 and 5 (B2) years resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1,

33 T3B2, T4B1, T4B2). Statistical analysis using a paired t-test at  $\alpha \leq 0.05$  showed that

34 treatments significantly affected the bioactive contents [(total phenol (TPC), total tannin

35 (TTC), total flavonoid (TFC)], antioxidant [(DPPH scavenging activity (DPPH) and ferric

36 reducing antioxidant power (FRAP)] potential and antidiabetic [( $\alpha$ -amylase (AA) and  $\alpha$ -

37 glucosidase (GA) inhibition)] properties of the *Pluchea* leaf infusion. TPC, TTC, DPPH,

38 and FRAP significantly increased for the storage period and the steeping temperatures.

39 Then, TFC decreased during the storage period but significantly increased at higher

40 steeping temperatures. The AA and GA of *Pluchea* leaf infusion increased until 70 °C of

41 the steeping temperature, but decreased until 95 °C. The DPPH and FRAP of the *Pluchea*

42 leaf infusion were strongly and positively correlated with TPC and TTC. The GA and AA

43 of *Pluchea* leaf infusion were not influenced by the TPC and TTC but were weakly and

44 positively correlated with TFC. The antioxidant activity of the *Pluchea* leaf infusion was

45 inversely proportional to the antidiabetic activity. The simple phenolic compounds derived

46 from *Pluchea* leaf infusion at different steeping temperatures and storage included gallic

47 acid, kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-caffeoylquinic acid, 3,5- di-  
48 O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid.

49

## 50 INTRODUCTION

51 Pluchea herbal tea is a product of dried Pluchea leaf processing introduced by  
52 world people (Srisook et al., 2012; Widyawati et al., 2016) because of the efficacy of the  
53 active components in Pluchea leaves, as a herbal plant that has been widely used for  
54 traditional medicine and food (Chan et al., 2022). Pluchea leaves are composed of many  
55 nutrients and bioactive compounds useful to body health. The nutrient compositions in  
56 the Pluchea leaves include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates,  
57 calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive compounds are comprised, i.e.,  
58 chlorogenic acid, caffeic acid, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-  
59 caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-  
60 caffeoylquinic acid, quercetin, myricetin, kaempferol, total anthocyanin,  $\beta$ -carotene, and  
61 total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et  
62 al., 2022, Chan et al., 2022).

63 The steeping process of Pluchea leaves can be performed with fresh or dry leaves  
64 in hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et al., 2020;  
65 Jayani et al., 2022). In Asia, especially in Indonesia, people usually consume the Pluchea  
66 infusion by steeping 2 g of powdered Pluchea leaves in a tea bag in 100 mL of hot or  
67 boiling water. Widyawati et al. (2016) claimed that steeping of 2 g of Pluchea leaf powder  
68 at 95 °C for 5 minutes exhibits total phenolic and total flavonoid contents, the ability to  
69 scavenge DPPH free radicals, and the capability to reduce ferric ions at 9.3 mg gallic acid  
70 equivalent (GAE)/g sample, 22.0 mg gallic acid equivalent (GAE)/g sample, 27.2 mg gallic



71 acid equivalent (GAE)/g sample, and 10.2 mg gallic acid equivalent (GAE)/g sample,  
72 respectively. Werdani and Widyawati (2018) reported that drinking *Pluchea* leaf powder  
73 infusion in the morning and evening regularly (2 g/100 mL) can decline blood sugar levels.

74 The steeping of *Pluchea* herbal tea with hot water at 95 °C for 5 minutes certainly  
75 determines the stability and amount of extracted bioactive compounds that  
76 influence the biological activity especially antioxidant and antidiabetic activities. Silva-  
77 Ramirez et al. (2020) reported that the infusion process can influence the content and  
78 composition of the bioactive compounds and antioxidant activity of tea. Acar et al. (2022)  
79 informed that the infusion quality of herbal tea extract depends on several factors, i.e.,  
80 storage and temperature. The polyphenol profile and antioxidant properties of herbal tea  
81 infusion decline with an increase in steeping/brewing and storage temperatures, and  
82 longer exposure periods.

83 Several studies have mentioned the effect of steeping temperature on the  
84 bioactive compound contents and antioxidant activity, such as some white and green teas  
85 are effective with hot water at 90 °C for 7 min (Castiglioni et al., 2015), on roseship tea is  
86 effective at infusion period around 6-8 min at temperatures of 84-86 °C (Ilyasoglu and  
87 Arpa, 2017), on the caffeine content extracted at the brewing temperature of coffee  
88 (Zarwinda and Sartika, 2018), and the high total phenol content and antioxidant activity  
89 of dark tea at 92 °C for 27 min (Wang et al., 2022). The study of the effect of steeping  
90 temperature on *Pluchea* infusion was carried out to afford information about the most  
91 efficient preparation of powdered *Pluchea* leaves to get higher bioactive compounds,  
92 antioxidant, and antidiabetic activities.

93 Storage period tea usually for several months to years *Pluchea* herbal tea also  
94 affects the levels of the bioactive compounds and biological activity (Jayani et al., 2022).  
95 Tea or herbal tea is generally stored at ambient temperature and packed in a tea bag or  
96 aluminum foil standing pouch or a combination of both. Many researchers reported that  
97 the storage period decreases the bioactive compounds, antioxidant and antidiabetic  
98 activities, i.e., juice from *Momordica charantia* L. (Lin et al., 2020), dried *Piper betle*  
99 extracts (Ali et al., 2018), white tea (Xu et al., 2019), kinnow-amlam beverages (Purewal et  
100 al., 2022), whole wheat flour (Zhang et al., 2021).

101 Therefore, this research studied the effect of steeping temperature and storage  
102 period on the bioactive compounds [total phenolic content (TPC), total flavonoid content  
103 (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging activity  
104 (DPPH), ferric reducing antioxidant power (FRAP)], and antidiabetic activities [( $\alpha$ -amylase  
105 (AA) and  $\alpha$ -glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea* leaves  
106 and on the phenolic compound profile.

107

## 108 MATERIALS AND METHODS

109

### 110 RAW MATERIALS AND PREPARATION

111 The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya,  
112 East Java, Indonesia. The *Pluchea* plants were included in the *Asteraceae* family with  
113 specifications according to the GBIF taxon ID number database:3132728 (Ferraris, 2023).  
114 *Pluchea* leaves at 1-6 levels of each branch from the shoot were collected, sorted,  
115 washed, and dried to get a moisture content of around  $11.16 \pm 0.09$  % dry basis  
116 (Widyawati et al., 2022). The dried *Pluchea* leaves was pulverized to a 45-mesh size

117 powder. The *Pluchea* leaf powder was dried in an oven (Binder, Merck KGaA, Darmstadt,  
118 Germany) at 120 °C for 10 min to reduce microbial organisms. Then, 2 g of the powder  
119 were packed into a paper filter infusion bag. Packed samples were stored for 0 (un-stored)  
120 and 5 (stored) years in standing pouch before analysis.

121 In the research, the one tea bag of *Pluchea* herbal tea that was stored 0 (B1) and  
122 5 (B2) year, was steeped with 100 mL hot water at various temperatures, including 60  
123 (T1), 70 (T2), 80 (T3), 95 (T4) °C for 5 min with infusion method obtaining 8 treatment  
124 combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2. After the  
125 temperature of *Pluchea* infusion similar to ambient temperature was analyzed further.

126

## 127 REAGENTS

128 The reagents used in the analysis include 2,2-diphenyl-1-picrylhydrazyl (DPPH),  
129 sodium carbonate, gallic acid,  $\alpha$ -amylase,  $\alpha$ -glucosidase, pNPG (p-nitrophenyl- $\alpha$ -  
130 glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic  
131 acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and (+)-catechin were  
132 purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, Folin–Ciocalteu’s Phenol,  
133 sodium nitric, aluminum chloride, ferric chloride, sodium dihydrogen phosphate, sodium  
134 phosphate, potassium ferricyanide, starch, acetic acid, and sodium hydroxide were  
135 purchased from Merck (Kenilworth, NJ, USA). All reagents used were of analytical grade  
136 except for distilled water which was purchased from PT Aqua Industry Surabaya.

137

## 138 METHODOLOGY

139

## ANALYSIS OF THE BIOACTIVE COMPOUNDS

140

141

### TOTAL PHENOLIC CONTENT ANALYSIS

142  
143 Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the  
144 technique by Gao et al. (2019). About 10  $\mu$ L *Pluchea* infusion and 1 mL Folin-Ciocalteu's  
145 phenol reagent 10 % were mixed in 10 mL volumetric flask and incubated for 5 min. And  
146 then 2 mL  $\text{Na}_2\text{CO}_3$  7.5 % was added and filled up to 10 mL volume with distilled water.  
147 The blue color intensity of the solution was measured in the spectrophotometer UV-Vis  
148 1800 (Shimadzu, Japan) at  $\lambda$  760 nm with gallic acid as the reference standard. The total  
149 phenolic content was calculated using the formula:  $y=0.00009x+0.008$  with  $R^2=0.9941$ .  
150 The results were expressed as mg gallic acid equivalent (GAE)/g samples.

151

### TOTAL FLAVONOID CONTENT ASSAY

152  
153 Total flavonoid content (TFC) of the samples was measured based on the reaction  
154 between  $\text{AlCl}_3$  and  $\text{NaNO}_2$  with the aromatic ring of flavonoid compounds, especially  
155 flavonol and flavon (Shraim et al., 2021). The reaction between  $\text{AlCl}_3$  and flavonoid  
156 compounds resulted in a yellow solution. About 30  $\mu$ L *Pluchea* infusion was mixed with  
157 0.3 mL  $\text{NaNO}_2$  5 % in 10 mL volumetric flask and incubated for 5 min. The mixture was  
158 added with 0.3 mL  $\text{AlCl}_3$  10 % for 5 min. Then, 2 mL  $\text{NaOH}$  1 M and distilled water were  
159 added until 10 mL volume. Then, the red solution was produced after  $\text{NaOH}$  solution  
160 addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800,  
161 Shimadzu, Japan) at  $\lambda$  510 nm with (+)-catechin as the reference standard compound,

162 and the results were expressed as mg catechin equivalents (CE)/g samples using the  
163 formula:  $y=0.00008x-0.0023$  with  $R^2= 0.9980$ .

164

#### 165 TOTAL TANNIN CONTENT ANALYSIS

166 Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method  
167 (Chandran and Indira, 2016). Approximately 10  $\mu$ L *Pluchea* infusion was added with 1 mL  
168 Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flask and incubated for 5 min.  
169 Then, the mixture was added with 2 mL  $\text{Na}_2\text{CO}_3$  7.5 % and filled up to 10 mL volume with  
170 distilled water. The blue dark color solution was measured in UV-Vis spectrophotometer  
171 1800 (Shimadzu, Japan) at  $\lambda$  760 nm with tannic acid as the reference standard.  
172 Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/g samples used  
173 the formula:  $y=0.00009x+0.0021$  with  $R^2=0.9993$

174

#### 175 ANALYSIS OF THE ANTIOXIDANT POTENTIAL

176

#### 177 DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

178 The DPPH free radical scavenging activity (DPPH) was measured by the  
179 spectrophotometric method (Widyawati et al., 2017) to determine the ability of the  
180 phytochemicals in the *Pluchea* leaf infusion to donate hydrogen atom to the nitrogen atom  
181 in DPPH resulting in the formation of DPPH-H compound exhibiting a yellow-colored  
182 solution. About 25  $\mu$ L *Pluchea* leaf infusion was poured into reaction tube into which was  
183 added 3 mL DPPH solution (4 mg/100 mL). After incubation for 15 min in a dark room,  
184 the absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis

185 1800, Shimadzu, Japan) at  $\lambda$ . 517 nm. The reference standard compound was gallic acid  
186 and the results of analysis were expressed as mg gallic acid equivalents (GAE)/g samples  
187 that calculated using formula:  $y=0.146x+1.7896$  with  $R^2=0.9975$ .

188

## 189 FERRIC REDUCING POWER ANALYSIS

190 Ferric-reducing power (FRAP) was determined following the method used by  
191 Widyawati et al. (2014) method. Approximately 10  $\mu$ L of samples were added to 2.5 mL  
192 phosphate buffer pH 6.6 and 2.5 mL of 1% potassium ferricyanide in the reaction tube.  
193 And then mixture was shaken and incubated for 20 min at 50 °C. Finally, 2.5 mL  
194 chloroacetic acid 10% (w/v) was added. Into the 2.5 mL supernatant was added 2.5 mL  
195 distilled water, 0.5 mL ferric chloride 0.1% w/v, and incubated for 10 min. Potency of the  
196 samples reducing iron (III) to iron (II) ion was indicated by the intensity of blue color  
197 formed that was measured using UV-Vis spectrophotometer (Spectrophotometer UV-Vis  
198 1800, Shimadzu, Japan) at  $\lambda$  700 nm. Intensity of the blue color indicated higher reducing  
199 capacity. The reducing power expressed as mg gallic acid equivalent (GAE)/g samples  
200 was calculated using the formula:  $y=0.0002x+0,0256$  with  $R^2=0,9906$ .

201

## 202 ANALYSIS OF THE ANTIDIABETIC PROPERTIES

203

### 204 $\alpha$ -AMYLASE ENZYME INHIBITION CAPACITY ASSAY

205 In vitro inhibition of  $\alpha$ -amylase enzyme (AA) followed the procedure as described  
206 by Widyawati et al. (2020). Each 500  $\mu$ L of samples, was mixed with starch 1 % (w/v) and  
207 sodium acetate buffer pH 5. Into a 250  $\mu$ L of the mixture was added an  $\alpha$ -amylase solution

208 (0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in 50 mL of 0.2 M sodium acetate  
209 pH 5. Mixture was shaken into which was and added 2 mL sodium hydroxide 1M. Before  
210 the analysis, this mixture was incubated at 37 °C for 10 min. Then, the capacity of the  $\alpha$ -  
211 amylase enzyme to hydrolyze the starch to release glucose was measured by UV-Vis  
212 spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda$  540 nm.  
213 The inhibition percentage of  $\alpha$ -amylase was assessed using the formula:  $(ACb - ACa) -$   
214  $(As - Ab) (ACb - ACa) \times 100 \%$ . Where, ACb is the absorbance of 100 % enzyme activity  
215 (solvent with the enzyme), ACa is the absorbance of 0 % enzyme activity (solvent without  
216 the enzyme), As is the absorbance of test sample with enzyme, Ab is absorbance of test  
217 sample without enzyme.

218

#### 219 $\alpha$ -GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

220 The analysis of the  $\alpha$ -glycosidase inhibitor activity (GA) was done by Widyawati et  
221 al. (2020) method with slight modification. About 150  $\mu$ L samples containing 100  $\mu$ L  
222 Pluchea infusion and 50  $\mu$ L pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH 7)  
223 were reacted with 50  $\mu$ L  $\alpha$ -glycosidase 2 mM (0.0833 unit/mL), and then the mixture was  
224 incubated at 37 °C for 15 min. The reaction was stopped with the addition of 1000  $\mu$ L  
225 sodium carbonate 0.2 M. The amount of these enzymes that didn't react with bioactive  
226 compounds of Pluchea infusion hydrolyzed p-nitrophenyl- $\alpha$ -D-glucopyranoside (pNPG)  
227 as a substrate to result in p-nitrophenol. The inhibition activity of the Pluchea infusion was  
228 measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu,  
229 Japan) at  $\lambda$  405 nm. The inhibition percentage of  $\alpha$ -glycosidase was calculated using  
230 formula:  $(ACb - ACa) - (As - Ab) (ACb - ACa) \times 100 \%$ . Where, ACb is the absorbance

231 of 100 % enzyme activity (solvent with enzyme), A<sub>Ca</sub> is the absorbance of 0 % enzyme  
232 activity (solvent without enzyme), A<sub>s</sub> is the absorbance of test sample with enzyme, A<sub>b</sub>  
233 is the absorbance of test sample without enzyme.

234

## 235 ANALYSIS OF PHENOLICS

236 The phenolic compounds of the samples were analyzed by HPLC based on  
237 Kongkiatpaiboon et al. (2018) method with modifications. Each *Pluchea* infusion was  
238 sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter  
239 syringe (Whatmann, 0.2 µm, NYL). About 20 µL of sample was injected in an HPLC  
240 (LC20AD series, Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC  
241 LC-20AD pump, CTO-30A column oven, CBM-20A/20 Alite system controller, and SPD-  
242 20A/20 AV UV-Vis detector. Separation of phenolic compounds in samples was carried  
243 out using a Shim-pack VP-ODS C18 column (ID 5 µm × 50 mm × 4.6 mm) with a GVP-  
244 ODS Cartridge guard column (2 pieces) (ID 10 mm × 4.6 mm). The mobile phase used  
245 consisted of a solution of (A) 0.5 % acetic acid in water and (B) absolute methanol.  
246 Analysis was carried out using a gradient system in the following order: initial conditions  
247 of 10 % B in A to 50 % B in A were maintained for 40 minutes; then 100 % B was  
248 maintained for 20 minutes. Next the column was re-equilibrated with 10 % B in A  
249 maintained for 10 minutes before analysis of the next sample. The sample flow rate was  
250 set at 1.0 ml/min with a controlled temperature at 40 °C. Detection was used at a  
251 wavelength of 280 nm. The reference standard used were gallic acid, (+)-catechin,  
252 myricetin, quercetin, kaempferol, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and



253 4,5-dicaffeoylquinic acid. All of reference standard was dissolved in distilled water and  
254 prepared similar to the samples before injected in HPLC.

255

## 256 EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

257 The research design used a randomized block design with two factors, i.e., the  
258 steeping temperature (T) and the storage period. *Pluchea* leaf blades were subjected to  
259 4 steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95 °C (T4), and  
260 the storage period of 0 year /un-stored (B1), and 5 year /stored (B2) resulting in 8  
261 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC  
262 analysis of phenolic was repeated six periods. The data analysis of samples was repeated  
263 for six periods. The data were analyzed using a paired t-test at  $\alpha \leq 0.05$ , treatment means  
264 of specific phenolic compounds that were identified were expressed as the mean  $\pm$  SD.  
265 The analysis used SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

266

## 267 RESULTS AND DISCUSSIONS

268

### 269 BIOACTIVE COMPOUNDS

270

#### 271 Phenolic Compounds

272 The bioactive compounds are active compounds in plants that are essential to  
273 protect a body health (Nguyen and Chuyen, 2020). These compounds usually have many  
274 biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer,  
275 antimicrobial, antibacterial, anti-cholesterol, and so on (Suriyaphan, 2014; Acar et al.,  
276 2022). Phenolic compounds have potential redox properties that can scavenge free

277 radicals that can cause a number of chronic diseases (Noreen et al., 2017; Aryal et al.,  
278 2019; Acar et al., 2022).

279 The total phenolic content (TPC) of *Pluchea* infusion at different steeping  
280 temperature and storage period generally significantly increased with increasing steeping  
281 temperature and storage period based on paired t-test at  $\alpha \leq 0.05$  (Figure 1a). Steeped  
282 and stored infusion had significantly higher amounts of phenolic compounds than the  
283 samples that were steeped and un-stored. Further, the highest total phenolic content was  
284 observed in samples infused at 95 °C and stored for 5 years (at 71.38±4.14 mg GAE/g  
285 sample) while the lowest was measured in the un-stored samples and infused at 60 °C  
286 (at 4.39±0.49 mg GAE/g sample). The phenolic content of stored samples that were  
287 steeped only at 60 and 95 °C showed a significant increase in their phenolic content. This  
288 implies that the steeping temperature and the storage periods significantly resulted in the  
289 high amounts of phenolic compounds in the infusions. Results also indicated that phenolic  
290 compounds were generally greater in the infusion at high steeping temperatures and long  
291 storage period. This could have been due to the fact that the steeping temperature and  
292 storage period could cause the process of degradation, oxidation, and leaching/release  
293 of phenolic compounds. Phenolic compounds are water soluble and thus soaking in hot  
294 water for a certain period of period as in steeping causes the migration process of more  
295 phenolic compounds to the water because of longer exposure of phenolic compounds to  
296 water (Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al. (2022). Su et al. (2019)  
297 reported that temperature treatment can stimulate the release of phenolic compounds  
298 and increase antioxidant activity of lychee juice stored at different temperatures of 4 and  
299 45 °C and different long storage (fresh and 72 hours).

300 Temperature treatment degrades (or hydrolyzes) the hydrogen bond between  
301 phenolic compounds and proteins resulting in an increase of phenolic compounds when  
302 exposed to higher temperatures (Ali et al. (2018); Jayani et al. (2022) and Ramphinwa et  
303 al. (2023)). Zhang et al. (2021) reported that phenolic compounds present in plants are  
304 not completely stable, but are easily degraded during storage after harvest. Reblova  
305 (2012) claimed that antioxidant compounds can be slowly degraded with increasing  
306 temperature. Fibrianto et al. (2021) also stated that the brewing temperature has an effect  
307 on the extracted antioxidant compounds, such as alkaloids, catechins, and tannins. Thus,  
308 there is an assumption that temperature and storage caused the degradation, oxidation,  
309 and hydrolysis of the phenolic compounds period resulting in the increased amount of the  
310 phenolic compounds at higher steeping temperature and longer storage period.

311 Simple phenolic compounds are identified in steeped and stored. Pluchea leaf  
312 infusion included gallic acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-  
313 O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic acids  
314 was showed in Table 1. The treatment effects using a t-test at  $\alpha \leq 0.05$  showed that gallic  
315 acid and kaempferol content were insignificantly different at various steeping  
316 temperatures and storage periods. The concentration of quercetin and 3,5-di-O-  
317 caffeoylquinic acid of the un-stored and stored Pluchea infusion was significantly different  
318 from the rest of the samples between 70 °C while (+)-catechin concentration of Pluchea  
319 infusion was only significantly different at 95 °C. The myricetin content was significantly  
320 different at 80 and 95 °C. The 3,4-di-O-caffeoylquinic acid content showed significant  
321 difference at 60, 80, and 95 °C while 4,5-di-O-caffeoylquinic acid content was only  
322 significantly different at 60 °C.

323 Results further showed that gallic acids and kaempferol were relatively stable as  
324 reflected by the insignificant changes when exposed to the different steeping temperature  
325 and storage period. Myricetin, (+)-catechin, and 3,4-di-O-caffeoylquinic acid showed a  
326 drastic increase at higher steeping temperatures and longer storage period implying that  
327 these compounds tended to be relatively labile. Quercetin, 3,5-di-O-caffeoylquinic acid,  
328 and 4,5-di-O-caffeoylquinic acid underwent moderate changes. Therefore, myricetin, (+)-  
329 catechin, and 3,4-di-O-caffeoylquinic acid were easier to dissolve or degrade to form  
330 simple phenolic acids at higher temperatures and storage period (Su et al. (2019, Ali et  
331 al. (2018); Jayani et al. (2022); Ramphinwa et al. (2023), and Zhang et al. (2021).  
332 Degradable polyphenol compounds have a simple structure and free hydroxyl groups that  
333 can react with Folin-Ciocalteu's Phenol reagent, resulting complex blue solution that can  
334 detected as total phenolic content.

335

### 336 Flavonoid Content (TFC)

337 Flavonoids are the major phenolic compounds that have potential chemical and  
338 biological activities, such as radical scavenging and antimicrobial activities (Ayele et al.,  
339 2022; Chandra et al., 2014) that can protect the human body from the oxidative stress  
340 caused by many degenerative diseases, especially cancer, cardiovascular problems and  
341 aging (Mathur and Vijayvergia, 2017). The total flavonoid content of steeped *Pluchea*  
342 infusion decreased with longer storage period. Un-stored samples exhibited higher  
343 flavonoid content than the stored samples. The statistical analysis using a paired t-test at  
344  $\alpha \leq 0.05$  showed that the total flavonoid content of *Pluchea* infusion was significantly  
345 different between the steeped un-stored and steeped stored samples (Figure 1b). The

346 highest total flavonoid content was exhibited by the un-stored samples steeped at 95°C  
347 at about 147.42±14.03 mg CE/g sample. Total flavonoid content was significantly lower  
348 in the stored samples than those of the un-stored samples implying that the increase in  
349 the flavonoid content of the infusion was affected primarily by the steeping temperature.

350

351

### Tannin Content (TTC)

352 Tannins are bioactive compounds that provide properties, such as astringent, anti-  
353 diarrheal, antibacterial and antioxidant (Malangngi et al., 2012). Generally, results  
354 indicated that the total tannin content of *Pluchea* infusion significantly increased with  
355 increasing steeping temperature and storage period (Figure 1c). Among, the un-stored  
356 steeped samples, the tannin content was significantly lowest in the samples infused at 60  
357 °C at about 4.81±0.58 to 17.42±1.04 mg TAE/g samples which was significantly different  
358 lower from that of the lowest tannin content of the stored samples. Among the stored and  
359 steeped samples, the highest tannin content was observed at samples steeped at 95 °C  
360 about 17.42 ± 1.04 mg TAE/g samples, and was significantly different from that of the  
361 highest tannin content of the un-stored steeped samples at 95 °C about 9.22 ± 1.48 mg  
362 TAE/g samples. Indicating that the tannin content was primarily affected by a longer  
363 storage period than high steeping temperature. The condensation of catechins to tannins  
364 is a dominant process occurring in tea leaves that is accelerated during the maceration  
365 of raw tea leaves (Kowalska et al., 2021) and could have had contributed to the observed  
366 increase in the tannin content in the treated samples.

367 Although, high temperature and long storage period can cause the degradation of  
368 tannins to catechins. Rusita et al. (2019) emphasized that tannins are polar thermostable

369 complex compounds, that are resistant to heating, indicating that even with the exposure  
370 to high temperature, the tannins still remained high in the treated samples period.

371

## 372 Antioxidant Activity

373 Antioxidant activity is capability of compounds to inhibit the oxidation of  
374 macromolecules from biological target that involve in oxidative chain reactions (Ali et al.,  
375 2005; Oh et al., 2013). The antioxidant activity assay was done in this research using  
376 DPPH Free Radical Scavenging Activity (DPPH) and ferric reducing power (FRAP)  
377 methods. The phenolic compounds are an active antioxidant that have antioxidant  
378 capability that depends on their redox properties. The structure of phenolic compounds  
379 determines the effectivity to donate hydrogen atom which is negatively correlated with the  
380 O-H phenolic bond strength. The antioxidant power of phenolic compounds is due to the  
381 weak hydrogen bonds in the OH group of the phenolic compound so that it is easier to  
382 donate hydrogen atoms (Kruk et al., 2022). The mechanism of phenolic compounds as  
383 antioxidants depends on their ability to donate hydrogen atoms and transfer electrons,  
384 and as reducing agents and singlet oxygen quenchers (Ali et al., 2005; Huang et al. 2005).

385

## 386 DPPH Free Radical Scavenging Activity

387 DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate  
388 antioxidant activity because this method is simple that is suitable to measure the donating  
389 hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of  
390 DPPH to change to yellow color (Munteanu and Apetrei, 2021; Baliyan et al., 2022).  
391 Figure 2a. shows that the free radical scavenging properties of the stored and steeped

392 samples were significantly higher than the un-stored steeped samples. It can also be  
393 observed that the free radical scavenging property was significantly different among the  
394 stored and steeped samples but insignificant among the un-stored and steeped sample  
395 period. *Pluchea* infusion stored at room temperature for 5 years resulted in high free  
396 radical scavenging activity by more than 10%. Steeping at higher temperatures  
397 significantly increased the DPPH free radical scavenging activity in stored *Pluchea*  
398 infusion by around 15 to 25 %. This implies that the higher free radical scavenging  
399 property was primarily affected by the storage period than the steeping temperature.  
400 During the storage process, it is possible to form complex phenolic compounds which  
401 provide a high ability to scavenge free radicals (Thanajiruschaya et al., 2010).

402           The scavenging activity of the samples was strongly and positively correlated with  
403 total phenolic and tannin contents, but inversely with total flavonoid levels (Table 2). The  
404 antioxidant activity was strongly and negatively correlated with flavonoid content. The  
405 storage period could be reduced flavonoid content. The study also demonstrated that  
406 longer storage period and higher infusion temperatures produced many simple phenolic  
407 compounds with free hydroxyl groups capable to donate hydrogen atoms to DPPH free  
408 radicals. Many phenolic acids, such as gallic acids, (+)-catechins, myricetins,  
409 kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 4,5-  
410 di-*O*-caffeoylquinic acids have established potential antioxidant activity (Kumar and Goel,  
411 2019) (Table 1). Kruk et al (2022) informed that the capability of phenolic compounds to  
412 donate hydrogen atom depends on chemical structure, number and position of hydroxyl  
413 groups attached to a benzene ring, a double bond between C2 and C3 rings, and a

414 carbonyl group (C=O) on the C ring at C4. The effectivity of antioxidant compounds to  
415 donate hydrogen atom is determined by O-H bond dissociation energy.

416 The free radical scavenging property observed in the study was not in consistent  
417 with the results of the study by Moraes-de-Souza et al. (2008). The research shows that  
418 total phenolic content of herbal infusion is low correlated with free radical scavenging  
419 activity. However, Dobrinas et al. (2021) informed that total phenolic content is positively  
420 and significantly correlated with the free radical scavenging property of tea infusion.

421

#### 422 Ferric Reducing Antioxidant Power (FRAP)

423 FRAP is an analysis of the antioxidant power of the phytochemical compounds  
424 that is based on the ability of antioxidant compounds to reduce iron ions of potassium  
425 ferricyanide ( $\text{Fe}^{3+}$ ) to potassium ferrocyanide ( $\text{Fe}^{2+}$ ). Potassium ferrocyanide reacts with  
426 ferric chloride to form a ferric-ferrous complex and results green color solution (Widyawati  
427 et al., 2017; Raharjo and Haryoto, 2019).

428 The results showed that the ferric reducing antioxidant power (FRAP) increased at  
429 higher steeping temperature and longer storage period. The lowest FRAP was observed  
430 in the un-stored samples which were steeped at 60 °C at  $3.95 \pm 0.17$  mg gallic acid  
431 equivalents (GAE)/g samples, and the highest was exhibited in *Pluchea* infusion which  
432 was stored for 5 years at 95 °C at  $48.63 \pm 10.83$  mg gallic acid equivalents (GAE)/g  
433 samples (Figure 2b). FRAP increased significantly as the steeping temperature was  
434 increased. FRAP of the samples stored for 5 years was also significantly higher than the  
435 un-stored samples at  $\alpha \leq 0.05$ .



436 This is in contrast with the study on the antioxidant activity of DPPH and FRAP of  
437 matcha. The longer storage period reduces the levels of catechin content due to the  
438 catechins, such as epigallocatechin gallat (EGCG), epicatechin gallat (ECG),  
439 epigallocatechin (EGC), and epicatechin (EC) which are bioactive compounds that have  
440 high antioxidant activity (Kim et al. 2020). The ferric-reducing capability of *Pluchea* could  
441 have been due to the presence of simple phenolic acid that can transfer electrons from  
442 their free hydroxyl groups of samples. The FRAP of *Pluchea* infusion was strongly and  
443 positively significantly correlated with the DPPH, TPC, and TTC, but inversely to TFC.

444

#### 445 ANTIDIABETIC ACTIVITY

446

##### 447 Alpha amylase enzyme inhibition activity (AA)

448 Antidiabetic activity is a measure of the potency of phenolic compounds to regulate  
449 the uptake of glucose by the cells from the blood through the mediation of 2-digestive  
450 enzymes i.e.,  $\alpha$ -amylase and  $\alpha$ -glucosidase, which are involved the control of dietary  
451 carbohydrate digestion and release in the postprandial blood glucose in human body (Fu  
452 et al., 2017). The phenolic compounds have the capability to bind with the protein  
453 component of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Martinez-Solis et al., 2022)  
454 resulting in the reduced activity of the enzymes. The results showed that lower steeping  
455 *Pluchea* leaf infusion was able to inhibit the action of the  $\alpha$ -amylase enzymes (Figure 3a).  
456 The *Pluchea* infusion exhibited a good  $\alpha$ -amylase enzyme inhibition activity of more than  
457 50 % and even almost 100 % in un-stored *Pluchea* infusion steeped at 60, 70, and 80 °C  
458 with the highest at 60 °C, and in stored *Pluchea* leaf infusion which was steeped at 60 °C.

459 The stored *Pluchea* leaf infusion steeped at 70, 80, and 95 °C for 5 minutes had lower  
460 enzyme inhibition activity of less than 50 % with the lowest at 95 °C around 13 %.  
461 Widyawati et al. (2017) found that the ability to inhibit the  $\alpha$ -amylase enzyme in un-stored  
462 *Pluchea* infusion steeped at 95 °C for 5 minutes was also low at 28.79 %. Increasing the  
463 steeping temperature and storage period reduced the ability of the phytochemicals in the  
464 *Pluchea* infusion to inhibit the  $\alpha$ -amylase enzyme activity period. Table 2 further shows  
465 that the AA of *Pluchea* infusion was strongly and negatively significantly correlated with  
466 TPC, TTC, DPPH, and FRAP, but it was weakly and positively significantly correlated with  
467 TFC.

468 This inhibitory activity was thought to be contributed by other bioactive compounds,  
469 besides phenolics which are sensitive to steeping temperature and storage period. Li et  
470 al. (2018) stated that there are flavonoid compounds that contribute to the ability to inhibit  
471 the  $\alpha$ -amylase enzyme. Akah et al. (2011) reported that phytochemical compounds, such  
472 as terpenoids, saponins, flavonoids, glycosides/carbohydrates, and alkaloids are good  
473 antidiabetic metabolites or  $\alpha$ -amylase enzyme activity inhibitors. Sangeetha and  
474 Vedesree (2012) explained that the ability of *Threspesia populnea* extract to inhibit the  $\alpha$ -  
475 amylase enzyme was determined of their phenolic compound content and protein.  
476 Moreover, the presence of  $\alpha$ -amylase enzyme inhibitor in this extract may be  
477 proteinaceous or nonproteinaceous in nature. It was assumed that this enzyme inhibitory  
478 activity in *Pluchea* infusion also was determined with their protein and polyphenolic  
479 content. Aleixandre et al. (2022) also stated that phenolic acids have inhibition activity to  
480  $\alpha$ -amylase enzyme depending on their structures. There are C=C double bonds  
481 conjugated with a carbonyl group of phenolic structures that stabilize the binding forces

482 to the active site of the  $\alpha$ -amylase. The hydroxyl groups can bind by non-covalent  
483 interaction (hydrogen bonding, cation- $\pi$  interactions, salt bridge interactions, ionic  
484 interactions, or electrostatic forces) with amino acid residue at the active site in  $\alpha$ -amylase  
485 enzyme. Elevated steeping temperature and longer storage period can easily cause the  
486 removal of the hydroxyl groups of phenolic compounds that can reduce their ability of  
487 enzyme inhibition. The phenolic acids with a greater number of hydroxyl groups exhibits  
488 stronger capability to obstruct the  $\alpha$ -amylase enzyme.

489

#### 490 Alpha glucosidase enzyme inhibition activity (GA)

491 Alpha glucosidase is an important enzyme in carbohydrate digestion, that catalysis  
492 the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and converts  
493 them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et al., 2014;  
494 Proenca et al., 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -glucosidase  
495 enzyme is used to determine their antidiabetic activity. This is supported by Werdani and  
496 Widyawati (2018) stated that Pluchea infusion has the potential as an antidiabetic agent.  
497 Widyawati et al. (2020) found that the steeping of un-stored Pluchea infusion at 95 °C for  
498 5 minutes has an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857 %.

499 Figure 3b shows that the ability of the Pluchea leaf infusion to inhibit the  $\alpha$ -  
500 glucosidase enzyme decreased with increasing steeping temperature and storage period.  
501 Steeping at 95 °C of the un-stored Pluchea leaf infusion obtained the lowest inhibitory  
502 ability, i.e.,  $48.32 \pm 1.27$  %, and the highest inhibitory activity was at 70 °C at  $95.11 \pm$   
503  $0.70$ %. The results of a paired t-test showed that GA of Pluchea infusion was significantly  
504 different between steeping temperature and long storage. Figure 3 further shows that the

505 ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher  
506 than the ability to inhibit the  $\alpha$ -amylase enzyme. Data analysis in Table 2. showed that  
507 the TFC of the *Pluchea* leaf infusion was influenced weakly and positively by GA and AA,  
508 but the GA and AA were not affected by TPC, TTC, DPPH, and FRAP. Li et al. (2018)  
509 stated that flavonoid compounds can inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -  
510 glucosidase enzymes. Dias et al. (2021) stated that flavonoid compounds, such as rutin,  
511 myricetin, kaempferol, and quercetin have antioxidant and antihyperglycemic activities.  
512 The ability to inhibit the action of enzymes from flavonoid compounds is determined by  
513 the position and number of hydroxyl groups, the number of double bonds in rings A and  
514 B, and the heterocyclic ring in ring C. Tadera et al. (2006) and Zhang et al. (2014) also  
515 explained that flavonoid compounds of samples significantly inhibit the  $\alpha$ -glucosidase  
516 enzyme activity.

517 The ability to inhibit the  $\alpha$ -glucosidase enzyme from *Pluchea* infusion was  
518 significantly affected by the steeping temperature and long storage. Figure 3 also showed  
519 that the capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater  
520 than the  $\alpha$ -amylase enzyme because the mechanism of the two enzymes was different,  
521 according to the opinion of McCue et al. (2005). The mechanism of the  $\alpha$ -glucosidase  
522 enzyme inhibitor includes making the sugar mimic structure, binding using ionic bonds  
523 with nucleophilic, making the transition state-like structure, binding hydrogen with catalytic  
524 acid residue, interacting ionic and hydrophobic with site other than the active site, and  
525 binding covalent with enzymes through an epoxy or aziridine group (Moorthy et al., 2012).  
526 Then, the mechanism of the  $\alpha$ -amylase enzyme inhibitor includes blocking carbohydrates,

527 limiting the digestibility and absorption of carbohydrates, and blocking the active centers  
528 of several subsites of the enzyme (Gong et al., 2020).

529 Widyawati et al. (2017) stated that phenolic and non-phenolic compounds can  
530 inhibit of the  $\alpha$ -glucosidase enzyme activity. The ability of bound phenolic compounds to  
531 inhibit  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. The presence  
532 of polymerization and degradation reactions, that may be occurred in *Pluchea* infusion  
533 during storage, affects the structure and profile of phenolic and non-phenolic compounds.  
534 Asriningtyas et al. (2014) explained that the methyl-esterified quinic acid with the caffeic  
535 groups, such as 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid methyl ester,  
536 3,4,5-tri-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid, and 1,3,4,5-  
537 tetra-*O*-caffeoylquinic acid of *Pluchea* leaves inhibits the  $\alpha$ -glucosidase enzyme activity.  
538 The resulting analysis of caffeoylquinic acids (3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-  
539 caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid in stored *Pluchea* leaf infusion higher  
540 concentration than in un-stored *Pluchea* infusion, and the concentrations of the simple  
541 phenolic compounds were increased at higher steeping temperature, but the  $\alpha$ -  
542 glucosidase inhibition activity of them was reduced. It means that the methyl-esterified  
543 quinic acid with the caffeic groups had more potential to inhibit  $\alpha$ -glucosidase enzyme  
544 than free caffeoylquinic acid.

545 This study showed that the increasing steeping temperature and storage period  
546 caused degradation of polyphenol compounds to produce simple phenolic compounds,  
547 such as gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,4-di-*O*-caffeoylquinic  
548 acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid that increased the total  
549 phenolic content and total tannin content. The increase in the simple phenolic

550 concentration of the *Pluchea* leaf infusion caused higher antioxidant activity and lower  
551 antidiabetic activity.

552

## 553 CONCLUSION

554 The Total Phenol (TPC) of *Pluchea* infusion at different steeping temperatures and  
555 storage periods generally significantly increased with increasing steeping temperature  
556 and storage periods. Steeped and stored infusion had significantly higher amounts of  
557 phenolic compounds than the samples that were steeped and un-stored. TPC was  
558 highest in the stored and steeped at 95°C and lowest in the un-stored and steeped at  
559 60°C. Un-stored steeped samples exhibited significantly higher flavonoid content than the  
560 stored steeped samples. The highest total flavonoid content was exhibited by the un-  
561 stored samples steeped at 95°C. The total tannin content of *Pluchea* leaf infusion  
562 significantly increased with increasing steeping temperature and storage period. Among  
563 the un-stored steeped samples, the tannin content was significantly lowest in the samples  
564 steeped at 60°C and highest in the samples steeped at 95°C.

565 The free radical scavenging property (DPPH) of the stored and steeped *Pluchea*  
566 leaf infusion was significantly higher than the un-stored steeped samples. The free radical  
567 scavenging property was highest in the stored samples steeped at 80 and 95°C. free  
568 radical scavenging activity of the samples was strongly and positively correlated with total  
569 phenolic and tannin contents, but inversely with total flavonoid levels. The ferric-reducing  
570 antioxidant power (FRAP) significantly increased with increasing steeping temperature  
571 and longer storage periods. The lowest FRAP was found in the un-stored samples which  
572 were steeped at 60°C and the highest was exhibited in *Pluchea* stored samples which

573 were stored for 5 years and steeped at 95°C. The FRAP of *Pluchea* leaf infusion was  
574 significantly strong and positively correlated with the free radical scavenging property,  
575 total phenolic, and total tannin content, but inversely with total flavonoid content. The  
576 inhibition of the  $\alpha$ -amylase activity was generally found to be higher at lower steeping  
577 temperatures of the un-stored *Pluchea* leaf infusion than at higher steeping temperatures  
578 of the stored sample. The  $\alpha$ -amylase enzyme inhibition capacity of the *Pluchea* leaf  
579 infusion showed a significantly strong and negative correlation with TPC, TTC, DPPH,  
580 and FRAP, but it was weakly and positively correlated significantly with TFC.

581 The ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme  
582 decreased at high steeping temperatures and long storage periods. The highest inhibitory  
583 activity was obtained in the un-stored *Pluchea* leaf infusion that was steeped at 70°C  
584 while the lowest was obtained in the un-stored sample that was steeped at 95°C. The  
585 ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher  
586 than the ability to inhibit the  $\alpha$ -amylase enzyme. The inhibition of the  $\alpha$ -glucosidase  
587 enzyme activity was significantly strong and negative TPC, TTC, DPPH, and FRAP, and  
588 it was weakly and positively correlated significantly with TFC.

589 The simple phenolic compounds identified in *Pluchea* leaf infusion may affect the  
590 presence of the bioactive compounds, antioxidant potential, and antidiabetic properties  
591 at different steeping temperatures and storage periods including gallic acids, (+)-  
592 catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-  
593 caffeoylquinic acids, 4,5-di-*O*-caffeoylquinic acids.

594

595 ACKNOWLEDGMENTS

596 The authors would like to thank the Ministry of Education and Culture of the Republic of  
597 Indonesia for a fundamental research grant to higher education institutions in 2022

598

#### 599 STATEMENT ON CONFLICT OF INTEREST

600 The authors declare no conflict of interest.

601

#### 602 NOTES ON APPENDICES (if any)

603 The complete appendices section of the study is accessible at

604 <http://philjournsci.dost.gov.ph>

#### 605 REFERENCES

606 ACAR A, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food  
607 wastes: evaluation of temperature and time effects on quality parameters. Applied Food  
608 Res. 2(100087):1-8.

609 ALEIXANDRE A et al. 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -  
610 glucosidase and influence of reaction conditions. Food Chem. 372(131231):1-9.

611 ALI et al. 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile.  
612 Food Chem. 89(2005):27-36.

613 ALI A. et al. 2018. Impact of storage conditions on the stability of predominant phenolic  
614 constituents and antioxidant activity of dried *Piper betle* extracts. Molecules.23(24):1-13.

615 AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and  
616 methanol extract and fractions of *Gongronema latifolium* (Asclepidaceae) leaves in  
617 alloxan diabetic rats. J Applied Pharm. Sci. 01(09): 99-102.



618 ARSININGTYAS IS. et al. 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves  
619 of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product  
620 use. *Natural Product Res.* 28(17):1350-1353.

621 ARYAL S et al. 2019. Total phenolic content, flavonoid content and antioxidant of wild  
622 vegetables from Western Nepal. *Plants.* 8(96): 1-12.

623 AYELE DT, AKELE ML, MELESE A.T. 2022. Analysis of total phenolic contents,  
624 favonoids, antioxidant and antibacterial activities of *Croton macrostachyus* root extracts.  
625 *BMC Chem.* 16(30): 1-9.

626 BALIYAN S et al. 2022. Determination of antioxidants by DPPH radical scavenging  
627 activity and quantitative phytochemical analysis of *Ficus religiosa*. *Molecules.* 27(1326):  
628 1-19.

629 CASTIGLIONI S et al. 2015. Influence of steeping conditions (time, temperature, and  
630 particle size) on antioxidant properties and sensory attributes of some white and green  
631 teas. *Intern. J. Food Sci. Nut.* Online 1-7.

632 Chan EWC et al. 2022. *Pluchea indica*: An updated review of its botany, uses, bioactive  
633 compounds and pharmacological properties. *Pharmaceutical Sciences Asia.* 49(1): 77-  
634 85.

635 CHANDRA S et al. 2014. Assessment of total phenolic and flavonoid content, antioxidant  
636 properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit  
637 crops: A Comparative study. Hindawi Publishing Corporation Evidence-Based  
638 Complementary Alter. Med. 2014:1-9 (2014)

639 CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic, flavonoids,  
640 tannin and chlorophyll content of leaves of *Strobilanthes Kunthiana (Neelakurinji)*. J.Med.  
641 Plants Studies. 4(4):282-286.

642 DIAS MC, PINTO DCGA, SILVA AMS. 2021. Plant flavonoids: chemical characteristics  
643 and biological activity. Molecules. 26(5377):1-16.

644 DOBRINAS S, SOCEANU A, POPESCU V, POPOVICI IC, JITARIU D. 2021.  
645 Relationship between total phenolic content, antioxidant capacity, Fe and Cu content from  
646 tea plant samples at different brewing times. Processes. 9(1311):1-11.c

647 FERRARIS Z. 2023. *Pluchea indica* Less. iNaturalist.org.  
648 <https://www.gbif.org/occurrence/4022273660>.

649 FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional  
650 properties and visual appearance of Dampit Robusta coffee leaves tea. Advances in Food  
651 Sci. Sustain. Agric. Agro-industrial Engineering. 4(1):25-32.

652 FU L et al. 2017. Total phenolic contents and antioxidant capacities of herbal and tea  
653 infusions. Intern. J. Molecular Sci. 12:2112-2124.

654 GAO [MR et al. 2019](#). A theoretical and experimental study: the influence of different  
655 standards on the determination of total phenol content in the Folin–Ciocalteu assay. J.  
656 Food Measurement and Characteristic. 13:1349–1356.

657 GONG L, FENG D, WANG T, REN Y, LIU Y, WANG J. 2020. Inhibitors of  $\alpha$ -amylase and  
658  $\alpha$ -glucosidase: Potential linkage for whole cereal foods on prevention of hyperglycemia.  
659 Food Sci. Nut. 2020(8):6320-6337.

660 HUANG D, OU B, PRIOR RL. 2005. The Chemistry behind antioxidant capacity assays.  
661 J. Agric. Food Chem. 53:1841-1856.

662 ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties  
663 of rosehip tea beverage: study by response surface methodology. J. Food Sci. Tech.  
664 54(11):3737–3743.

665 JAYANI NIE et al. 2022. Evaluation of stability and quality characteristics of moringa  
666 (*Moringa oleifera*) herbal tea during storage. Food Res. 6(4):399-406.

667 KIM JM et al. 2020. Effect of storage temperature on the antioxidant activity and catechins  
668 stability of Matcha (*Camellia sinensis*). Food Science and Biotechnology. 29(9):1261–  
669 1271.

670 KILIC et al. 2017. Antioxidant properties of some herbal teas (green tea, senna, corn silk,  
671 rosmery) brewed at different temperatures. Intern. J. Secondary Metabolite. 4(3):142-248  
672 Special Issue 1.

673 KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of  
674 extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves  
675 from different provenances in Thailand. Revista Brasileira de Farmacognosia. 28(2018):  
676 145–150.

677 KOWALSKA J et al. 2021. Influence of tea brewing parameters on the antioxidant  
678 potential of infusions and extracts depending on the degree of processing of the leaves  
679 of *Camellia sinensis*. Molecules.26(4773):1-17.

680 KRUK J, ABOUL-ENAIN BH, DUCHNIK E, MARCHLEWICZ M. 2022. Antioxidative  
681 properties of phenolic compounds and their effect on oxidative stress induced by severe  
682 physical exercise. J. Physiol. Sci. 72(19):1-24.

683 LI K et al. 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the flavonoids  
684 rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity

685 relationship of its eight flavonoids by a refined assign score method. Chem. Central J.  
686 12(82):1-11.

687 LIN YS. et al. 2020. Effects of storage time and temperature on antioxidants in juice from  
688 *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser. Molecules.  
689 25(3614): 1-12.

690 MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content  
691 and antioxidant activity test of avocado seed extract (*Persea Americana* Mill). Jurnal MIPA  
692 Unsrat Online. 1(1): 5-10. [https://media.neliti.com/media/publications/116214-ID-](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf)  
693 [penentuan-kandungan-tanin-dan-uji-aktivi.pdf](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf) [In Bahasa Indonesia]

694 MARTINEZ-SOLIS J et al. 2022. Antidiabetic and toxicological effect of the tea infusion  
695 of summer collection from *Annona cherimola* Miller leaves. Plants. 11(3224):1-17.

696 MATHUR R, VIJAYVERGIA R. 2017. Determination of total flavonoid and phenol content  
697 in *Mimusops Elengi* Linn. Intern. J. Pharm. Sci. Res. 8(12): 5282-5285.

698 MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of  
699 sprouted and solid-state bioprocessed soybean. Asia Pacific J. Clinical Nut. 14(2): 145-  
700 152.

701 MOORTHY NS, RAMOS MJ, A FERNANDES P. 2012. Studies on  $\alpha$ -glucosidase  
702 inhibitors development: Magic molecules for the treatment of carbohydrate mediated  
703 diseases. Mini Rev Med Chem. 12:713–720.

704 MORAES-DE-SOUZA RA, OLDONI TLC, REGITANO-D'ARCE MAB, ALENCAR SM.  
705 2008. Antioxidant activity and phenolic composition of herbal infusions consumed in  
706 Brazil. Cienc Tecnol. Aliment. 6(1):41-47.

707 MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant  
708 activity: A Review. *Intern. J. Molecular Sci.* 22(3380):1-30.

709 NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus*  
710 *sabdariffa* L.): Effect of dying temperature and brewing conditions on total soluble solid,  
711 phenolic content, antioxidant capacity and sensory quality. *Beverages*.6(2):1-11 (2020).

712 NOREEN H. et al. 2017. Measurement of total phenolic content and antioxidant activity  
713 of aerial parts of medicinal plant *Coronopus didymus*. *Asian Pacific J. Tropical Med.* 10(8):  
714 792–801.

715 NURCHOLIS GW et al. 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity in-  
716 vitro and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum pictum*  
717 (L.). *Current Biochem.* 1(2): 58-65.

718 OH J et al. 2013. Antioxidant and antimicrobial activities of various leafy herbal teas. *Food*  
719 *Control.* 31(2013):403-409.

720 PROENCA C et al. 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an in vitro and in silico  
721 structure–activity relationship study. *J. Enzyme Inhibition Med. Chem.* 32(1): 1216–1228.

722 PUREWAL SS et al. 2022. Unraveling the effect of storage duration on antioxidant  
723 properties, physicochemical and sensorial parameters of ready to serve Kinnow-aml  
724 beverages. *Applied Food Res.* 2(1): 1-8.

725 RAHARJO D, HARYOTO. 2019. Antioxidant activity of mangrove *Sonneratia caseolaris*  
726 L. using the FRAP method. *ISETH.* 2019: 623-629.

727 RAMPHINWA ML et al. 2023. Eco-physiological response of secondary metabolites of  
728 teas: review of quality attributes of herbal tea. *Frontiers in Sustain. Food Systems.*  
729 7(990334):1-14.

730 REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids.  
731 Czech J. Food Sci. 30(2):171-177.

732 RUAN J et al. 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea*  
733 *indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel  
734 column chromatography fractions. Molecules. 24(2784):1-20.

735 RUSITA YD et al. 2019. Comparison of tannin concentration on fresh and dried tea leaf  
736 (*Camelia sinensis* L.) extract using UV-visible spectrophotometric method. 1st  
737 International Conference of Health, Science & Technology (ICOHETECH) 2019 E-ISBN:  
738 978-623-92207-0-9. November 16<sup>th</sup>, 2019.LPPM Universitas Duta Bangsa Surakarta,  
739 Surakarta.

740 SANGEETHA R, VEDASREE N. 2012. In Vitro  $\alpha$ -Amylase inhibitory activity of the leaves  
741 of *Thespesia populnea*. Intern. Scholarly Res.Network. 2012: 1-4 (2012)

742 SHRAIM AM et al. 2021. Determination of total flavonoid content by aluminum chloride  
743 assay: A critical evaluation. LWT Food Sci. Tech. 150(111932):1-11.

744 SILVA-RAMIREZ KA et al. 2020. Effect of drying and steeping temperature on the  
745 phenolic content, antioxidant activity, aromatic compounds and sensory properties of  
746 *Culila polyantha* Benth. Infusions. Processes. 8(1378):2-20.

747 SRISOOK K et al. 2012. Antioxidant and anti-inflammatory activities of hot water extract  
748 from *Pluchea indica* Less. herbal tea. J. Med. Plants Res. 6(23):4077-4081.

749 SURIYAPHAN O. 2014. Nutrition, health benefits and applications of *Pluchea indica* (L)  
750 Less leaves. Pharm. Sci. Asia. 41:1–10.

751 SU D et al. 2019. Impact of thermal processing and storage temperature on phenolic  
752 profile and antioxidant activity of different varieties of lychee juice. LWT-Food Tech.  
753 116(108578):1-9.

754 TADERA K, MINAMI Y, TAKAMATSU K, MATSUOKA T. 2006. Inhibition of alpha  
755 glucosidase and alpha-amylase by flavonoids. J Nutr Sci Vitaminol. 52:149–153.

756 THANAJIRUSCHAYA P et al. 2010. Effect of storage period and temperature on  
757 antioxidant components and properties of milled rice. Khon Kaen Univ. Res. J.15(9):843-  
758 851.

759 VONGSAK B et al. 2018. Comparison of active constituents, antioxidant capacity, and  
760 alpha-glucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. Food  
761 Biosci. 25: 68–73.

762 WANG SC. et al. 2022. Effect of brewing conditions on polyphenols in the dark tea  
763 (*Camellia sinensis* L.) infusions: content, composition and antioxidant activities. Food  
764 Sci.Tech. 42(e36322):1-9.

765 WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica*  
766 Less as functional beverage in diabetic patients. ASSEHR. 98:164-167.

767 WIDYAWATI PS et al. 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract  
768 as antioxidant and anti-warmed-over flavor (WOF) of duck meat. International Congress  
769 on Challenges of Biotechnological Research in Food and Health. November 15<sup>th</sup>  
770 2014.Agricultural Technology Faculty, Slamet Riyadi University, Surakarta.

771 WIDYAWATI PS et al. 2016. The physicochemical and antioxidant properties of *Pluchea*  
772 *indica* Less drink in tea bag packaging. IJFNS. 5: 13–120.

773 WIDYAWATI PS et al. 2017. In vitro antioxidant capacities and antidiabetic properties of  
774 pluchea leaves. IJPPS. 9(8): 203-208.

775 WIDYAWATI PS et al. 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica*  
776 Less beverage. CJFST.12(4): 125-139.

777 WIDYAWATI PS ET AL. 2022. The effect of κ-carrageenan proportion and hot water  
778 extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of stink  
779 lily (*Amorphophallus muelleri*) wet noodles. Molecules. 27(5062):1-16.

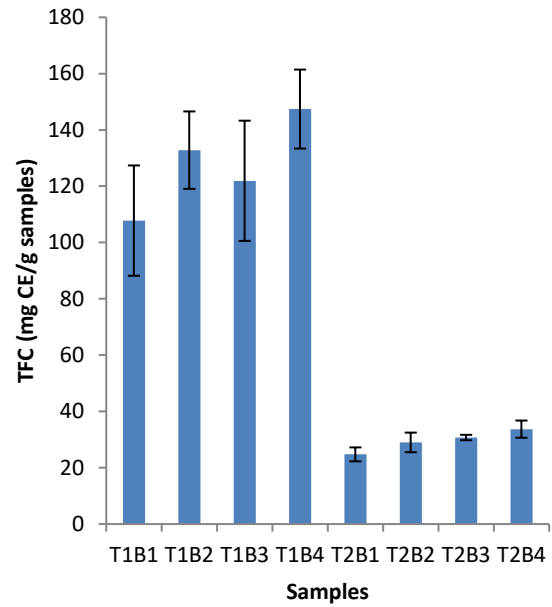
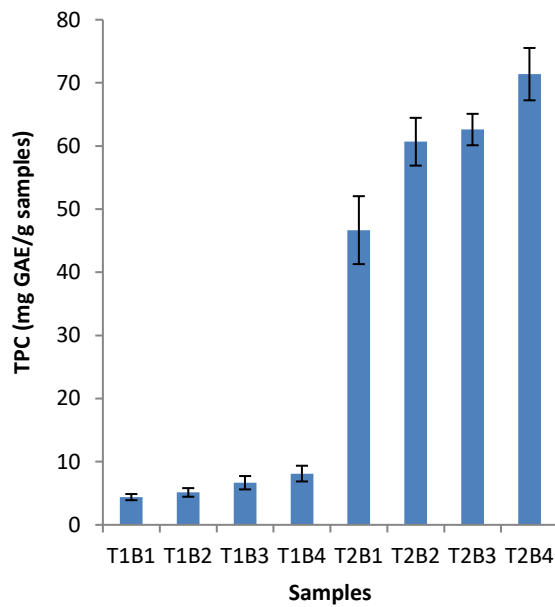
780 XU P, CHEN L, WANG Y. 2019. Effect of storage period on antioxidant activity and  
781 inhibition on α-amylase and α-glucosidase of white tea. Food Sci. Nut. 7(2): 636-644.

782 ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction period on caffeine  
783 in coffee. Lantanida J. 6(2):103-202. [https://jurnal.ar-](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811)  
784 [raniry.ac.id/index.php/lantanida/article/view/3811](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811). [In Bahasa Indonesia]

785 Zhang H, Wang G, Dong J. 2014. Inhibitory properties of aqueous ethanol extracts of  
786 propolis on alpha-glucosidase. J Evid Based Complement Alternat Med. 2015:1–8.

787 ZHANG Y et al. 2021. Effect of storage conditions and period on the polyphenol content  
788 of wheat flours. Processes. 9(248):1-11.



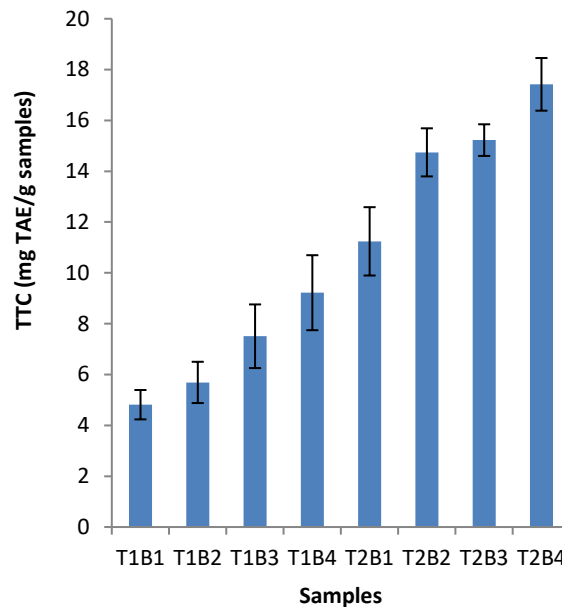


789

790

(a)

(b)



791

792

(c)

793 Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping  
 794 temperature and storage period (a) Total phenolic content (b) Total flavonoid  
 795 content (c) Total tannin content. Data analysis using ANOVA at  $\alpha \leq 0.05$   
 796 continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as  
 797 mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored;

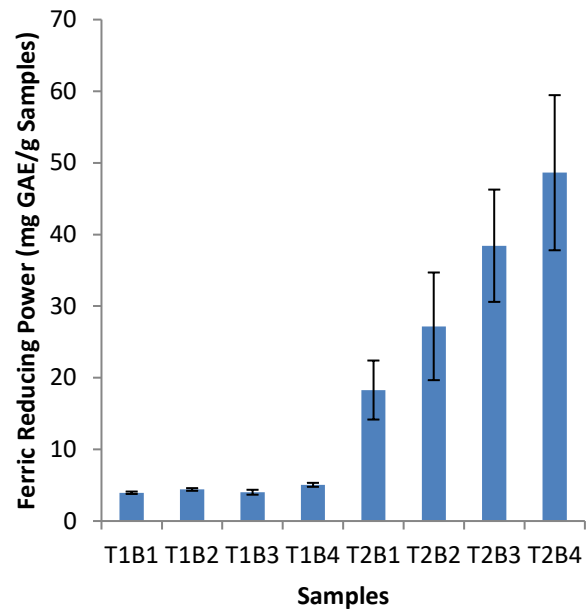
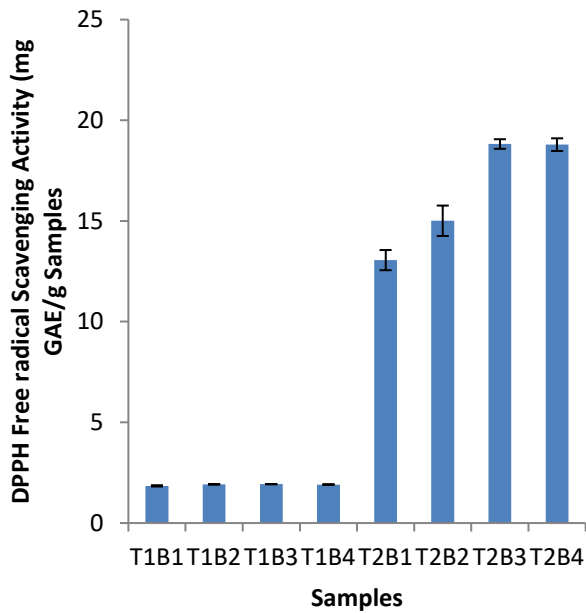
798 T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-  
799 steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-  
800 steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5  
801 years; T3B4-steeped at 95 °C, stored for 5 years.

802 Table 1. Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperature and storage period

Phenolic Compounds	Steeping Temperature (°C)	Mean±SD Un-stored	Mean±SD Stored	Mean difference ±SD	Sig (2-tailed)
Gallic Acid (µg/g samples)	60	0.2132±0.0027	0.2364±0.0015	0.0375±0.0175	0.2030
	70	0.2157±0.0013	0.2324±0.0214	0.0167±0.0227	0.4870
	80	0.2234±0.0122	0.2347±0.0078	0.0386±0.0264	0.2870
	95	0.2316±0.0104	0.2402±0.0169	0.0086±0.1990	0.8500
(+) -Catechin (µg/g samples)	60	0.3425±0.0110	0.5085±0.0111	-0.1576±0.0885	0.241
	70	0.3260±0.0265	0.5448±0.0006	-0.2188±0.0259	0.053
	80	0.3240±0.0222	0.5023±0.0773	-0.1451±0.0248	0.077
	95	0.4039±0.0320	0.5995±0.0372	-0.2049±0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756±0.1234	1.4762±0.0271	-1.2887±0.3222	0.111
	70	0.2587±0.0160	1.4245±0.2526	-1.1657±0.2695	0.103
	80	0.4175±0.0104	1.4570±0.0925	-1.0391±0.0841	0.036*
	95	0.8786±0.0434	2.6138±0.0695	-1.1735±0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220±0.0268	0.6220±0.0706	-0.5999±0.9733	0.544
	70	0.1530±0.0511	1.0708±0.0289	-0.9177±0.0222	0.011*
	80	0.3666±0.0103	0.8629±0.0815	-0.1082±0.4462	0.790
	95	0.6559±0.0570	2.0230±0.0573	-1.4123±0.3203	0.101
Kaempferol (µg/g samples)	60	0.1394±0.0202	0.3675±0.0183	-0.3207±0.1122	0.154
	70	0.0514±0.0037	0.3726±0.0944	0.3213±0.0907	0.125
	80	0.3699±0.0924	0.7966±0.0366	-0.4267±0.2727	0.271
	95	0.5913±0.0239	0.9478±0.0287	-0.3565±0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103±0.0628	2.4863±0.0270	-1.8760±0.2074	0.050*
	70	0.6271±0.0099	2.3403±0.0325	-1.7131±0.3152	0.082
	80	0.7967±0.03060	2.6278±0.0211	-1.8311±0.0095	0.002*
	95	1.5386±0.0668	4.0211±0.0851	-2.4825±0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635±0.0628	0.9449±0.0501	-0.2814±0.4458	0.536
	70	0.6162±0.0099	0.9485±0.0794	-0.3323±0.0301	0.041*
	80	0.6601±0.0306	0.9099±0.0387	-0.2498±0.3127	0.461
	95	0.6642±0.0668	1.3156±0.0166	-0.6514±0.2666	0.179

4,5-di-O-Caffeoylquinic acid ( $\mu\text{g/g}$ samples)	60	0.4906 $\pm$ 0.0060	1.1842 $\pm$ 0.0120	-0.6886 $\pm$ 0.2723	0.018*
	70	0.4807 $\pm$ 0.0034	1.0089 $\pm$ 0.0736	-0.5281 $\pm$ 0.0702	0.060
	80	0.5299 $\pm$ 0.0053	1.2382 $\pm$ 0.1435	-0.7082 $\pm$ 0.1489	0.094
	95	1.0018 $\pm$ 0.0526	1.3797 $\pm$ 0.2170	-0.3086 $\pm$ 0.3086	0.333

803 Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  
804  $\pm$ standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped  
805 at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped at 70 °C,  
806 stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-steeped at 95 °C, stored for 5 years.  
807



808

(a)

(b)

809

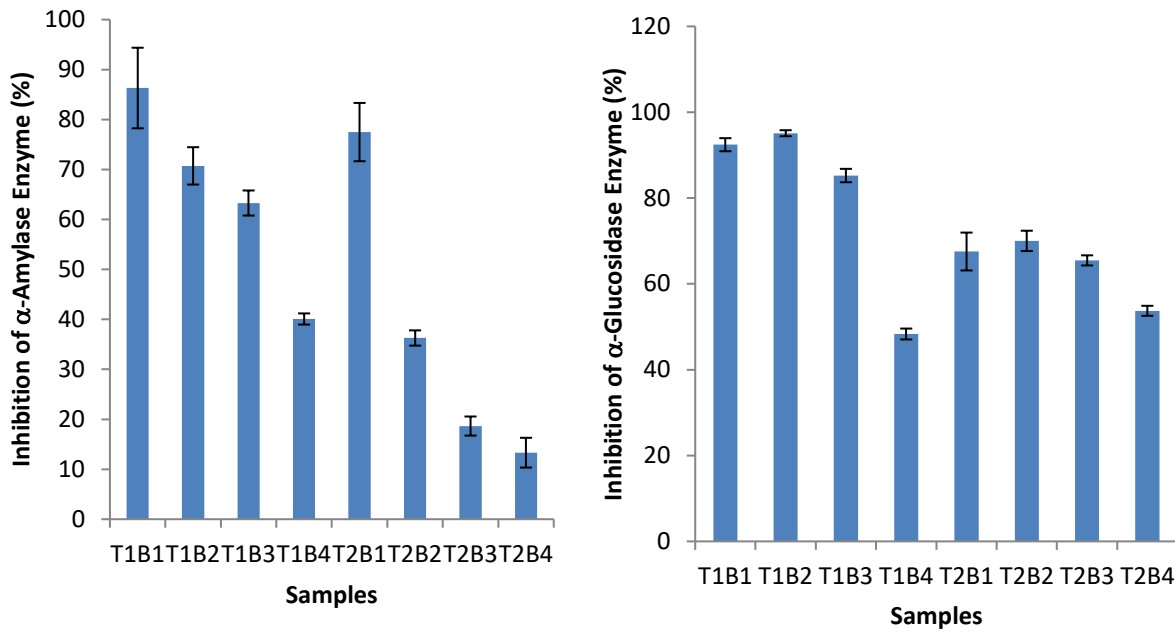
810 Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage  
 811 period (a) DPPH (b) FRAP. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued  
 812 analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  
 813  $\pm$ standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-  
 814 steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped  
 815 at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-steeped  
 816 at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5 years; T3B4-  
 817 steeped at 95 °C, stored for 5 years.

818

819

820

821



822

823

(a)

(b)

824 Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage  
 825 period (a)  $\alpha$ -amylase (b)  $\alpha$ -glucosidase. Data analysis using ANOVA at  $\alpha \leq 0.05$   
 826 continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as  
 827 mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored;  
 828 T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-  
 829 steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 years; T3B2-  
 830 steeped at 70 °C, stored for 5 years; T3B3-steeped at 80 °C, stored for 5  
 831 years; T3B4-steeped at 95 °C, stored for 5 years.

832 Table 2. Pearson correlation coefficients between bioactive contents (TPC, TFC and TAC), antioxidant activity (DPPH and  
 833 FRAP) and antidiabetic activity (AA and GA)

	<i>TPC</i>	<i>TFC</i>	<i>TTC</i>	<i>DPPH</i>	<i>FRAP</i>	<i>Alpha Glucosidase</i>	<i>Alpha Amylase</i>
TPC	1						
TFC	-0.93589	1					
TTC	0.960028	-0.81321	1				
DPPH	0.992776	-0.93992	0.942273	1			
FRAP	0.953366	-0.82636	0.947778	0.956242	1		
Alpha Glucosidase	-0.55512	0.349873	-0.71534	-0.5272	-0.55947	1	
Alpha Amylase	-0.70842	0.429393	-0.8569	-0.69579	-0.80548	0.725161631	1

834 Significant at the 0.05 level (2-tailed)

835

836



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**Fwd: Comments on PJS Paper Ms 23-158**

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Fri, Jun 14, 2024 at 9:31 AM

Dear Ms CARYL MARIA MINETTE I. ULAY

Greetings,

I have seen that my manuscript entitled Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of Pluchea indica Less Tea has been published in the June 2024 edition ([#28] [23-158][#28] [23-158 ] Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of Pluchea indica Less Tea [Research Note] PS Widyawati and YR Wilianto), but I don't get any information, how can I access the manuscript, please provide information.

Regards

Paini SW

[Quoted text hidden]





Paini Sri Widyawati <paini@ukwms.ac.id>

---

## Manuscript has been formatted

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>

Sat, Jun 15, 2024 at 1:17 PM

To: Paini Sri Widyawati <paini@ukwms.ac.id>

Dear Dr. Widyawati,

Greetings! We wish to guarantee that your peer-reviewed article shall remain open-access to all online users once published by the PJS Editorial Office.

Moreover, we the editors alone shall implement the open-access setup on your behalf as the author. Thank you!

Sincerely,

Mr. ALLYSTER A. ENDOZO

Managing Editor

[Quoted text hidden]



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**Manuscript has been formatted**

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Sat, Jun 15, 2024 at 12:54 PM

Dear Mr ALLYSTER A. ENDOZO  
Managing Editor

Greetings,

Is it possible after the manuscript is published that readers can view the contents of the manuscript (open access). If the manuscript can be made open access, what do I have to do or fulfill it? Please help, because the publication of the manuscript is very important for the leadership to know our performance.

Thank You for attention

Regards

Paini Sri Widyawati  
[Quoted text hidden]



Paini Sri Widyawati <paini@ukwms.ac.id>

---

## Manuscript has been formatted

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Sat, Jun 15, 2024 at 1:17 PM

Dear Dr. Widyawati,

Greetings! We wish to guarantee that your peer-reviewed article shall remain open-access to all online users once published by the PJS Editorial Office.

Moreover, we the editors alone shall implement the open-access setup on your behalf as the author. Thank you!

Sincerely,  
Mr. ALLYSTER A. ENDOZO  
Managing Editor  
[Quoted text hidden]



Paini Sri Widyawati <paini@ukwms.ac.id>

---

## Manuscript has been formatted

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Sat, Jun 15, 2024 at 4:05 PM

Dear Mr. ALLYSTER A. ENDOZO  
Managing Editor

Greetings,

Thanks for your attention, I am glad to hear that. I hope this manuscript can be published in June or July.

Regards

Paini SW  
[Quoted text hidden]

7. Paper Accepted (15-6-2024)

- Correspondence
- Copyediting Process
- Document



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

## Copyediting of PJS Paper Ms 23-158

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Sat, Jun 15, 2024 at 11:28 AM

Dear Dr. Widyawati,

Greetings! We acknowledge the receipt of the accepted Ms 23-158 paper.

Before we commence the copyediting process, kindly revise key sections in accordance with our formatting style as per the following guidelines:

- [main content] the Latin phrase "*et al.*" must be italicized;
- [main content] each of the time units must be indicated briefly (*i.e.* s, min, h, d, wk, mo, yr); and
- [references] the in-text citation "Kumar and Goel (2019)" must be corroborated by a reference entry.

We hope to receive your revised manuscript soon. Thank you!

Sincerely,  
Mr. ALLYSTER A. ENDOZO  
Managing Editor



**Philippine Journal of Science**  
**Science and Technology Information Institute**  
**Department of Science and Technology**  
**DOST Complex, Gen. Santos Ave., Bicutan 1631**  
**Taguig City, Metro Manila, Philippines**  
Telephone no. : 837 - 2191  
Email: [pjs@stii.dost.gov.ph](mailto:pjs@stii.dost.gov.ph); [philjournsci@gmail.com](mailto:philjournsci@gmail.com)  
Website: <https://philjournalsci.dost.gov.ph>  
Scopus: <https://www.scopus.com/sourceid/19700175735>



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

## Copyediting of PJS Paper Ms 23-158

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Sat, Jun 15, 2024 at 4:02 PM

Dear Mr. ALLYSTER A. ENDOZO  
Managing Editor


Greetings,

I have sent revised manuscript according to recommendations. If something is still incorrect, please don't hesitate to contact me.  
Thank You for attention.

Regards

Paini SW  
[Quoted text hidden]

---

 **PJS paper Ms 23-158-Effect of Brewing Temperature and Storage Time on Antioxidant and Antidiabetic Properties of Pluchea Tea (Final).docx**  
86K

1 **Effect of Steeping Temperature and Storage Period on the Bioactive Compounds,**  
2 **Antioxidant and Antidiabetic Activities of Infusion from Powdered Pluchea Indica**  
3 **Less**

4 **Paini Sri Widyawati<sup>1\*</sup>, Yufita Ratnasari Wilianto<sup>2</sup>**

5 <sup>1</sup>Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala  
6 Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

7 <sup>2</sup>Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic  
8 University, Kalisari Street Number 1, Surabaya 60272, Indonesia

9 Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature,  
10 Pluchea indica Less, storage period

11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23



24

25

26 ABSTRACT

27 This study was done to determine the effects of steeping temperature and storage period  
28 on the bioactive contents, antioxidant and antidiabetic activities of *Pluchea* leaf infusion.

29 The research used a randomized block design with two factors, i.e., steeping temperature  
30 (T) and storage period (B). The *Pluchea* leaf blades were exposed to 4 steeping

31 temperatures of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) °C with the storage period of 0 (B1)

32 and 5 (B2) yr resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1,

33 T3B2, T4B1, T4B2). Statistical analysis using a paired t-test at  $\alpha \leq 0.05$  showed that

34 treatments significantly affected the bioactive contents [(total phenol (TPC), total tannin

35 (TTC), total flavonoid (TFC)], antioxidant [(DPPH scavenging activity (DPPH) and ferric

36 reducing antioxidant power (FRAP)] potential and antidiabetic [( $\alpha$ -amylase (AA) and  $\alpha$ -

37 glucosidase (GA) inhibition)] properties of the *Pluchea* leaf infusion. TPC, TTC, DPPH,

38 and FRAP significantly increased for the storage period and the steeping temperatures.

39 Then, TFC decreased during the storage period but significantly increased at higher

40 steeping temperatures. The AA and GA of *Pluchea* leaf infusion increased until 70 °C of

41 the steeping temperature, but decreased until 95 °C. The DPPH and FRAP of the *Pluchea*

42 leaf infusion were strongly and positively correlated with TPC and TTC. The GA and AA

43 of *Pluchea* leaf infusion were not influenced by the TPC and TTC but were weakly and

44 positively correlated with TFC. The antioxidant activity of the *Pluchea* leaf infusion was

45 inversely proportional to the antidiabetic activity. The simple phenolic compounds derived

46 from *Pluchea* leaf infusion at different steeping temperatures and storage included gallic

47 acid, kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-caffeoylquinic acid, 3,5- di-  
48 O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid.

49

## 50 INTRODUCTION

51 Pluchea herbal tea is a product of dried Pluchea leaf processing introduced by  
52 world people (Srisook *et al.*, 2012; Widyawati *et al.*, 2016) because of the efficacy of the  
53 active components in Pluchea leaves, as a herbal plant that has been widely used for  
54 traditional medicine and food (Chan *et al.*, 2022). Pluchea leaves are composed of many  
55 nutrients and bioactive compounds useful to body health. The nutrient compositions in  
56 the Pluchea leaves include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates,  
57 calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive compounds are comprised, i.e.,  
58 chlorogenic acid, caffeic acid, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-  
59 caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-  
60 caffeoylquinic acid, quercetin, myricetin, kaempferol, total anthocyanin,  $\beta$ -carotene, and  
61 total carotenoid (Suriyaphan, 2014; Vongsak *et al.*, 2018; Ruan *et al.*, 2019; Widyawati *et*  
62 *al.*, 2022, Chan *et al.*, 2022).

63 The steeping process of Pluchea leaves can be performed with fresh or dry leaves  
64 in hot or boiling water for a few min (Suriyaphan, 2014; Silva-Ramirez *et al.*, 2020; Jayani  
65 *et al.*, 2022). In Asia, especially in Indonesia, people usually consume the Pluchea  
66 infusion by steeping 2 g of powdered Pluchea leaves in a tea bag in 100 mL of hot or  
67 boiling water. Widyawati *et al.* (2016) claimed that steeping of 2 g of Pluchea leaf powder  
68 at 95 °C for 5 min exhibits total phenolic and total flavonoid contents, the ability to  
69 scavenge DPPH free radicals, and the capability to reduce ferric ions at 9.3 mg gallic acid  
70 equivalent (GAE)/g sample, 22.0 mg gallic acid equivalent (GAE)/g sample, 27.2 mg gallic

71 acid equivalent (GAE)/g sample, and 10.2 mg gallic acid equivalent (GAE)/g sample,  
72 respectively. Werdani and Widyawati (2018) reported that drinking *Pluchea* leaf powder  
73 infusion in the morning and evening regularly (2 g/100 mL) can decline blood sugar levels.

74 The steeping of *Pluchea* herbal tea with hot water at 95 °C for 5 min certainly  
75 determines the stability and amount of extracted bioactive compounds that  
76 influence the biological activity especially antioxidant and antidiabetic activities. Silva-  
77 Ramirez *et al.* (2020) reported that the infusion process can influence the content and  
78 composition of the bioactive compounds and antioxidant activity of tea. Acar *et al.* (2022)  
79 informed that the infusion quality of herbal tea extract depends on several factors, i.e.,  
80 storage and temperature. The polyphenol profile and antioxidant properties of herbal tea  
81 infusion decline with an increase in steeping/brewing and storage temperatures, and  
82 longer exposure periods.

83 Several studies have mentioned the effect of steeping temperature on the  
84 bioactive compound contents and antioxidant activity, such as some white and green teas  
85 are effective with hot water at 90 °C for 7 min (Castiglioni *et al.*, 2015), on rosheship tea is  
86 effective at infusion period around 6-8 min at temperatures of 84-86 °C (Ilyasoglu and  
87 Arpa, 2017), on the caffeine content extracted at the brewing temperature of coffee  
88 (Zarwinda and Sartika, 2018), and the high total phenol content and antioxidant activity  
89 of dark tea at 92 °C for 27 min (Wang *et al.*, 2022). The study of the effect of steeping  
90 temperature on *Pluchea* infusion was carried out to afford information about the most  
91 efficient preparation of powdered *Pluchea* leaves to get higher bioactive compounds,  
92 antioxidant, and antidiabetic activities.

93 Storage period tea usually for several months to years *Pluchea* herbal tea also  
94 affects the levels of the bioactive compounds and biological activity (Jayani *et al.*, 2022).  
95 Tea or herbal tea is generally stored at ambient temperature and packed in a tea bag or  
96 aluminum foil standing pouch or a combination of both. Many researchers reported that  
97 the storage period decreases the bioactive compounds, antioxidant and antidiabetic  
98 activities, i.e., juice from *Momordica charantia* L. (Lin *et al.*, 2020), dried *Piper betle*  
99 extracts (Ali *et al.*, 2018), white tea (Xu *et al.*, 2019), kinnow-amlam beverages (Purewal *et*  
100 *al.*, 2022), whole wheat flour (Zhang *et al.*, 2021).

101 Therefore, this research studied the effect of steeping temperature and storage  
102 period on the bioactive compounds [total phenolic content (TPC), total flavonoid content  
103 (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging activity  
104 (DPPH), ferric reducing antioxidant power (FRAP)], and antidiabetic activities [( $\alpha$ -amylase  
105 (AA) and  $\alpha$ -glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea* leaves  
106 and on the phenolic compound profile.

107

## 108 MATERIALS AND METHODS

109

### 110 RAW MATERIALS AND PREPARATION

111 The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya,  
112 East Java, Indonesia. The *Pluchea* plants were included in the *Asteraceae* family with  
113 specifications according to the GBIF taxon ID number database:3132728 (Ferraris, 2023).  
114 *Pluchea* leaves at 1-6 levels of each branch from the shoot were collected, sorted,  
115 washed, and dried to get a moisture content of around  $11.16 \pm 0.09$  % dry basis  
116 (Widyawati *et al.*, 2022). The dried *Pluchea* leaves was pulverized to a 45-mesh size

117 powder. The *Pluchea* leaf powder was dried in an oven (Binder, Merck KGaA, Darmstadt,  
118 Germany) at 120 °C for 10 min to reduce microbial organisms. Then, 2 g of the powder  
119 were packed into a paper filter infusion bag. Packed samples were stored for 0 (un-stored)  
120 and 5 (stored) yr in standing pouch before analysis.

121 In the research, the one tea bag of *Pluchea* herbal tea that was stored 0 (B1) and  
122 5 (B2) yr, was steeped with 100 mL hot water at various temperatures, including 60 (T1),  
123 70 (T2), 80 (T3), 95 (T4) °C for 5 min with infusion method obtaining 8 treatment  
124 combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2. After the  
125 temperature of *Pluchea* infusion similar to ambient temperature was analyzed further.

126

## 127 REAGENTS

128 The reagents used in the analysis include 2,2-diphenyl-1-picrylhydrazyl (DPPH),  
129 sodium carbonate, gallic acid,  $\alpha$ -amylase,  $\alpha$ -glucosidase, pNPG (p-nitrophenyl- $\alpha$ -  
130 glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-O-caffeoylquinic  
131 acid, 4,5-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and (+)-catechin were  
132 purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, Folin–Ciocalteu’s Phenol,  
133 sodium nitric, aluminum chloride, ferric chloride, sodium dihydrogen phosphate, sodium  
134 phosphate, potassium ferricyanide, starch, acetic acid, and sodium hydroxide were  
135 purchased from Merck (Kenilworth, NJ, USA). All reagents used were of analytical grade  
136 except for distilled water which was purchased from PT Aqua Industry Surabaya.

137

## 138 METHODOLOGY

139

140

## ANALYSIS OF THE BIOACTIVE COMPOUNDS

141

### 142 TOTAL PHENOLIC CONTENT ANALYSIS

143 Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the  
144 technique by Gao *et al.* (2019). About 10  $\mu$ L *Pluchea* infusion and 1 mL Folin-Ciocalteu's  
145 phenol reagent 10 % were mixed in 10 mL volumetric flask and incubated for 5 min. And  
146 then 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5 % was added and filled up to 10 mL volume with distilled water.  
147 The blue color intensity of the solution was measured in the spectrophotometer UV-Vis  
148 1800 (Shimadzu, Japan) at  $\lambda$  760 nm with gallic acid as the reference standard. The total  
149 phenolic content was calculated using the formula:  $y=0.00009x+0.008$  with  $R^2=0.9941$ .  
150 The results were expressed as mg gallic acid equivalent (GAE)/g samples.

151

### 152 TOTAL FLAVONOID CONTENT ASSAY

153 Total flavonoid content (TFC) of the samples was measured based on the reaction  
154 between AlCl<sub>3</sub> and NaNO<sub>2</sub> with the aromatic ring of flavonoid compounds, especially  
155 flavonol and flavon (Shraim *et al.*, 2021). The reaction between AlCl<sub>3</sub> and flavonoid  
156 compounds resulted in a yellow solution. About 30  $\mu$ L *Pluchea* infusion was mixed with  
157 0.3 mL NaNO<sub>2</sub> 5 % in 10 mL volumetric flask and incubated for 5 min. The mixture was  
158 added with 0.3 mL AlCl<sub>3</sub> 10 % for 5 min. Then, 2 mL NaOH 1 M and distilled water were  
159 added until 10 mL volume. Then, the red solution was produced after NaOH solution  
160 addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800,  
161 Shimadzu, Japan) at  $\lambda$  510 nm with (+)-catechin as the reference standard compound,

162 and the results were expressed as mg catechin equivalents (CE)/g samples using the  
163 formula:  $y=0.00008x-0.0023$  with  $R^2= 0.9980$ .

164

#### 165 TOTAL TANNIN CONTENT ANALYSIS

166 Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method  
167 (Chandran and Indira, 2016). Approximately 10  $\mu$ L *Pluchea* infusion was added with 1 mL  
168 Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flask and incubated for 5 min.  
169 Then, the mixture was added with 2 mL  $\text{Na}_2\text{CO}_3$  7.5 % and filled up to 10 mL volume with  
170 distilled water. The blue dark color solution was measured in UV-Vis spectrophotometer  
171 1800 (Shimadzu, Japan) at  $\lambda$  760 nm with tannic acid as the reference standard.  
172 Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/g samples used  
173 the formula:  $y=0.00009x+0.0021$  with  $R^2=0.9993$

174

#### 175 ANALYSIS OF THE ANTIOXIDANT POTENTIAL

176

#### 177 DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

178 The DPPH free radical scavenging activity (DPPH) was measured by the  
179 spectrophotometric method (Widyawati *et al.*, 2017) to determine the ability of the  
180 phytochemicals in the *Pluchea* leaf infusion to donate hydrogen atom to the nitrogen atom  
181 in DPPH resulting in the formation of DPPH-H compound exhibiting a yellow-colored  
182 solution. About 25  $\mu$ L *Pluchea* leaf infusion was poured into reaction tube into which was  
183 added 3 mL DPPH solution (4 mg/100 mL). After incubation for 15 min in a dark room,  
184 the absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis

185 1800, Shimadzu, Japan) at  $\lambda$ . 517 nm. The reference standard compound was gallic acid  
186 and the results of analysis were expressed as mg gallic acid equivalents (GAE)/g samples  
187 that calculated using formula:  $y=0.146x+1.7896$  with  $R^2=0.9975$ .

188

## 189 FERRIC REDUCING POWER ANALYSIS

190 Ferric-reducing power (FRAP) was determined following the method used by  
191 Widyawati *et al.* (2014) method. Approximately 10  $\mu$ L of samples were added to 2.5 mL  
192 phosphate buffer pH 6.6 and 2.5 mL of 1% potassium ferricyanide in the reaction tube.  
193 And then mixture was shaken and incubated for 20 min at 50 °C. Finally, 2.5 mL  
194 chloroacetic acid 10% (w/v) was added. Into the 2.5 mL supernatant was added 2.5 mL  
195 distilled water, 0.5 mL ferric chloride 0.1% w/v, and incubated for 10 min. Potency of the  
196 samples reducing iron (III) to iron (II) ion was indicated by the intensity of blue color  
197 formed that was measured using UV-Vis spectrophotometer (Spectrophotometer UV-Vis  
198 1800, Shimadzu, Japan) at  $\lambda$  700 nm. Intensity of the blue color indicated higher reducing  
199 capacity. The reducing power expressed as mg gallic acid equivalent (GAE)/g samples  
200 was calculated using the formula:  $y=0.0002x+0,0256$  with  $R^2=0,9906$ .

201

## 202 ANALYSIS OF THE ANTIDIABETIC PROPERTIES

203

### 204 $\alpha$ -AMYLASE ENZYME INHIBITION CAPACITY ASSAY

205 In vitro inhibition of  $\alpha$ -amylase enzyme (AA) followed the procedure as described  
206 by Widyawati *et al.* (2020). Each 500  $\mu$ L of samples, was mixed with starch 1 % (w/v) and  
207 sodium acetate buffer pH 5. Into a 250  $\mu$ L of the mixture was added an  $\alpha$ -amylase solution



208 (0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in 50 mL of 0.2 M sodium acetate  
209 pH 5. Mixture was shaken into which was and added 2 mL sodium hydroxide 1M. Before  
210 the analysis, this mixture was incubated at 37 °C for 10 min. Then, the capacity of the α-  
211 amylase enzyme to hydrolyze the starch to release glucose was measured by UV-Vis  
212 spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ 540 nm.  
213 The inhibition percentage of α-amylase was assessed using the formula:  $(ACb - ACa) -$   
214  $(As - Ab) (ACb - ACa) \times 100 \%$ . Where, ACb is the absorbance of 100 % enzyme activity  
215 (solvent with the enzyme), ACa is the absorbance of 0 % enzyme activity (solvent without  
216 the enzyme), As is the absorbance of test sample with enzyme, Ab is absorbance of test  
217 sample without enzyme.

218

#### 219 α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

220 The analysis of the α-glycosidase inhibitor activity (GA) was done by Widyawati *et*  
221 *al.* (2020) method with slight modification. About 150 µL samples containing 100 µL  
222 *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH 7)  
223 were reacted with 50 µL α-glycosidase 2 mM (0.0833 unit/mL), and then the mixture was  
224 incubated at 37 °C for 15 min. The reaction was stopped with the addition of 1000 µL  
225 sodium carbonate 0.2 M. The amount of these enzymes that didn't react with bioactive  
226 compounds of *Pluchea* infusion hydrolyzed p-nitrophenyl-α-D-glucopyranoside (pNPG)  
227 as a substrate to result in p-nitrophenol. The inhibition activity of the *Pluchea* infusion was  
228 measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu,  
229 Japan) at λ 405 nm. The inhibition percentage of α-glycosidase was calculated using  
230 formula:  $(ACb - ACa) - (As - Ab) (ACb - ACa) \times 100 \%$ . Where, ACb is the absorbance

231 of 100 % enzyme activity (solvent with enzyme), A<sub>Ca</sub> is the absorbance of 0 % enzyme  
232 activity (solvent without enzyme), A<sub>s</sub> is the absorbance of test sample with enzyme, A<sub>b</sub>  
233 is the absorbance of test sample without enzyme.

234

## 235 ANALYSIS OF PHENOLICS

236 The phenolic compounds of the samples were analyzed by HPLC based on  
237 Kongkiatpaiboon *et al.* (2018) method with modifications. Each *Pluchea* infusion was  
238 sonicated for 15 min (Branson 1510) and then the sample was filtered using a filter syringe  
239 (Whatmann, 0.2 µm, NYL). About 20 µL of sample was injected in an HPLC (LC20AD  
240 series, Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC LC-20AD  
241 pump, CTO-30A column oven, CBM-20A/20 Alite system controller, and SPD-20A/20 AV  
242 UV-Vis detector. Separation of phenolic compounds in samples was carried out using a  
243 Shim-pack VP-ODS C18 column (ID 5 µm × 50 mm × 4.6 mm) with a GVP-ODS Cartridge  
244 guard column (2 pieces) (ID 10 mm × 4.6 mm). The mobile phase used consisted of a  
245 solution of (A) 0.5 % acetic acid in water and (B) absolute methanol. Analysis was carried  
246 out using a gradient system in the following order: initial conditions of 10 % B in A to 50  
247 % B in A were maintained for 40 min; then 100 % B was maintained for 20 min. Next the  
248 column was re-equilibrated with 10 % B in A maintained for 10 min before analysis of the  
249 next sample. The sample flow rate was set at 1.0 ml/min with a controlled temperature at  
250 40 °C. Detection was used at a wavelength of 280 nm. The reference standard used were  
251 gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,5-dicaffeoylquinic acid, 3,4-  
252 dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid. All of reference standard was  
253 dissolved in distilled water and prepared similar to the samples before injected in HPLC.

254

## 255 EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

256 The research design used a randomized block design with two factors, i.e., the  
257 steeping temperature (T) and the storage period. *Pluchea* leaf blades were subjected to  
258 4 steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95 °C (T4), and  
259 the storage period of 0 yr /un-stored (B1), and 5 yr /stored (B2) resulting in 8 treatment  
260 combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis  
261 of phenolic was repeated six periods. The data analysis of samples was repeated for six  
262 periods. The data were analyzed using a paired t-test at  $\alpha \leq 0.05$ , treatment means of  
263 specific phenolic compounds that were identified were expressed as the mean  $\pm$  SD. The  
264 analysis used SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

265

## 266 RESULTS AND DISCUSSIONS

267

### 268 BIOACTIVE COMPOUNDS

269

270

#### Phenolic Compounds

271 The bioactive compounds are active compounds in plants that are essential to  
272 protect a body health (Nguyen and Chuyen, 2020). These compounds usually have many  
273 biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer,  
274 antimicrobial, antibacterial, anti-cholesterol, and so on (Suriyaphan, 2014; Acar *et al.*,  
275 2022). Phenolic compounds have potential redox properties that can scavenge free  
276 radicals that can cause a number of chronic diseases (Noreen *et al.*, 2017; Aryal *et al.*,  
277 2019; Acar *et al.*, 2022).

Corresponding Author: paini@ukwms.ac.id

278 The total phenolic content (TPC) of *Pluchea* infusion at different steeping  
279 temperature and storage period generally significantly increased with increasing steeping  
280 temperature and storage period based on paired t-test at  $\alpha \leq 0.05$  (Figure 1a). Steeped  
281 and stored infusion had significantly higher amounts of phenolic compounds than the  
282 samples that were steeped and un-stored. Further, the highest total phenolic content was  
283 observed in samples infused at 95 °C and stored for 5 yr (at 71.38±4.14 mg GAE/g  
284 sample) while the lowest was measured in the un-stored samples and infused at 60 °C  
285 (at 4.39±0.49 mg GAE/g sample). The phenolic content of stored samples that were  
286 steeped only at 60 and 95 °C showed a significant increase in their phenolic content. This  
287 implies that the steeping temperature and the storage periods significantly resulted in the  
288 high amounts of phenolic compounds in the infusions. Results also indicated that phenolic  
289 compounds were generally greater in the infusion at high steeping temperatures and long  
290 storage period. This could have been due to the fact that the steeping temperature and  
291 storage period could cause the process of degradation, oxidation, and leaching/release  
292 of phenolic compounds. Phenolic compounds are water soluble and thus soaking in hot  
293 water for a certain period of period as in steeping causes the migration process of more  
294 phenolic compounds to the water because of longer exposure of phenolic compounds to  
295 water (Castiglioni *et al.*, 2015; Kilic *et al.*, 2017; Acar *et al.*, 2022). Su *et al.* (2019) reported  
296 that temperature treatment can stimulate the release of phenolic compounds and  
297 increase antioxidant activity of lychee juice stored at different temperatures of 4 and 45  
298 °C and different long storage (fresh and 72 h).

299 Temperature treatment degrades (or hydrolyzes) the hydrogen bond between  
300 phenolic compounds and proteins resulting in an increase of phenolic compounds when

301 exposed to higher temperatures (Ali *et al.*, 2018; Jayani *et al.*, 2022, Ramphinwa *et al.*,  
302 2023). Zhang *et al.* (2021) reported that phenolic compounds present in plants are not  
303 completely stable, but are easily degraded during storage after harvest. Reblova (2012)  
304 claimed that antioxidant compounds can be slowly degraded with increasing temperature.  
305 Fibrianto *et al.* (2021) also stated that the brewing temperature has an effect on the  
306 extracted antioxidant compounds, such as alkaloids, catechins, and tannins. Thus, there  
307 is an assumption that temperature and storage caused the degradation, oxidation, and  
308 hydrolysis of the phenolic compounds period resulting in the increased amount of the  
309 phenolic compounds at higher steeping temperature and longer storage period.

310 Simple phenolic compounds are identified in steeped and stored. *Pluchea* leaf  
311 infusion included gallic acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-  
312 O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic acids  
313 was showed in Table 1. The treatment effects using a t-test at  $\alpha \leq 0.05$  showed that gallic  
314 acid and kaempferol content were insignificantly different at various steeping  
315 temperatures and storage periods. The concentration of quercetin and 3,5-di-O-  
316 caffeoylquinic acid of the un-stored and stored *Pluchea* infusion was significantly different  
317 from the rest of the samples between 70 °C while (+)-catechin concentration of *Pluchea*  
318 infusion was only significantly different at 95 °C. The myricetin content was significantly  
319 different at 80 and 95 °C. The 3,4-di-O-caffeoylquinic acid content showed significant  
320 difference at 60, 80, and 95 °C while 4,5-di-O-caffeoylquinic acid content was only  
321 significantly different at 60 °C.

322 Results further showed that gallic acids and kaempferol were relatively stable as  
323 reflected by the insignificant changes when exposed to the different steeping temperature

324 and storage period. Myricetin, (+)-catechin, and 3,4-di-O-caffeoylquinic acid showed a  
325 drastic increase at higher steeping temperatures and longer storage period implying that  
326 these compounds tended to be relatively labile. Quercetin, 3,5-di-O-caffeoylquinic acid,  
327 and 4,5-di-O-caffeoylquinic acid underwent moderate changes. Therefore, myricetin, (+)-  
328 catechin, and 3,4-di-O-caffeoylquinic acid were easier to dissolve or degrade to form  
329 simple phenolic acids at higher temperatures and storage period (Su *et al.*, 2019; Ali *et*  
330 *al.*, 2018; Jayani *et al.*, 2022; Ramphinwa *et al.*, 2023; Zhang *et al.*, 2021). Degradable  
331 polyphenol compounds have a simple structure and free hydroxyl groups that can react  
332 with Folin-Ciocalteu's Phenol reagent, resulting complex blue solution that can detected  
333 as total phenolic content.

334

#### 335 Flavonoid Content (TFC)

336 Flavonoids are the major phenolic compounds that have potential chemical and  
337 biological activities, such as radical scavenging and antimicrobial activities (Ayele *et al.*,  
338 2022; Chandra *et al.*, 2014) that can protect the human body from the oxidative stress  
339 caused by many degenerative diseases, especially cancer, cardiovascular problems and  
340 aging (Mathur and Vijayvergia, 2017). The total flavonoid content of steeped *Pluchea*  
341 infusion decreased with longer storage period. Un-stored samples exhibited higher  
342 flavonoid content than the stored samples. The statistical analysis using a paired t-test at  
343  $\alpha \leq 0.05$  showed that the total flavonoid content of *Pluchea* infusion was significantly  
344 different between the steeped un-stored and steeped stored samples (Figure 1b). The  
345 highest total flavonoid content was exhibited by the un-stored samples steeped at 95°C  
346 at about 147.42±14.03 mg CE/g sample. Total flavonoid content was significantly lower

347 in the stored samples than those of the un-stored samples implying that the increase in  
348 the flavonoid content of the infusion was affected primarily by the steeping temperature.

349

### 350 Tannin Content (TTC)

351 Tannins are bioactive compounds that provide properties, such as astringent, anti-  
352 diarrheal, antibacterial and antioxidant (Malangngi *et al.*, 2012). Generally, results  
353 indicated that the total tannin content of *Pluchea* infusion significantly increased with  
354 increasing steeping temperature and storage period (Figure 1c). Among, the un-stored  
355 steeped samples, the tannin content was significantly lowest in the samples infused at 60  
356 °C at about  $4.81 \pm 0.58$  to  $17.42 \pm 1.04$  mg TAE/g samples which was significantly different  
357 lower from that of the lowest tannin content of the stored samples. Among the stored and  
358 steeped samples, the highest tannin content was observed at samples steeped at 95 °C  
359 about  $17.42 \pm 1.04$  mg TAE/g samples, and was significantly different from that of the  
360 highest tannin content of the un-stored steeped samples at 95 °C about  $9.22 \pm 1.48$  mg  
361 TAE/g samples. Indicating that the tannin content was primarily affected by a longer  
362 storage period than high steeping temperature. The condensation of catechins to tannins  
363 is a dominant process occurring in tea leaves that is accelerated during the maceration  
364 of raw tea leaves (Kowalska *et al.*, 2021) and could have had contributed to the observed  
365 increase in the tannin content in the treated samples.

366 Although, high temperature and long storage period can cause the degradation of  
367 tannins to catechins. Rusita *et al.* (2019) emphasized that tannins are polar thermostable  
368 complex compounds, that are resistant to heating, indicating that even with the exposure  
369 to high temperature, the tannins still remained high in the treated samples period.

370

371

## Antioxidant Activity

372 Antioxidant activity is capability of compounds to inhibit the oxidation of  
373 macromolecules from biological target that involve in oxidative chain reactions (Ali *et al.*,  
374 2005; Oh *et al.*, 2013). The antioxidant activity assay was done in this research using  
375 DPPH Free Radical Scavenging Activity (DPPH) and ferric reducing power (FRAP)  
376 methods. The phenolic compounds are an active antioxidant that have antioxidant  
377 capability that depends on their redox properties. The structure of phenolic compounds  
378 determines the effectivity to donate hydrogen atom which is negatively correlated with the  
379 O-H phenolic bond strength. The antioxidant power of phenolic compounds is due to the  
380 weak hydrogen bonds in the OH group of the phenolic compound so that it is easier to  
381 donate hydrogen atoms (Kruk *et al.*, 2022). The mechanism of phenolic compounds as  
382 antioxidants depends on their ability to donate hydrogen atoms and transfer electrons,  
383 and as reducing agents and singlet oxygen quenchers (Ali *et al.*, 2005; Huang *et al.* 2005).

384

385

## DPPH Free Radical Scavenging Activity

386 DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate  
387 antioxidant activity because this method is simple that is suitable to measure the donating  
388 hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of  
389 DPPH to change to yellow color (Munteanu and Apetrei, 2021; Baliyan *et al.*, 2022).  
390 Figure 2a. shows that the free radical scavenging properties of the stored and steeped  
391 samples were significantly higher than the un-stored steeped samples. It can also be  
392 observed that the free radical scavenging property was significantly different among the



393 stored and steeped samples but insignificant among the un-stored and steeped sample  
394 period. *Pluchea* infusion stored at room temperature for 5 yr resulted in high free radical  
395 scavenging activity by more than 10%. Steeping at higher temperatures significantly  
396 increased the DPPH free radical scavenging activity in stored *Pluchea* infusion by around  
397 15 to 25 %. This implies that the higher free radical scavenging property was primarily  
398 affected by the storage period than the steeping temperature. During the storage process,  
399 it is possible to form complex phenolic compounds which provide a high ability to  
400 scavenge free radicals (Thanajiruschaya *et al.*, 2010).

401 The scavenging activity of the samples was strongly and positively correlated with  
402 total phenolic and tannin contents, but inversely with total flavonoid levels (Table 2). The  
403 antioxidant activity was strongly and negatively correlated with flavonoid content. The  
404 storage period could be reduced flavonoid content. The study also demonstrated that  
405 longer storage period and higher infusion temperatures produced many simple phenolic  
406 compounds with free hydroxyl groups capable to donate hydrogen atoms to DPPH free  
407 radicals. Many phenolic acids, such as gallic acids, (+)-catechins, myricetins,  
408 kaempferols, quercetins, 3,5-di-O-caffeoylquinic acids, 3,4-di-O-caffeoylquinic acids, 4,5-  
409 di-O-caffeoylquinic acids have established potential antioxidant activity (Kumar and Goel,  
410 2019) (Table 1). Kruk *et al.* (2022) informed that the capability of phenolic compounds  
411 to donate hydrogen atom depends on chemical structure, number and position of hydroxyl  
412 groups attached to a benzene ring, a double bond between C2 and C3 rings, and a  
413 carbonyl group (C=O) on the C ring at C4. The effectivity of antioxidant compounds to  
414 donate hydrogen atom is determined by O-H bond dissociation energy.

415 The free radical scavenging property observed in the study was not in consistent  
416 with the results of the study by Moraes-de-Souza *et al.* (2008). The research shows that  
417 total phenolic content of herbal infusion is low correlated with free radical scavenging  
418 activity. However, Dobrinas *et al.* (2021) informed that total phenolic content is positively  
419 and significantly correlated with the free radical scavenging property of tea infusion.

420

#### 421 Ferric Reducing Antioxidant Power (FRAP)

422 FRAP is an analysis of the antioxidant power of the phytochemical compounds  
423 that is based on the ability of antioxidant compounds to reduce iron ions of potassium  
424 ferricyanide ( $\text{Fe}^{3+}$ ) to potassium ferrocyanide ( $\text{Fe}^{2+}$ ). Potassium ferrocyanide reacts with  
425 ferric chloride to form a ferric-ferrous complex and results green color solution (Widyawati  
426 *et al.*, 2017; Raharjo and Haryoto, 2019).

427 The results showed that the ferric reducing antioxidant power (FRAP) increased at  
428 higher steeping temperature and longer storage period. The lowest FRAP was observed  
429 in the un-stored samples which were steeped at 60 °C at  $3.95 \pm 0.17$  mg gallic acid  
430 equivalents (GAE)/g samples, and the highest was exhibited in *Pluchea* infusion which  
431 was stored for 5 yr at 95 °C at  $48.63 \pm 10.83$  mg gallic acid equivalents (GAE)/g samples  
432 (Figure 2b). FRAP increased significantly as the steeping temperature was increased.  
433 FRAP of the samples stored for 5 years was also significantly higher than the un-stored  
434 samples at  $\alpha \leq 0.05$ .

435 This is in contrast with the study on the antioxidant activity of DPPH and FRAP of  
436 matcha. The longer storage period reduces the levels of catechin content due to the  
437 catechins, such as epigallocatechin gallat (EGCG), epicatechin gallat (ECG),

438 epigallocatechin (EGC), and epicatechin (EC) which are bioactive compounds that have  
439 high antioxidant activity (Kim *et al.*, 2020). The ferric-reducing capability of *Pluchea* could  
440 have been due to the presence of simple phenolic acid that can transfer electrons from  
441 their free hydroxyl groups of samples. The FRAP of *Pluchea* infusion was strongly and  
442 positively significantly correlated with the DPPH, TPC, and TTC, but inversely to TFC.

443

#### 444 ANTIDIABETIC ACTIVITY

445

##### 446 Alpha amylase enzyme inhibition activity (AA)

447 Antidiabetic activity is a measure of the potency of phenolic compounds to regulate  
448 the uptake of glucose by the cells from the blood through the mediation of 2-digestive  
449 enzymes i.e.,  $\alpha$ -amylase and  $\alpha$ -glucosidase, which are involved the control of dietary  
450 carbohydrate digestion and release in the postprandial blood glucose in human body (Fu  
451 *et al.*, 2017). The phenolic compounds have the capability to bind with the protein  
452 component of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Martinez-Solis *et al.*, 2022)  
453 resulting in the reduced activity of the enzymes. The results showed that lower steeping  
454 *Pluchea* leaf infusion was able to inhibit the action of the  $\alpha$ -amylase enzymes (Figure 3a).  
455 The *Pluchea* infusion exhibited a good  $\alpha$ -amylase enzyme inhibition activity of more than  
456 50 % and even almost 100 % in un-stored *Pluchea* infusion steeped at 60, 70, and 80 °C  
457 with the highest at 60 °C, and in stored *Pluchea* leaf infusion which was steeped at 60 °C.  
458 The stored *Pluchea* leaf infusion steeped at 70, 80, and 95 °C for 5 minutes had lower  
459 enzyme inhibition activity of less than 50 % with the lowest at 95 °C around 13 %.  
460 Widyawati *et al.* (2017) found that the ability to inhibit the  $\alpha$ -amylase enzyme in un-stored

461 *Pluchea* infusion steeped at 95 °C for 5 min was also low at 28.79 %. Increasing the  
462 steeping temperature and storage period reduced the ability of the phytochemicals in the  
463 *Pluchea* infusion to inhibit the  $\alpha$ -amylase enzyme activity period. Table 2 further shows  
464 that the AA of *Pluchea* infusion was strongly and negatively significantly correlated with  
465 TPC, TTC, DPPH, and FRAP, but it was weakly and positively significantly correlated with  
466 TFC.

467 This inhibitory activity was thought to be contributed by other bioactive compounds,  
468 besides phenolics which are sensitive to steeping temperature and storage period. Li *et*  
469 *al.* (2018) stated that there are flavonoid compounds that contribute to the ability to inhibit  
470 the  $\alpha$ -amylase enzyme. Akah *et al.* (2011) reported that phytochemical compounds, such  
471 as terpenoids, saponins, flavonoids, glycosides/carbohydrates, and alkaloids are good  
472 antidiabetic metabolites or  $\alpha$ -amylase enzyme activity inhibitors. Sangeetha and  
473 Vedesree (2012) explained that the ability of *Threspesia populnea* extract to inhibit the  $\alpha$ -  
474 amylase enzyme was determined of their phenolic compound content and protein.  
475 Moreover, the presence of  $\alpha$ -amylase enzyme inhibitor in this extract may be  
476 proteinaceous or nonproteinaceous in nature. It was assumed that this enzyme inhibitory  
477 activity in *Pluchea* infusion also was determined with their protein and polyphenolic  
478 content. Aleixandre *et al.* (2022) also stated that phenolic acids have inhibition activity to  
479  $\alpha$ -amylase enzyme depending on their structures. There are C=C double bonds  
480 conjugated with a carbonyl group of phenolic structures that stabilize the binding forces  
481 to the active site of the  $\alpha$ -amylase. The hydroxyl groups can bind by non-covalent  
482 interaction (hydrogen bonding, cation- $\pi$  interactions, salt bridge interactions, ionic  
483 interactions, or electrostatic forces) with amino acid residue at the active site in  $\alpha$ -amylase

484 enzyme. Elevated steeping temperature and longer storage period can easily cause the  
485 removal of the hydroxyl groups of phenolic compounds that can reduce their ability of  
486 enzyme inhibition. The phenolic acids with a greater number of hydroxyl groups exhibits  
487 stronger capability to obstruct the  $\alpha$ -amylase enzyme.

488

#### 489 Alpha glucosidase enzyme inhibition activity (GA)

490 Alpha glucosidase is an important enzyme in carbohydrate digestion, that catalysis  
491 the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and converts  
492 them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis *et al.*, 2014;  
493 Proenca *et al.*, 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -glucosidase  
494 enzyme is used to determine their antidiabetic activity. This is supported by Werdani and  
495 Widyawati (2018) stated that *Pluchea* infusion has the potential as an antidiabetic agent.  
496 Widyawati *et al.* (2020) found that the steeping of un-stored *Pluchea* infusion at 95 °C for  
497 5 min has an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857 %.

498 Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -  
499 glucosidase enzyme decreased with increasing steeping temperature and storage period.  
500 Steeping at 95 °C of the un-stored *Pluchea* leaf infusion obtained the lowest inhibitory  
501 ability, i.e.,  $48.32 \pm 1.27$  %, and the highest inhibitory activity was at 70 °C at  $95.11 \pm$   
502  $0.70$ %. The results of a paired t-test showed that GA of *Pluchea* infusion was significantly  
503 different between steeping temperature and long storage. Figure 3 further shows that the  
504 ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher  
505 than the ability to inhibit the  $\alpha$ -amylase enzyme. Data analysis in Table 2. showed that  
506 the TFC of the *Pluchea* leaf infusion was influenced weakly and positively by GA and AA,

507 but the GA and AA were not affected by TPC, TTC, DPPH, and FRAP. Li *et al.* (2018)  
508 stated that flavonoid compounds can inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -  
509 glucosidase enzymes. Dias *et al.* (2021) stated that flavonoid compounds, such as rutin,  
510 myricetin, kaempferol, and quercetin have antioxidant and antihyperglycemic activities.  
511 The ability to inhibit the action of enzymes from flavonoid compounds is determined by  
512 the position and number of hydroxyl groups, the number of double bonds in rings A and  
513 B, and the heterocyclic ring in ring C. Tadera *et al.* (2006) and Zhang *et al.* (2014) also  
514 explained that flavonoid compounds of samples significantly inhibit the  $\alpha$ -glucosidase  
515 enzyme activity.

516 The ability to inhibit the  $\alpha$ -glucosidase enzyme from *Pluchea* infusion was  
517 significantly affected by the steeping temperature and long storage. Figure 3 also showed  
518 that the capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater  
519 than the  $\alpha$ -amylase enzyme because the mechanism of the two enzymes was different,  
520 according to the opinion of McCue *et al.* (2005). The mechanism of the  $\alpha$ -glucosidase  
521 enzyme inhibitor includes making the sugar mimic structure, binding using ionic bonds  
522 with nucleophilic, making the transition state-like structure, binding hydrogen with catalytic  
523 acid residue, interacting ionic and hydrophobic with site other than the active site, and  
524 binding covalent with enzymes through an epoxy or aziridine group (Moorthy *et al.*, 2012).  
525 Then, the mechanism of the  $\alpha$ -amylase enzyme inhibitor includes blocking carbohydrates,  
526 limiting the digestibility and absorption of carbohydrates, and blocking the active centers  
527 of several subsites of the enzyme (Gong *et al.*, 2020).

528 Widyawati *et al.* (2017) stated that phenolic and non-phenolic compounds can  
529 inhibit of the  $\alpha$ -glucosidase enzyme activity. The ability of bound phenolic compounds to

530 inhibit  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. The presence  
531 of polymerization and degradation reactions, that may be occurred in *Pluchea* infusion  
532 during storage, affects the structure and profile of phenolic and non-phenolic compounds.  
533 Asriningtyas *et al.* (2014) explained that the methyl-esterified quinic acid with the caffeic  
534 groups, such as 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid methyl ester,  
535 3,4,5-tri-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid, and 1,3,4,5-  
536 tetra-*O*-caffeoylquinic acid of *Pluchea* leaves inhibits the  $\alpha$ -glucosidase enzyme activity.  
537 The resulting analysis of caffeoylquinic acids (3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-  
538 caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid in stored *Pluchea* leaf infusion higher  
539 concentration than in un-stored *Pluchea* infusion, and the concentrations of the simple  
540 phenolic compounds were increased at higher steeping temperature, but the  $\alpha$ -  
541 glucosidase inhibition activity of them was reduced. It means that the methyl-esterified  
542 quinic acid with the caffeic groups had more potential to inhibit  $\alpha$ -glucosidase enzyme  
543 than free caffeoylquinic acid.

544 This study showed that the increasing steeping temperature and storage period  
545 caused degradation of polyphenol compounds to produce simple phenolic compounds,  
546 such as gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,4-di-*O*-caffeoylquinic  
547 acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid that increased the total  
548 phenolic content and total tannin content. The increase in the simple phenolic  
549 concentration of the *Pluchea* leaf infusion caused higher antioxidant activity and lower  
550 antidiabetic activity.

551

552

553 CONCLUSION

554 The Total Phenol (TPC) of *Pluchea* infusion at different steeping temperatures  
555 and storage periods generally significantly increased with increasing steeping  
556 temperature and storage periods. Steeped and stored infusion had significantly higher  
557 amounts of phenolic compounds than the samples that were steeped and un-stored. TPC  
558 was highest in the stored and steeped at 95°C and lowest in the un-stored and steeped  
559 at 60°C. Un-stored steeped samples exhibited significantly higher flavonoid content than  
560 the stored steeped samples. The highest total flavonoid content was exhibited by the un-  
561 stored samples steeped at 95°C. The total tannin content of *Pluchea* leaf infusion  
562 significantly increased with increasing steeping temperature and storage period. Among  
563 the un-stored steeped samples, the tannin content was significantly lowest in the samples  
564 steeped at 60°C and highest in the samples steeped at 95°C.

565 The free radical scavenging property (DPPH) of the stored and steeped *Pluchea*  
566 leaf infusion was significantly higher than the un-stored steeped samples. The free radical  
567 scavenging property was highest in the stored samples steeped at 80 and 95°C. Free  
568 radical scavenging activity of the samples was strongly and positively correlated with total  
569 phenolic and tannin contents, but inversely with total flavonoid levels. The ferric-reducing  
570 antioxidant power (FRAP) significantly increased with increasing steeping temperature  
571 and longer storage periods. The lowest FRAP was found in the un-stored samples which  
572 were steeped at 60°C and the highest was exhibited in *Pluchea* stored samples which  
573 were stored for 5 yr and steeped at 95°C. The FRAP of *Pluchea* leaf infusion was  
574 significantly strong and positively correlated with the free radical scavenging property,  
575 total phenolic, and total tannin content, but inversely with total flavonoid content. The



576 inhibition of the  $\alpha$ -amylase activity was generally found to be higher at lower steeping  
577 temperatures of the un-stored *Pluchea* leaf infusion than at higher steeping temperatures  
578 of the stored sample. The  $\alpha$ -amylase enzyme inhibition capacity of the *Pluchea* leaf  
579 infusion showed a significantly strong and negative correlation with TPC, TTC, DPPH,  
580 and FRAP, but it was weakly and positively correlated significantly with TFC.

581 The ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme  
582 decreased at high steeping temperatures and long storage periods. The highest inhibitory  
583 activity was obtained in the un-stored *Pluchea* leaf infusion that was steeped at 70°C  
584 while the lowest was obtained in the un-stored sample that was steeped at 95°C. The  
585 ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher  
586 than the ability to inhibit the  $\alpha$ -amylase enzyme. The inhibition of the  $\alpha$ -glucosidase  
587 enzyme activity was significantly strong and negative TPC, TTC, DPPH, and FRAP, and  
588 it was weakly and positively correlated significantly with TFC.

589 The simple phenolic compounds identified in *Pluchea* leaf infusion may affect the  
590 presence of the bioactive compounds, antioxidant potential, and antidiabetic properties  
591 at different steeping temperatures and storage periods including gallic acids, (+)-  
592 catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-  
593 caffeoylquinic acids, 4,5-di-*O*-caffeoylquinic acids.

594

## 595 ACKNOWLEDGMENTS

596 The authors would like to thank the Ministry of Education and Culture of the Republic of  
597 Indonesia for a fundamental research grant to higher education institutions in 2022

598

## 599 STATEMENT ON CONFLICT OF INTEREST

Corresponding Author: [paini@ukwms.ac.id](mailto:paini@ukwms.ac.id)

600 The authors declare no conflict of interest.

601

602 NOTES ON APPENDICES (if any)

603 The complete appendices section of the study is accessible at

604 <http://philjournsci.dost.gov.ph>

605 REFERENCES

606 ACAR A, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food  
607 wastes: evaluation of temperature and time effects on quality parameters. Applied Food  
608 Res. 2(100087):1-8.

609 ALEIXANDRE A *et al.* 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -  
610 glucosidase and influence of reaction conditions. Food Chem. 372(131231):1-9.

611 ALI *et al.* 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile.  
612 Food Chem. 89(2005):27-36.

613 ALI A. *et al.* 2018. Impact of storage conditions on the stability of predominant phenolic  
614 constituents and antioxidant activity of dried *Piper betle* extracts. Molecules.23(24):1-13.

615 AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and  
616 methanol extract and fractions of *Gongronema latifolium* (*Asclepidaceae*) leaves in  
617 alloxan diabetic rats. J Applied Pharm. Sci. 01(09): 99-102.

618 ARSININGTYAS IS. *et al.* 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves  
619 of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product  
620 use. Natural Product Res. 28(17):1350-1353.

621 ARYAL S *et al.* 2019. Total phenolic content, flavonoid content and antioxidant of wild  
622 vegetables from Western Nepal. Plants. 8(96): 1-12.

Corresponding Author: paini@ukwms.ac.id

623 AYELE DT, AKELE ML, MELESE A.T. 2022. Analysis of total phenolic contents,  
624 favonoids, antioxidant and antibacterial activities of *Croton macrostachyus* root extracts.  
625 BMC Chem. 16(30): 1-9.

626 BALIYAN S *et al.* 2022. Determination of antioxidants by DPPH radical scavenging  
627 activity and quantitative phytochemical analysis of *Ficus religiosa*. Molecules. 27(1326):  
628 1-19.

629 CASTIGLIONI S *et al.* 2015. Influence of steeping conditions (time, temperature, and  
630 particle size) on antioxidant properties and sensory attributes of some white and green  
631 teas. Intern. J. Food Sci. Nut. Online 1-7.

632 Chan EWC *et al.* 2022. *Pluchea indica*: An updated review of its botany, uses, bioactive  
633 compounds and pharmacological properties. Pharmaceutical Sciences Asia. 49(1): 77-  
634 85.

635 CHANDRA S *et al.* 2014. Assessment of total phenolic and flavonoid content, antioxidant  
636 properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit  
637 crops: A Comparative study. Hindawi Publishing Corporation Evidence-Based  
638 Complementary Alter. Med. 2014:1-9 (2014)

639 CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic, flavonoids,  
640 tannin and chlorophyll content of leaves of *Strobilanthes Kunthiana (Neelakurinji)*. J.Med.  
641 Plants Studies. 4(4):282-286.

642 DIAS MC, PINTO DCGA, SILVA AMS. 2021. Plant flavonoids: chemical characteristics  
643 and biological activity. Molecules. 26(5377):1-16.

644 DOBRINAS S, SOCEANU A, POPESCU V, POPOVICI IC, JITARIU D. 2021.  
645 Relationship between total phenolic content, antioxidant capacity, Fe and Cu content from  
646 tea plant samples at different brewing times. *Processes*. 9(1311):1-11.c

647 FERRARIS Z. 2023. *Pluchea indica* Less. iNaturalist.org.  
648 <https://www.gbif.org/occurrence/4022273660>.

649 FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional  
650 properties and visual appearance of Dampit Robusta coffee leaves tea. *Advances in Food*  
651 *Sci. Sustain. Agric. Agro-industrial Engineering*. 4(1):25-32.

652 FU L *et al.* 2017. Total phenolic contents and antioxidant capacities of herbal and tea  
653 infusions. *Intern. J. Molecular Sci.* 12:2112-2124.

654 GAO MR *et al.* 2019. A theoretical and experimental study: the influence of different  
655 standards on the determination of total phenol content in the Folin–Ciocalteu assay. *J.*  
656 *Food Measurement and Characteristic*. 13:1349–1356.

657 GONG L, FENG D, WANG T, REN Y, LIU Y, WANG J. 2020. Inhibitors of  $\alpha$ -amylase and  
658  $\alpha$ -glucosidase: Potential linkage for whole cereal foods on prevention of hyperglycemia.  
659 *Food Sci. Nut.* 2020(8):6320-6337.

660 HUANG D, OU B, PRIOR RL. 2005. The Chemistry behind antioxidant capacity assays.  
661 *J. Agric. Food Chem.* 53:1841-1856.

662 ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties  
663 of rosehip tea beverage: study by response surface methodology. *J. Food Sci. Tech.*  
664 54(11):3737–3743.

665 JAYANI NIE *et al.* 2022. Evaluation of stability and quality characteristics of moringa  
666 (*Moringa oleifera*) herbal tea during storage. *Food Res.* 6(4):399-406.

667 KIM JM *et al.* 2020. Effect of storage temperature on the antioxidant activity and catechins  
668 stability of Matcha (*Camellia sinensis*). Food Science and Biotechnology. 29(9):1261–  
669 1271.

670 KILIC *et al.* 2017. Antioxidant properties of some herbal teas (green tea, senna, corn silk,  
671 rosmery) brewed at different temperatures. Intern. J. Secondary Metabolite. 4(3):142-248  
672 Special Issue 1.

673 KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of  
674 extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves  
675 from different provenances in Thailand. Revista Brasileira de Farmacognosia. 28(2018):  
676 145–150.

677 KOWALSKA J *et al.* 2021. Influence of tea brewing parameters on the antioxidant  
678 potential of infusions and extracts depending on the degree of processing of the leaves  
679 of *Camellia sinensis*. Molecules.26(4773):1-17.

680 KRUK J, ABOUL-ENAIN BH, DUCHNIK E, MARCHLEWICZ M. 2022. Antioxidative  
681 properties of phenolic compounds and their effect on oxidative stress induced by severe  
682 physical exercise. J. Physiol. Sci. 72(19):1-24.

683 KUMAR, N. AND GOEL, N. 2019. Phenolic acids: natural versatile molecules with  
684 promising therapeutic applications. Biotechnology Reports. 24(e00370):1-10.

685 LI K *et al.* 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the flavonoids  
686 rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity  
687 relationship of its eight flavonoids by a refined assign score method. Chem. Central J.  
688 12(82):1-11.

689 LIN YS *et al.* 2020. Effects of storage time and temperature on antioxidants in juice from  
690 *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser. *Molecules*.  
691 25(3614): 1-12.

692 MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content  
693 and antioxidant activity test of avocado seed extract (*Persea Americana* Mill). *Jurnal MIPA*  
694 *Unsrat Online*. 1(1): 5-10. [https://media.neliti.com/media/publications/116214-ID-](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf)  
695 [penentuan-kandungan-tanin-dan-uji-aktivi.pdf](https://media.neliti.com/media/publications/116214-ID-penentuan-kandungan-tanin-dan-uji-aktivi.pdf) [In Bahasa Indonesia]

696 MARTINEZ-SOLIS J *et al.* 2022. Antidiabetic and toxicological effect of the tea infusion  
697 of summer collection from *Annona cherimola* Miller leaves. *Plants*. 11(3224):1-17.

698 MATHUR R, VIJAYVERGIA R. 2017. Determination of total flavonoid and phenol content  
699 in *Mimusops Elengi* Linn. *Intern. J. Pharm. Sci. Res.* 8(12): 5282-5285.

700 MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of  
701 sprouted and solid-state bioprocessed soybean. *Asia Pacific J. Clinical Nut.* 14(2): 145-  
702 152.

703 MOORTHY NS, RAMOS MJ, A FERNANDES P. 2012. Studies on  $\alpha$ -glucosidase  
704 inhibitors development: Magic molecules for the treatment of carbohydrate mediated  
705 diseases. *Mini Rev Med Chem.* 12:713–720.

706 MORAES-DE-SOUZA RA, OLDONI TLC, REGITANO-D'ARCE MAB, ALENCAR SM.  
707 2008. Antioxidant activity and phenolic composition of herbal infusions consumed in  
708 Brazil. *Cienc Tecnol. Aliment.* 6(1):41-47.

709 MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant  
710 activity: A Review. *Intern. J. Molecular Sci.* 22(3380):1-30.

711 NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus*  
712 *sabdariffa* L.): Effect of dying temperature and brewing conditions on total soluble solid,  
713 phenolic content, antioxidant capacity and sensory quality. *Beverages*.6(2):1-11 (2020).

714 NOREEN H *et al.* 2017. Measurement of total phenolic content and antioxidant activity of  
715 aerial parts of medicinal plant *Coronopus didymus*. *Asian Pacific J. Tropical Med.* 10(8):  
716 792–801.

717 NURCHOLIS GW *et al.* 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity in-  
718 vitro and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum pictum*  
719 (L.). *Current Biochem.* 1(2): 58-65.

720 OH J *et al.* 2013. Antioxidant and antimicrobial activities of various leafy herbal teas. *Food*  
721 *Control.* 31(2013):403-409.

722 PROENCA C *et al.* 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an in vitro and in silico  
723 structure–activity relationship study. *J. Enzyme Inhibition Med. Chem.* 32(1): 1216–1228.

724 PUREWAL SS *et al.* 2022. Unraveling the effect of storage duration on antioxidant  
725 properties, physicochemical and sensorial parameters of ready to serve Kinnow-amla  
726 beverages. *Applied Food Res.* 2(1): 1-8.

727 RAHARJO D, HARYOTO. 2019. Antioxidant activity of mangrove *Sonneratia caseolaris*  
728 L. using the FRAP method. *ISETH.* 2019: 623-629.

729 RAMPHINWA ML *et al.* 2023. Eco-physiological response of secondary metabolites of  
730 teas: review of quality attributes of herbal tea. *Frontiers in Sustain. Food Systems.*  
731 7(990334):1-14.

732 REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids.  
733 *Czech J. Food Sci.* 30(2):171-177.

734 RUAN J *et al.* 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea*  
735 *indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel  
736 column chromatography fractions. *Molecules*. 24(2784):1-20.

737 RUSITA YD *et al.* 2019. Comparison of tannin concentration on fresh and dried tea leaf  
738 (*Camelia sinensis* L.) extract using UV-visible spectrophotometric method. 1st  
739 International Conference of Health, Science & Technology (ICOHETECH) 2019 E-ISBN:  
740 978-623-92207-0-9. November 16<sup>th</sup>, 2019.LPPM Universitas Duta Bangsa Surakarta,  
741 Surakarta.

742 SANGEETHA R, VEDASREE N. 2012. In Vitro  $\alpha$ -Amylase inhibitory activity of the leaves  
743 of *Thespesia populnea*. *Intern. Scholarly Res.Network*. 2012: 1-4 (2012)

744 SHRAIM AM *et al.* 2021. Determination of total flavonoid content by aluminum chloride  
745 assay: A critical evaluation. *LWT Food Sci. Tech*. 150(111932):1-11.

746 SILVA-RAMIREZ KA *et al.* 2020. Effect of drying and steeping temperature on the  
747 phenolic content, antioxidant activity, aromatic compounds and sensory properties of  
748 *Culila polyantha* Benth. *Infusions. Processes*. 8(1378):2-20.

749 SRISOOK K *et al.* 2012. Antioxidant and anti-inflammatory activities of hot water extract  
750 from *Pluchea indica* Less. herbal tea. *J. Med. Plants Res*. 6(23):4077-4081.

751 SURIYAPHAN O. 2014. Nutrition, health benefits and applications of *Pluchea indica* (L)  
752 Less leaves. *Pharm. Sci. Asia*. 41:1–10.

753 SU D *et al.* 2019. Impact of thermal processing and storage temperature on phenolic  
754 profile and antioxidant activity of different varieties of lychee juice. *LWT-Food Tech*.  
755 116(108578):1-9.



756 TADERA K, MINAMI Y, TAKAMATSU K, MATSUOKA T. 2006. Inhibition of alpha  
757 glucosidase and alpha-amylase by flavonoids. J Nutr Sci Vitaminol. 52:149–153.

758 THANAJIRUSCHAYA P *et al.* 2010. Effect of storage period and temperature on  
759 antioxidant components and properties of milled rice. Khon Kaen Univ. Res. J.15(9):843-  
760 851.

761 VONGSAK B *et al.* 2018. Comparison of active constituents, antioxidant capacity, and  
762 αglucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. Food  
763 Biosci. 25: 68–73.

764 WANG SC *et al.* 2022. Effect of brewing conditions on polyphenols in the dark tea  
765 (*Camellia sinensis* L.) infusions: content, composition and antioxidant activities. Food  
766 Sci.Tech. 42(e36322):1-9.

767 WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica*  
768 Less as functional beverage in diabetic patients. ASSEHR. 98:164-167.

769 WIDYAWATI PS *et al.* 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract  
770 as antioxidant and anti-warmed-over flavor (WOF) of duck meat. International Congress  
771 on Challenges of Biotechnological Research in Food and Health. November 15<sup>th</sup>  
772 2014.Agricultural Technology Faculty, Slamet Riyadi University, Surakarta.

773 WIDYAWATI PS *et al.* 2016. The physicochemical and antioxidant properties of *Pluchea*  
774 *indica* Less drink in tea bag packaging. IJFNS. 5: 13–120.

775 WIDYAWATI PS *et al.* 2017. In vitro antioxidant capacities and antidiabetic properties of  
776 pluchea leaves. IJPPS. 9(8): 203-208.

777 WIDYAWATI PS *et al.* 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica*  
778 Less beverage. CJFST.12(4): 125-139.

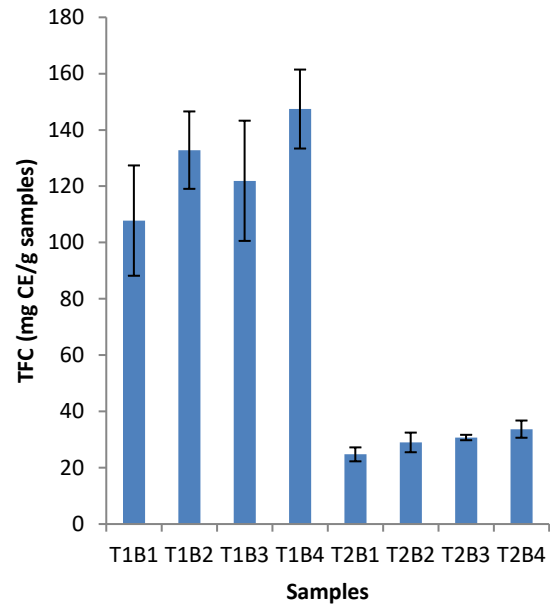
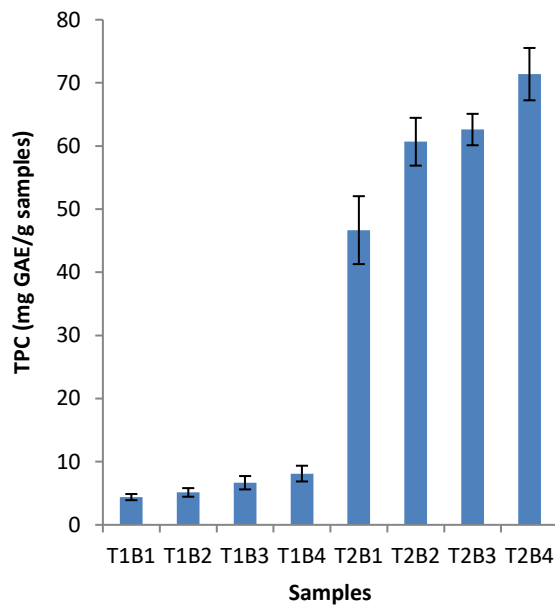
779 WIDYAWATI PS *et al.* 2022. The effect of  $\kappa$ -carrageenan proportion and hot water  
780 extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of stink  
781 lily (*Amorphophallus muelleri*) wet noodles. *Molecules*. 27(5062):1-16.

782 XU P, CHEN L, WANG Y. 2019. Effect of storage period on antioxidant activity and  
783 inhibition on  $\alpha$ -amylase and  $\alpha$ -glucosidase of white tea. *Food Sci. Nut.* 7(2): 636-644.

784 ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction period on caffeine  
785 in coffee. *Lantanida J.* 6(2):103-202. [https://jurnal.ar-](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811)  
786 [raniry.ac.id/index.php/lantanida/article/view/3811](https://jurnal.ar-raniry.ac.id/index.php/lantanida/article/view/3811). [In Bahasa Indonesia]

787 Zhang H, Wang G, Dong J. 2014. Inhibitory properties of aqueous ethanol extracts of  
788 propolis on alpha-glucosidase. *J Evid Based Complement Alternat Med.* 2015:1–8.

789 ZHANG Y *et al.* 2021. Effect of storage conditions and period on the polyphenol content  
790 of wheat flours. *Processes*. 9(248):1-11.

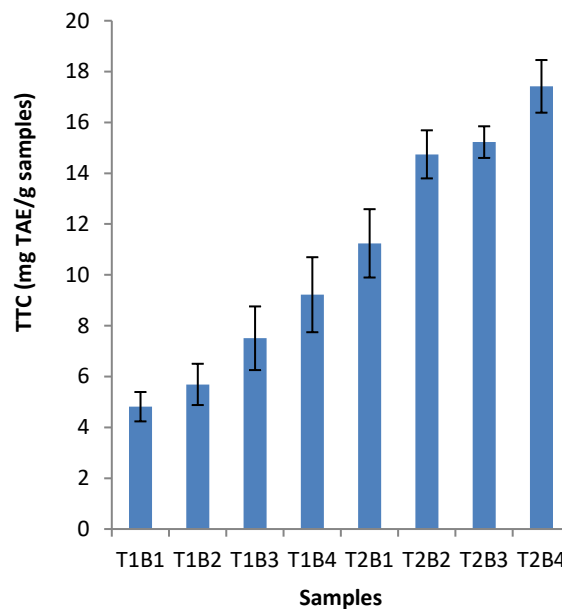


791

792

(a)

(b)



793

794

(c)

795 Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping  
 796 temperature and storage period (a) Total phenolic content (b) Total flavonoid  
 797 content (c) Total tannin content. Data analysis using ANOVA at  $\alpha \leq 0.05$   
 798 continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as  
 799 mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored;

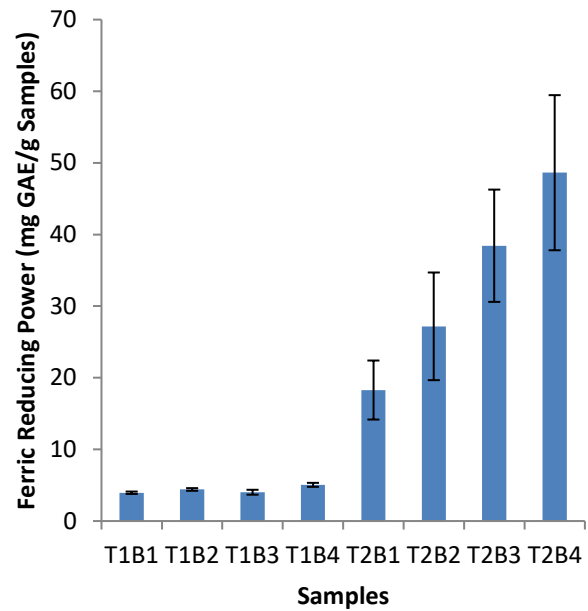
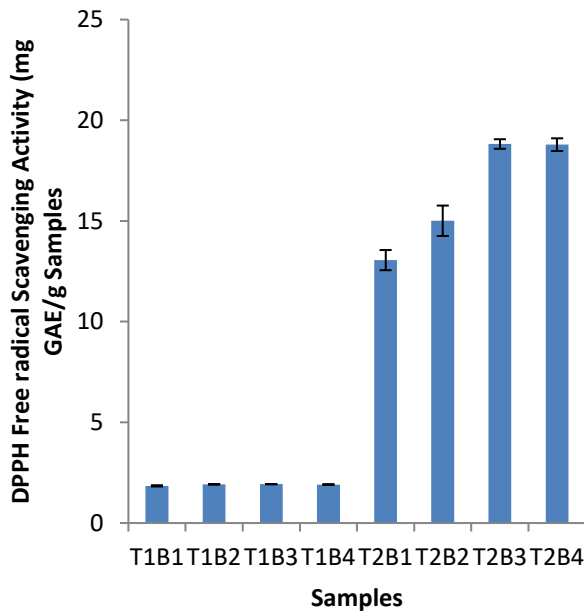
800 T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-  
801 steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 yr; T3B2-  
802 steeped at 70 °C, stored for 5 yr; T3B3-steeped at 80 °C, stored for 5 yr;  
803 T3B4-steeped at 95 °C, stored for 5 yr.

804 Table 1. Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperature and storage period

Phenolic Compounds	Steeping Temperature (°C)	Mean±SD Un-stored	Mean±SD Stored	Mean difference ±SD	Sig (2-tailed)
Gallic Acid (µg/g samples)	60	0.2132±0.0027	0.2364±0.0015	0.0375±0.0175	0.2030
	70	0.2157±0.0013	0.2324±0.0214	0.0167±0.0227	0.4870
	80	0.2234±0.0122	0.2347±0.0078	0.0386±0.0264	0.2870
	95	0.2316±0.0104	0.2402±0.0169	0.0086±0.1990	0.8500
(+) -Catechin (µg/g samples)	60	0.3425±0.0110	0.5085±0.0111	-0.1576±0.0885	0.241
	70	0.3260±0.0265	0.5448±0.0006	-0.2188±0.0259	0.053
	80	0.3240±0.0222	0.5023±0.0773	-0.1451±0.0248	0.077
	95	0.4039±0.0320	0.5995±0.0372	-0.2049±0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756±0.1234	1.4762±0.0271	-1.2887±0.3222	0.111
	70	0.2587±0.0160	1.4245±0.2526	-1.1657±0.2695	0.103
	80	0.4175±0.0104	1.4570±0.0925	-1.0391±0.0841	0.036*
	95	0.8786±0.0434	2.6138±0.0695	-1.1735±0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220±0.0268	0.6220±0.0706	-0.5999±0.9733	0.544
	70	0.1530±0.0511	1.0708±0.0289	-0.9177±0.0222	0.011*
	80	0.3666±0.0103	0.8629±0.0815	-0.1082±0.4462	0.790
	95	0.6559±0.0570	2.0230±0.0573	-1.4123±0.3203	0.101
Kaempferol (µg/g samples)	60	0.1394±0.0202	0.3675±0.0183	-0.3207±0.1122	0.154
	70	0.0514±0.0037	0.3726±0.0944	0.3213±0.0907	0.125
	80	0.3699±0.0924	0.7966±0.0366	-0.4267±0.2727	0.271
	95	0.5913±0.0239	0.9478±0.0287	-0.3565±0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103±0.0628	2.4863±0.0270	-1.8760±0.2074	0.050*
	70	0.6271±0.0099	2.3403±0.0325	-1.7131±0.3152	0.082
	80	0.7967±0.03060	2.6278±0.0211	-1.8311±0.0095	0.002*
	95	1.5386±0.0668	4.0211±0.0851	-2.4825±0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635±0.0628	0.9449±0.0501	-0.2814±0.4458	0.536
	70	0.6162±0.0099	0.9485±0.0794	-0.3323±0.0301	0.041*
	80	0.6601±0.0306	0.9099±0.0387	-0.2498±0.3127	0.461
	95	0.6642±0.0668	1.3156±0.0166	-0.6514±0.2666	0.179

4,5-di-O-Caffeoylquinic acid ( $\mu\text{g/g}$ samples)	60	0.4906 $\pm$ 0.0060	1.1842 $\pm$ 0.0120	-0.6886 $\pm$ 0.2723	0.018*
	70	0.4807 $\pm$ 0.0034	1.0089 $\pm$ 0.0736	-0.5281 $\pm$ 0.0702	0.060
	80	0.5299 $\pm$ 0.0053	1.2382 $\pm$ 0.1435	-0.7082 $\pm$ 0.1489	0.094
	95	1.0018 $\pm$ 0.0526	1.3797 $\pm$ 0.2170	-0.3086 $\pm$ 0.3086	0.333

805 Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  
806  $\pm$ standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-steeped at 70 °C, un-stored; T1B3-steeped  
807 at 80 °C, un-stored; T1B4-steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 yr; T3B2-steeped at 70 °C,  
808 stored for 5 yr; T3B3-steeped at 80 °C, stored for 5 yr; T3B4-steeped at 95 °C, stored for 5 yr.  
809



810

811

(a)

(b)

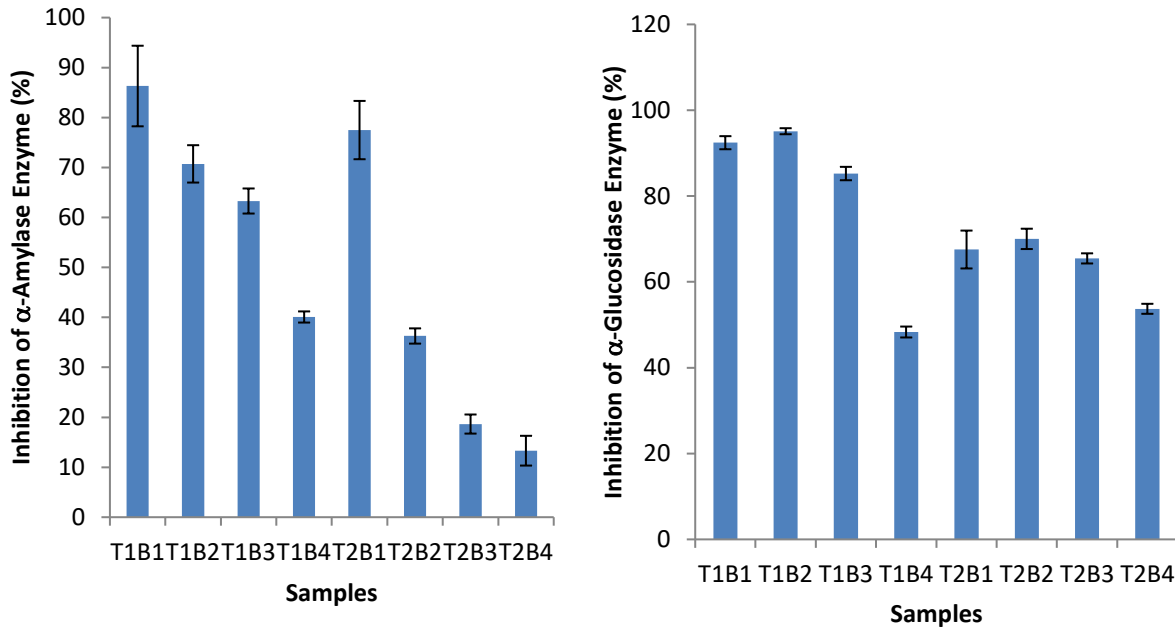
812 Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage  
 813 period (a) DPPH (b) FRAP. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued  
 814 analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  
 815  $\pm$ standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored; T1B2-  
 816 steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-steeped  
 817 at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 yr; T2B2-steeped at  
 818 70 °C, stored for 5 yr; T2B3-steeped at 80 °C, stored for 5 yr; T2B4-steeped  
 819 at 95 °C, stored for 5 yr.

820

821

822

823



824

825

(a)

(b)

826 Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage  
 827 period (a) α-amylase (b) α-glucosidase. Data analysis using ANOVA at  $\alpha \leq 0.05$   
 828 continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as  
 829 mean  $\pm$  standard deviation (n=6). Samples : T1B1-steeped at 60 °C, un-stored;  
 830 T1B2-steeped at 70 °C, un-stored; T1B3-steeped at 80 °C, un-stored; T1B4-  
 831 steeped at 95 °C, un-stored; T2B1-steeped at 60 °C, stored for 5 yr; T3B2-  
 832 steeped at 70 °C, stored for 5 yr; T3B3-steeped at 80 °C, stored for 5 yr; T3B4-  
 833 steeped at 95 °C, stored for 5 yr.



834 Table 2. Pearson correlation coefficients between bioactive contents (TPC, TFC and TAC), antioxidant activity (DPPH and  
 835 FRAP) and antidiabetic activity (AA and GA)

	<i>TPC</i>	<i>TFC</i>	<i>TTC</i>	<i>DPPH</i>	<i>FRAP</i>	<i>Alpha Glucosidase</i>	<i>Alpha Amylase</i>
TPC	1						
TFC	-0.93589	1					
TTC	0.960028	-0.81321	1				
DPPH	0.992776	-0.93992	0.942273	1			
FRAP	0.953366	-0.82636	0.947778	0.956242	1		
Alpha Glucosidase	-0.55512	0.349873	-0.71534	-0.5272	-0.55947	1	
Alpha Amylase	-0.70842	0.429393	-0.8569	-0.69579	-0.80548	0.725161631	1

836 Significant at the 0.05 level (2-tailed)

837

838



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

## Copyediting of PJS Paper Ms 23-158

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Mon, Jun 17, 2024 at 2:58 PM

Dear Mr. ALLYSTER A. ENDOZO  
Managing Editor

Greetings,

I have corrected and reviewed the manuscript that was sent to me. Basically I have agreed and there are some things I have corrected and highlighted. Thank you for your attention

Regards

Paini SW

[Quoted text hidden]



**[PRO] 23-158 - Widyawati and Wilianto - Manuscript (Edited) (15 Jun 2024).docx**  
121K

1 **Effect of Steeping Temperature and Storage Period on the Bioactive Compounds plus**  
2 **Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea indica* Less**

3

4 **Paini Sri Widyawati<sup>1\*</sup> and Yufita Ratnasari Wilianto<sup>2</sup>**

5 <sup>1</sup>Food Technology Study Program, Agricultural Technology Faculty,  
6 Widya Mandala Surabaya Catholic University, Surabaya 60265 Indonesia

7 <sup>2</sup>Pharmacy Study Program, Pharmacy Faculty,  
8 Widya Mandala Surabaya Catholic University, Surabaya 60272 Indonesia

9

10 Keywords: antioxidant, antidiabetic, bioactive compound,  
11 *Pluchea indica* Less, steeping temperature, storage period

12

Formatted: Centered

13 **ABSTRACT**

14 This study was done to determine the effects of steeping temperature and storage  
15 period on the bioactive contents plus antioxidant and antidiabetic activities of Pluchea  
16 leaf infusion. The research used a randomized block design with two factors, *i.e.* steeping  
17 temperature (T) and storage period (B). The *Pluchea* leaf blades were exposed to four  
18 steeping temperatures of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) °C with the storage period  
19 of 0 (B1) and 5 (B2) yr resulting in eight treatment combinations (T1B1, T1B2, T2B1,  
20 T2B2, T3B1, T3B2, T4B1, T4B2). Statistical analysis using a paired t-test at  $\alpha \leq 0.05$   
21 showed that treatments significantly affected the bioactive contents [total phenol (TPC),  
22 total tannin (TTC), and total flavonoid (TFC)], antioxidant [DPPH scavenging activity

Formatted: No underline

23 (DPPH) and ferric reducing antioxidant power (FRAP)] potential and antidiabetic [ ~~$\alpha$ - $\alpha$~~   
24 amylase (AA) and  ~~$\alpha$ - $\alpha$~~ glucosidase (GA) inhibition] properties of the *Pluchea* leaf  
25 infusion. TPC, TTC, DPPH, and FRAP significantly increased for the storage period and  
26 the steeping temperatures. Then, TFC decreased during the storage period but  
27 significantly increased at higher steeping temperatures. The AA and GA of *Pluchea* leaf  
28 infusion increased until 70 °C of the steeping temperature, but decreased until 95 °C. The  
29 DPPH and FRAP of the *Pluchea* leaf infusion were strongly and positively correlated with  
30 TPC and TTC. The GA and AA of *Pluchea* leaf infusion were not influenced by the TPC  
31 and TTC but were weakly and positively correlated with TFC. The antioxidant activity of  
32 the *Pluchea* leaf infusion was inversely proportional to the antidiabetic activity. The simple  
33 phenolic compounds derived from *Pluchea* leaf infusion at different steeping  
34 temperatures and storage included gallic acid, kaempferol, myricetin, (+)-catechin,  
35 quercetin, 3,4-di-O-caffeoylquinic acid, 3,5- di-O-caffeoylquinic acid, and 4,5-di-O-  
36 caffeoylquinic acid.

Formatted: No underline

Formatted: No underline

Formatted: No underline

Formatted: No underline

## 38 INTRODUCTION

39 *Pluchea* herbal tea is a product of dried *Pluchea* leaf processing introduced by  
40 world people (Srisook *et al.* 2012; Widyawati *et al.* 2016) because of the efficacy of the  
41 active components in *Pluchea* leaves, as a herbal plant that has been widely used for  
42 traditional medicine and food (Chan *et al.* 2022). *Pluchea* leaves are composed of many  
43 nutrients and bioactive compounds useful to body health. The nutrient compositions in  
44 the *Pluchea* leaves include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates,  
45 calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive compounds are comprised, *i.e.*

Formatted: No underline

Formatted: No underline

Formatted: Highlight

Formatted: Highlight

Formatted: No underline

Formatted: Highlight

Formatted: No underline

Formatted: No underline

46 chlorogenic acid, caffeic acid, 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-  
47 caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-  
48 caffeoylquinic acid, quercetin, myricetin, kaempferol, total anthocyanin,  $\beta$ -carotene, and  
49 total carotenoid (Suriyaphan 2014; Vongsak et al. 2018; Ruan et al. 2019; Widyawati et  
50 al. 2022; Chan et al. 2022).

51 The steeping process of *Pluchea* leaves can be performed with fresh or dry leaves  
52 in hot or boiling water for a few min (Suriyaphan 2014; Silva-Ramirez et al. 2020; Jayanti  
53 et al. 2022). In Asia, especially in Indonesia, people usually consume the *Pluchea* infusion  
54 by steeping 2 g of powdered *Pluchea* leaves in a tea bag in 100 mL of hot or boiling water.  
55 Widyawati et al. (2016) claimed that steeping of 2 g of *Pluchea* leaf powder at 95 °C for  
56 5 min exhibits total phenolic and total flavonoid contents, the ability to scavenge DPPH  
57 free radicals, and the capability to reduce ferric ions at 9.3 mg gallic acid equivalent  
58 (GAE)/g sample, 22.0 mg gallic acid equivalent (GAE)/g sample, 27.2 mg gallic acid  
59 equivalent (GAE)/g sample, and 10.2 mg gallic acid equivalent (gallic acid equivalent  
60 (GAE))/g sample, respectively. Werdani and Widyawati (2018) reported that drinking  
61 *Pluchea* leaf powder infusion in the morning and evening regularly (2 g/ 100 mL) can  
62 decline blood sugar levels.

63 The steeping of *Pluchea* herbal tea with hot water at 95 °C for 5 min certainly  
64 determines the stability and amount of extracted bioactive compounds that influence the  
65 biological activity especially antioxidant and antidiabetic activities. Silva-Ramirez et al.  
66 (2020) reported that the infusion process can influence the content and composition of  
67 the bioactive compounds and antioxidant activity of tea. Acar et al. (2022) informed stated  
68 that the infusion quality of herbal tea extract depends on several a number of factors,

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: No underline

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: No underline

Formatted: No underline

Formatted: Highlight

Formatted: No underline

Formatted: Highlight

Formatted: No underline

Formatted: No underline

Formatted: Highlight

Formatted: Highlight

69 *i.e.* storage and temperature. The polyphenol profile and antioxidant properties of herbal  
70 tea infusion decline with an increase in steeping ~~or~~ brewing and storage temperatures,  
71 ~~as well as~~ longer exposure periods.

72 Several studies have mentioned the effect of steeping temperature on the bioactive  
73 compound contents and antioxidant activity, ~~such~~ as some white and green teas are  
74 effective with hot water at 90 °C for 7 min (Castiglioni *et al.* 2015), on roseship tea is  
75 effective at infusion period around 6–8 min at temperatures of 84–86 °C (Ilyasoglu and  
76 Arpa 2017), on the caffeine content extracted at the brewing temperature of coffee  
77 (Zarwinda and Sartika 2018), and the high total phenol content and antioxidant activity of  
78 dark tea at 92 °C for 27 min (Wang *et al.* 2022). The study of the effect of steeping  
79 temperature on *Pluchea* infusion was carried out to afford information about the most  
80 efficient preparation of powdered *Pluchea* leaves to get higher bioactive compounds,  
81 antioxidant, and antidiabetic activities.

82 Storage period tea usually for several months to yr *Pluchea* herbal tea also affects  
83 the levels of the bioactive compounds and biological activity (Jayani *et al.* 2022). Tea or  
84 herbal tea is generally stored at ambient temperature and packed in a tea bag or  
85 aluminum foil standing pouch or a combination of both. Many researchers reported that  
86 the storage period decreases the bioactive compounds ~~plus~~, antioxidant and antidiabetic  
87 activities, *i.e.* juice from *Momordica charantia* L. (Lin *et al.* 2020), dried *Piper betle* extracts  
88 (Ali *et al.* 2018), white tea (Xu *et al.* 2019), Kkinnow-Aamla beverages (Purewal *et al.*  
89 2022), ~~and~~ whole-wheat flour (Zhang *et al.* 2021).

90 Therefore, this research studied the effect of steeping temperature and storage  
91 period on the bioactive compounds [total phenolic content (TPC), total flavonoid content

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: No underline

Formatted: No underline

Formatted: No underline

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

92 (TFC), and total tannin content (TTC)], antioxidant [~~(DPPH~~ free radical scavenging activity  
93 (DPPH) and, ferric reducing antioxidant power (FRAP)], and antidiabetic activities [~~( $\alpha$ -~~  
94 amylase (AA) and  $\alpha$ -glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea*  
95 leaves and on the phenolic compound profile.

Formatted: No underline

96

## 97 MATERIALS AND METHODS

### 98 Raw Materials and Preparation

99 The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya,  
100 East Java, Indonesia. The *Pluchea* plants were included in the Asteraceae family with  
101 specifications according to the GBIF taxon ID number database:3132728 (Ferraris 2023).

Formatted: No underline

Formatted: No underline

Formatted: Font: Not Italic

Formatted: Highlight

102 *Pluchea* leaves at 1–6 levels of each branch from the shoot were collected, sorted,  
103 washed, and dried to get a moisture content of around  $11.16 \pm 0.09\%$  dry basis  
104 (Widyawati *et al.* 2022). The dried *Pluchea* leaves ~~was~~ were pulverized to a 45-mesh size

Formatted: No underline

Formatted: Highlight

Formatted: No underline

Formatted: No underline

105 powder. The *Pluchea* leaf powder was dried in an oven (Binder, Merck KGaA, Darmstadt,  
106 Germany) at 120 °C for 10 min to reduce microbial organisms. Then, 2 g of the powder  
107 ~~were~~ was packed into a paper filter infusion bag. Packed samples were stored for 0  
108 (unstored) and 5 (stored) yr in a standing pouch before analysis.

109 In the research, ~~the~~ one tea bag of *Pluchea* herbal tea that was stored for 0 (B1)  
110 and 5 (B2) year, was steeped with 100 mL hot water at various temperatures —, including  
111 60 (T1), 70 (T2), 80 (T3), and 95 (T4) °C for 5 min — with infusion method obtaining 8  
112 eight treatment combinations — namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1,  
113 and T4B2. After the temperature of *Pluchea* infusion similar to ambient temperature was  
114 analyzed further.

Formatted: No underline

## 115 Reagents

116 The reagents used in the analysis include 2,2-diphenyl-1-picrylhydrazyl (DPPH),  
117 sodium carbonate, gallic acid,  $\alpha$ -amylase,  $\alpha$ -glucosidase, ~~pNPG~~ (p-nitrophenyl- $\alpha$ -  
118 glucopyranoside pNPG), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-O-  
119 caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and (+)-  
120 catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, Folin-  
121 Ciocalteu's ~~p~~Phenol, sodium nitric, aluminum chloride, ferric chloride, sodium dihydrogen  
122 phosphate, sodium phosphate, potassium ferricyanide, starch, acetic acid, and sodium  
123 hydroxide were purchased from Merck (Kenilworth, NJ, USA). All reagents used were of  
124 analytical grade except for distilled water which was purchased from PT Aqua Industry  
125 Surabaya.

## 126 Analysis of the Bioactive Compounds

127 **Total phenolic content (TPC) analysis.** ~~The total phenolic content (TPC)~~ of  
128 treated *Pluchea* infusion was carried out using the technique by **Gao *et al.* (2019)**. About  
129 10  $\mu$ L *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10-% were mixed in  
130 10-mL volumetric flask and incubated for 5 min. ~~And then,~~ 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5-% was  
131 added and filled up to 10 mL volume with distilled water. The blue color intensity of the  
132 solution was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at  $\lambda$  =  
133 760 nm, with gallic acid as the reference standard. The ~~total phenolic content~~ TPC was  
134 calculated using the following formula:  $y = 0.00009x + 0.008$ , with  $R^2 = 0.9941$ . The results  
135 were expressed as mg ~~gallic acid equivalent (GAE)~~ /g samples.

136 **Total flavonoid content (TFC) assay.** ~~The total flavonoid content (TFC)~~ of the  
137 samples was measured based on the reaction between AlCl<sub>3</sub> and NaNO<sub>2</sub> with the

Formatted: No underline

Formatted: Highlight

Formatted: No underline



138 aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim *et al.* 2021).

Formatted: Highlight

139 The reaction between  $\text{AlCl}_3$  and flavonoid compounds resulted in a yellow solution. About

140 30- $\mu\text{L}$  *Pluchea* infusion was mixed with 0.3 mL  $\text{NaNO}_2$  5-% in 10-mL volumetric flask

Formatted: No underline

141 and incubated for 5 min. The mixture was added with 0.3 mL  $\text{AlCl}_3$  10-% for 5 min. Then,

142 2-mL NaOH 1 M and distilled water were added until to a 10-mL volume. Then, the red

143 solution was produced after NaOH solution addition that was measured by a

144 spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda = 510$  nm,

145 with (+)-catechin as the reference standard compound, and the results were expressed

146 as mg catechin equivalents (CE)/g samples using the following formula:  $y = 0.00008x -$

147  $-0.0023$ , with  $R^2 = 0.9980$ .

148 **Total tannin content (TTC) analysis.** ~~The total tannin content (TTC)~~ of the

149 samples was analyzed by using the Folin-Ciocalteu method (Chandran and Indira 2016).

Formatted: Highlight

150 Approximately 10- $\mu\text{L}$  *Pluchea* infusion was added with 1-mL Folin-Ciocalteu's phenol

Formatted: No underline

151 reagent 10-% in 10-mL volumetric flask and incubated for 5 min. Then, the mixture was

152 added with 2-mL  $\text{Na}_2\text{CO}_3$  7.5-% and filled up to 10-mL volume with distilled water. The

153 blue dark color solution was measured in UV-Vis spectrophotometer 1800 (Shimadzu,

154 Japan) at  $\lambda = 760$  nm, with tannic acid as the reference standard. Calculation of TTC was

155 expressed as mg tannic acid equivalents (TAE)/g samples used using the following

156 formula:  $y = 0.00009x + 0.0021$ , with  $R^2 = 0.9993$

### 157 Analysis of the Antioxidant Potential

158 **DPPH free radical scavenging activity assay.** The DPPH free radical

159 scavenging activity (DPPH) was measured by the spectrophotometric method (Widyawati

Formatted: Highlight

160 *et al.* 2017) to determine the ability of the phytochemicals in the *Pluchea* leaf infusion to

Formatted: No underline

161 donate hydrogen atoms to the nitrogen atom in DPPH, resulting in the formation of DPPH-  
162 H compound exhibiting a yellow-colored solution. About 25  $\mu$ L *Pluchea* leaf infusion was  
163 poured into the reaction tube, into which ~~was added~~ 3 mL DPPH solution (4 mg/100 mL)  
164 ~~was added~~. After incubation for 15 min in a dark room, the absorbance was measured by  
165 a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda = 517$   
166 nm. The reference standard compound was gallic acid, and the results of the analysis  
167 were expressed as mg ~~gallic acid equivalents~~ (GAE)/g samples ~~that~~ calculated using the  
168 ~~following~~ formula:  $y = 0.146x + 1.7896$ , with  $R^2 = 0.9975$ .

Formatted: No underline

169 **Ferric-reducing power (FRAP) analysis.** ~~Ferric-reducing power~~ (FRAP) was  
170 determined following the method used by **Widyawati *et al.* (2014)** method. Approximately  
171 10  $\mu$ L of samples were added to 2.5 mL phosphate buffer pH 6.6 and 2.5 mL of 1%  
172 potassium ferricyanide in the reaction tube. ~~And~~ ~~then~~, the mixture was shaken and  
173 incubated for 20 min at 50 °C. Finally, 2.5 mL chloroacetic acid 10% (w/v) was added.  
174 Into the 2.5 mL supernatant, ~~was added~~ 2.5 mL distilled water ~~and~~, 0.5 mL ferric chloride  
175 0.1% w/v ~~were added~~, and ~~the mixture was~~ incubated for 10 min. ~~The p~~ Potency of the  
176 samples reducing iron (III) to iron (II) ion was indicated by the intensity of blue color  
177 formed that was measured using UV-Vis spectrophotometer (Spectrophotometer UV-Vis  
178 1800, Shimadzu, Japan) at  $\lambda = 700$  nm. ~~The i~~ Intensity of the blue color indicated a higher  
179 reducing capacity. The reducing power, expressed as mg ~~gallic acid equivalent~~ (GAE)/g  
180 samples, was calculated using the ~~following~~ formula:  $y = 0.0002x + 0.0256$ , with  $R^2 =$   
181 ~~0.9906~~.

Formatted: Highlight

## 182 Analysis of the Antidiabetic Properties

183  **$\alpha$ -amylase enzyme inhibition (AA) capacity assay.** *In vitro* ~~inhibition of  $\alpha$ -~~  
184 ~~amylase enzyme (AA)~~ followed the procedure, as described by **Widyawati *et al.* (2020)**.  
185 Each 500  $\mu$ L of ~~the~~ samples, was mixed with starch 1-% (w/v) and sodium acetate buffer  
186 pH 5. Into ~~a~~-250  $\mu$ L of the mixture, ~~was added~~ an  ~~$\alpha$~~ -amylase solution (0.1 g of this  
187 enzyme 12.5 unit/mL) was added and then, ~~was~~ dissolved in 50 mL of 0.2 M sodium  
188 acetate pH 5. ~~The m~~Mixture was shaken, into which ~~was and added~~ 2- mL sodium  
189 hydroxide 1M was added. Before the analysis, this mixture was incubated at 37 °C for 10  
190 min. Then, the capacity of the  $\alpha$ -amylase enzyme to hydrolyze the starch to release  
191 glucose was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800,  
192 Shimadzu, Japan) at  $\lambda$  = 540 nm. The inhibition percentage of  $\alpha$ -amylase was assessed  
193 using the formula:  $(ACb - ACa) - (As - Ab) / (ACb - ACa) \times 100\%$  ~~where~~, ACb is the  
194 absorbance of 100-% enzyme activity (solvent with the enzyme), ACa is the absorbance  
195 of 0 % enzyme activity (solvent without the enzyme), As is the absorbance of ~~the~~ test  
196 sample with enzyme, and Ab is ~~the~~ absorbance of test sample without enzyme.

197  **$\alpha$ -glucosidase enzyme inhibition (GA) capacity assay.** The analysis of the  $\alpha$ -  
198 glycosidase inhibitor activity (GA) was done ~~by using the method of~~ **Widyawati *et al.***  
199 **(2020)** ~~method~~ with slight modifications. About 150- $\mu$ L samples containing 100- $\mu$ L  
200 *Pluchea* infusion and 50  $\mu$ L pNPG (0.0150 g in 100- mL sodium phosphate 0.2 M at pH  
201 7) were reacted with 50- $\mu$ L  $\alpha$ -glycosidase 2 mM (0.0833 unit/mL); ~~and then~~ the mixture  
202 was incubated at 37 °C for 15 min. The reaction was stopped with the addition of 1000-  
203  $\mu$ L sodium carbonate 0.2 M. The amount of these enzymes that did ~~no~~t react with  
204 bioactive compounds of *Pluchea* infusion hydrolyzed ~~p-nitrophenyl- $\alpha$ -D-glucopyranoside~~  
205 (~~pNPG~~) as a substrate to result in p-nitrophenol. The inhibition activity of the *Pluchea*

Formatted: Highlight

Formatted: Highlight

Formatted: No underline

Formatted: No underline

Formatted: No underline

206 infusion was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis-1800,  
207 Shimadzu, Japan) at  $\lambda = 405$  nm. The inhibition percentage of  $\alpha$ -glycosidase was  
208 calculated using the formula:  $(ACb - ACa) - (As - Ab) / (ACb - ACa) \times 100\%$ , where,  
209 ACb is the absorbance of 100% enzyme activity (solvent with enzyme), ACa is the  
210 absorbance of 0% enzyme activity (solvent without enzyme), As is the absorbance of  
211 test sample with enzyme, and Ab is the absorbance of test sample without enzyme.

### 212 Analysis of Phenolics

213 The phenolic compounds of the samples were analyzed by using high-  
214 performance liquid chromatography (HPLC) based on the method of Kongkiatpaiboon  
215 *et al.* (2018) method with modifications. Each *Pluchea* infusion was sonicated for 15 min  
216 (Branson 1510); and then, the sample was filtered using a filter syringe (Whatmann, 0.2  
217  $\mu$ m, NYL). About 20  $\mu$ L of the sample was injected in an HPLC (LC20AD series,  
218 Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC LC-20AD pump,  
219 CTO-30A column oven, CBM-20A/20 Alite system controller, and SPD-20A/20 AV UV-  
220 Vis detector. Separation of phenolic compounds in samples was carried out using a Shim-  
221 pack VP-ODS C18 column (ID 5  $\mu$ m x 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard  
222 column (2 two pieces) (ID 10 mm x 4.6 mm). The mobile phase used consisted of a  
223 solution of [A] 0.5% acetic acid in water and [B] absolute methanol. Analysis was  
224 carried out using a gradient system in the following order: initial conditions of 10% B in A  
225 to 50% B in A were maintained for 40 minutes; then, 100% B was maintained for 20  
226 minutes. Next, the column was re-equilibrated with 10% B in A and maintained for 10  
227 min before analysis of the next sample. The sample flow rate was set at 1.0 mL/min with  
228 a controlled temperature at of 40 °C. Detection was used at a wavelength of 280 nm. The

Formatted: Highlight

Formatted: No underline

229 reference standard used were gallic acid, (+)-catechin, myricetin, quercetin, kaempferol,  
230 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid. All of ~~the~~  
231 reference standard was dissolved in distilled water and prepared similarly to the samples  
232 before ~~being~~ injected in HPLC.

233 **Experiment design and statistical analysis.** The research design used a  
234 randomized block design with two factors, *i.e.* the steeping temperature (T) and the  
235 storage period. *Pluchea* leaf blades were subjected to ~~four~~ 4-steeping temperatures,  
236 namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95 °C (T4), and the storage period of 0  
237 ~~year~~ /unstored (B1), and 5 ~~year~~ /stored (B2) resulting in 8 treatment combinations  
238 (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic  
239 was repeated ~~for~~ six periods. The data analysis of samples was repeated for six periods.  
240 The data were analyzed using a paired t-test at  $\alpha \leq 0.05$ , treatment means of specific  
241 phenolic compounds that were identified were expressed as the mean  $\pm$  SD. The analysis  
242 used SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

243

## 244 RESULTS AND DISCUSSIONS

### 245 Bioactive Compounds

246 **Phenolic compounds.** ~~B-The~~ bioactive compounds are active compounds in  
247 plants that are essential to protect ~~a~~-body health (Nguyen and Chuyen 2020). These  
248 compounds usually have many biological activities, such as antioxidant, antidiabetic, anti-  
249 inflammatory, anticancer, antimicrobial, antibacterial, anti-cholesterol, and so on  
250 (Suriyaphan 2014; Acar *et al.* 2022). Phenolic compounds have potential redox properties

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

251 that can scavenge free radicals that can cause a number of chronic diseases (Noreen *et*  
252 *al.* 2017; Aryal *et al.* 2019; Acar *et al.* 2022).

253 The ~~total phenolic content~~ (TPC) of *Pluchea* infusion at different steeping  
254 temperatures and storage periods generally significantly increased with increasing  
255 steeping temperature and storage period based on paired t-test at  $\alpha \leq 0.05$  (Figure 1a).

256 Steeped and stored infusion had significantly higher amounts of phenolic compounds  
257 than the samples that were steeped and unstored. Further, the highest ~~total phenolic~~  
258 ~~content~~ TPC was observed in samples infused at 95 °C and stored for 5 yr (at 71.38 ±  
259 4.14 mg GAE/g sample), ~~while~~ ~~whereas~~ the lowest was measured in the unstored  
260 samples and infused at 60 °C (at 4.39 ± 0.49 mg GAE/g sample). The phenolic content  
261 of stored samples that were steeped only at 60 and 95 °C showed a significant increase  
262 in their phenolic content. This implies that the steeping temperature and the storage  
263 periods significantly resulted in the high amounts of phenolic compounds in the infusions.

264 Results also indicated that phenolic compounds were generally greater in the infusion at  
265 high steeping temperatures and long storage periods. This could have been due to the  
266 fact that the steeping temperature and storage period could cause the process of  
267 degradation, oxidation, and leaching ~~or~~ /release of phenolic compounds. Phenolic  
268 compounds are water-soluble and, thus, soaking in hot water for a certain period ~~of~~  
269 ~~period~~ as ~~in~~ steeping causes the migration process of more phenolic compounds to the  
270 water because of longer exposure of phenolic compounds to water (Castiglioni *et al.*

271 2015); Kilic *et al.* 2017; and Acar *et al.* 2022). Su *et al.* (2019) reported that  
272 temperature treatment can stimulate the release of phenolic compounds and increase ~~the~~

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: No underline

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

273 antioxidant activity of lychee juice stored at different temperatures of 4 and 45 °C and  
274 different long storage (fresh and 72 hours).

275 Temperature treatment degrades (or hydrolyzes) the hydrogen bond between  
276 phenolic compounds and proteins, resulting in an increase of phenolic compounds when  
277 exposed to higher temperatures (Ali *et al.* 2018; Jayani *et al.* 2022; and Ramphinwa  
278 *et al.* 2023). Zhang *et al.* (2021) reported that phenolic compounds present in plants are  
279 not completely stable, but are easily degraded during storage after harvest. Reblova  
280 (2012) claimed that antioxidant compounds can be slowly degraded with increasing  
281 temperature. Fibrianto *et al.* (2021) also stated that the brewing temperature has an effect  
282 on the extracted antioxidant compounds, such as alkaloids, catechins, and tannins. Thus,  
283 there is an assumption that temperature and storage caused the degradation, oxidation,  
284 and hydrolysis of the phenolic compounds period, resulting in the increased amount of  
285 the phenolic compounds at higher steeping temperatures and longer storage periods.

286 Simple phenolic compounds are identified in steeped and stored. *Pluchea* leaf  
287 infusion included gallic acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-  
288 O-caffeoylquinic acids, 3,5-di-O-caffeoylquinic acids, and 4,5-di-O-caffeoylquinic acids  
289 ~~was is showed shown~~ in Table 1. The treatment effects using the a-t-test at  $\alpha \leq 0.05$   
290 showed that gallic acid and kaempferol content were insignificantly different at various  
291 steeping temperatures and storage periods. The concentration of quercetin and 3,5-di-O-  
292 caffeoylquinic acid of the unstored and stored *Pluchea* infusion was significantly different  
293 from the rest of the samples between 70 °C, while whereas (+)-catechin concentration of  
294 *Pluchea* infusion was only significantly different at 95 °C. The myricetin content was  
295 significantly different at 80 and 95 °C. The 3,4-di-O-caffeoylquinic acid content showed

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: No underline

Formatted: Highlight

Formatted: No underline

Formatted: No underline

296 significant difference at 60, 80, and 95 °C, ~~while~~ ~~whereas~~ 4,5-di-O-caffeoylquinic acid  
297 content was only significantly different at 60 °C.

298 Results further showed that gallic acids and kaempferol were relatively stable, as  
299 reflected by the insignificant changes when exposed to the different steeping  
300 temperatures and storage periods. Myricetin, (+)-catechin, and 3,4-di-O-caffeoylquinic  
301 acid showed a drastic increase at higher steeping temperatures and longer storage  
302 periods, implying that these compounds tended to be relatively labile. Quercetin, 3,5-di-  
303 O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid underwent moderate changes.  
304 Therefore, myricetin, (+)-catechin, and 3,4-di-O-caffeoylquinic acid were easier to  
305 dissolve or degrade to form simple phenolic acids at higher temperatures and storage  
306 period (Su *et al.* 2019; Ali *et al.* 2018; Jayani *et al.* 2022; Ramphinwa *et al.* 2023;  
307 Zhang *et al.* (2021). Degradable polyphenol compounds have a simple structure and free  
308 hydroxyl groups that can react with Folin-Ciocalteu's ~~Phenol-phenol~~ reagent, resulting in  
309 a complex blue solution that can detected as ~~total phenolic content~~ TPC.

310 **Flavonoid content (TFC).** Flavonoids are the major phenolic compounds that  
311 have potential chemical and biological activities, such as radical scavenging and  
312 antimicrobial activities (Ayele *et al.* 2022; Chandra *et al.* 2014) that can protect the human  
313 body from the oxidative stress caused by many degenerative diseases ~~—~~, especially  
314 cancer, cardiovascular problems, and aging (Mathur and Vijayvergia 2017). The ~~total~~  
315 ~~flavonoid content~~ TFC of steeped *Pluchea* infusion decreased with a longer storage  
316 period. Unstored samples exhibited higher flavonoid content than the stored samples.  
317 The statistical analysis using a paired t-test at  ~~$\alpha \leq$~~  0.05 showed that the ~~TFC~~ total  
318 ~~flavonoid content~~ of *Pluchea* infusion was significantly different between the steeped

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: No underline

Formatted: No underline



319 unstored and steeped stored samples (Figure 1b). The highest ~~TFC~~total flavonoid content  
320 was exhibited by the unstored samples steeped at 95 °C at about 147.42 ± 14.03 mg  
321 CE/g sample. ~~The TFC~~Total flavonoid content was significantly lower in the stored  
322 samples than those of the unstored samples, implying that the increase in the flavonoid  
323 content of the infusion was affected primarily by the steeping temperature.

Formatted: Highlight

324 **Tannin content (TTC).** Tannins are bioactive compounds that provide properties,  
325 such as astringent, anti-diarrheal, antibacterial, and antioxidant (Malangngi *et al.* 2012).

Formatted: Highlight

326 Generally, results indicated that the ~~total tannin content~~TTC of *Pluchea* infusion  
327 significantly increased with increasing steeping temperature and storage period (Figure

Formatted: No underline

328 1c). Among, the unstored steeped samples, the tannin content was significantly lowest in  
329 the samples infused at 60 °C at about 4.81 ± 0.58 to 17.42 ± 1.04 mg TAE/g samples,

Formatted: Highlight

330 which was significantly different lower from that of the lowest tannin content of the stored  
331 samples. Among the stored and steeped samples, the highest tannin content was

332 observed at samples steeped at 95 °C about 17.42 ± 1.04 mg TAE/g samples, and was  
333 significantly different from that of the highest tannin content of the unstored steeped

334 samples at 95 °C about 9.22 ± 1.48 mg TAE/g samples. Indicating that the tannin content  
335 was primarily affected by a longer storage period than high steeping temperature. The

336 condensation of catechins to tannins is a dominant process occurring in tea leaves that  
337 is accelerated during the maceration of raw tea leaves (Kowalska *et al.* 2021) and could

Formatted: Highlight

338 have ~~had~~ contributed to the observed increase in the tannin content in the treated  
339 samples.

340 ~~Although~~Nonetheless, high temperatures and long storage periods can cause the  
341 degradation of tannins to catechins. Rusita *et al.* (2019) emphasized that tannins are polar

Formatted: Highlight

thermostable complex compounds, that are resistant to heating, indicating that even with the exposure to high temperature, the tannins still remained high in the treated samples period.

**Antioxidant activity.** Antioxidant activity is the capability of compounds to inhibit the oxidation of macromolecules from biological targets that are involved in oxidative chain reactions (Ali *et al.* 2005; Oh *et al.* 2013). The antioxidant activity assay was done in this research using DPPH Free Radical Scavenging Activity (DPPH) and ferric reducing power (FRAP) methods. The phenolic compounds are an active antioxidants that have with antioxidant capability that depends on their redox properties. The structure of phenolic compounds determines the effectivity to donate hydrogen atoms, which is negatively correlated with the O-H phenolic bond strength. The antioxidant power of phenolic compounds is due to the weak hydrogen bonds in the OH group of the phenolic compound, so that it is easier to donate hydrogen atoms (Kruk *et al.* 2022). The mechanism of phenolic compounds as antioxidants depends on their ability to donate hydrogen atoms and transfer electrons, and as well as reducing agents and singlet oxygen quenchers (Ali *et al.* 2005; Huang *et al.* 2005).

**DPPH free radical scavenging activity (DPPH).** DPPH (2,2-diphenyl-1-picrylhydrazyl) is a free radical, that is often used to evaluate antioxidant activity because this method is simple that and is suitable for measuring the donating hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of DPPH to change to a yellow color (Munteanu and Apetrei 2021; Baliyan *et al.* 2022). Figure 2a shows that the free radical scavenging properties of the stored and steeped samples were significantly higher than the unsteeped samples. It can also be observed that the

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

365 free radical scavenging property was significantly different among the stored and steeped  
366 samples but insignificant among the unsteeped and steeped sample period. *Pluchea*  
367 infusion stored at room temperature for 5 yr resulted in high free radical scavenging  
368 activity by more than 10%. Steeping at higher temperatures significantly increased the  
369 DPPH free radical scavenging activity in stored *Pluchea* infusion by around 15~~–~~to 25%.

Formatted: No underline

370 This implies that the higher free radical scavenging property was primarily affected by the  
371 storage period than the steeping temperature. During the storage process, it is possible  
372 to form complex phenolic compounds ~~which that~~ provide a high ability to scavenge free  
373 radicals (Thanajiruschaya *et al.* 2010).

Formatted: Highlight

374 The scavenging activity of the samples was strongly and positively correlated with  
375 total phenolic and tannin contents, but inversely with total flavonoid levels (Table 2). The  
376 antioxidant activity was strongly and negatively correlated with flavonoid content. The  
377 storage period could be reduced flavonoid content. The study also demonstrated that  
378 longer storage period and higher infusion temperatures produced many simple phenolic  
379 compounds with free hydroxyl groups capable to donate hydrogen atoms to DPPH free  
380 radicals. Many phenolic acids, such as gallic acids, (+)-catechins, myricetins,  
381 kaempferols, quercetins, 3,5-di-O-caffeoylquinic acids, 3,4-di-O-caffeoylquinic acids, and  
382 4,5-di-O-caffeoylquinic acids have established potential antioxidant activity (Kumar and  
383 Goel 2019) (Table 1). Kruk *et al.* (2022) informed that the capability of phenolic  
384 compounds to donate hydrogen atom depends on the chemical structure, number, and  
385 position of hydroxyl groups attached to a benzene ring, a double bond between C2 and  
386 C3 rings, and a carbonyl group (C=O) on the C ring at C4. The effectivity of antioxidant  
387 compounds to donate hydrogen atoms is determined by O-H bond dissociation energy.

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Font: Italic, Highlight

Formatted: Highlight

388 The free radical scavenging property observed in the study was not ~~in~~-consistent  
389 with the results of the study by ~~Moraes de Souza et al. (2008)~~. The research shows that  
390 ~~the total phenolic content~~TPC of herbal infusion is low~~ly~~ correlated with free radical  
391 scavenging activity. However, ~~Dobrin~~~~as et al. (2021)~~ informed that ~~total phenolic~~  
392 ~~content~~TPC is positively and significantly correlated with the free radical scavenging  
393 property of tea infusion.

394 **Ferric reducing antioxidant power (FRAP).** FRAP is an analysis of the  
395 antioxidant power of the phytochemical compounds that is based on the ability of  
396 antioxidant compounds to reduce iron ions of potassium ferricyanide ( $\text{Fe}^{3+}$ ) to potassium  
397 ferrocyanide ( $\text{Fe}^{2+}$ ). Potassium ferrocyanide reacts with ferric chloride to form a ferric-  
398 ferrous complex and results green color solution (~~Widyawati et al. 2017; Raharjo and~~  
399 ~~Haryoto 2019~~).

400 The results showed that the ~~ferric-reducing-antioxidant power (FRAP)~~ increased at  
401 higher steeping temperatures~~s~~ and longer storage periods~~s~~. The lowest FRAP was  
402 observed in the unstored samples, which were steeped at 60 °C at 3.95 ± 0.17 mg ~~gallic~~  
403 ~~acid-equivalents (GAE)~~/g samples, and the highest was exhibited in *Pluchea* infusion  
404 which was stored for 5 yr at 95 °C at 48.63 ± 10.83 mg ~~gallic-acid-equivalents (GAE)~~/g  
405 samples (~~Figure 2b~~). FRAP increased significantly as the steeping temperature was  
406 increased. FRAP of the samples stored for 5 yr was also significantly higher than the  
407 unstored samples at  ~~$\alpha \leq 0.05$~~ .

408 This is in contrast with the study on the antioxidant activity of DPPH and FRAP of  
409 matcha. The longer storage period reduces the levels of catechin content due to the  
410 catechins, such as epigallocatechin gallate~~e (EGCG)~~, epicatechin gallate~~e (ECG)~~,

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: No underline

Formatted: Highlight

411 epigallocatechin (~~EGC~~), and epicatechin (~~EC~~), which are bioactive compounds that have  
412 high antioxidant activity (Kim *et al.* 2020). The ferric-reducing capability of *Pluchea* could  
413 have been due to the presence of simple phenolic acid that can transfer electrons from  
414 their free hydroxyl groups of samples. The FRAP of *Pluchea* infusion was strongly and  
415 positively significantly correlated with the DPPH, TPC, and TTC, but inversely to TFC.

#### 416 Antidiabetic Activity

417  ~~$\alpha$ Alpha~~-amylase enzyme inhibition activity (AA). Antidiabetic activity is a  
418 measure of the potency of phenolic compounds to regulate the uptake of glucose by the  
419 cells from the blood through the mediation of ~~two~~-digestive enzymes, *i.e.*  $\alpha$ -amylase  
420 and  $\alpha$ -glucosidase, which are involved in the control of dietary carbohydrate digestion and  
421 release in the postprandial blood glucose in human body (Fu *et al.* 2017). The phenolic  
422 compounds have the capability to bind with the protein component of  $\alpha$ -amylase and  $\alpha$ -  
423 glucosidase enzymes (Martinez-Solis *et al.* 2022), resulting in the reduced activity of the  
424 enzymes. The results showed that lower steeping *Pluchea* leaf infusion was able to inhibit  
425 the action of the  $\alpha$ -amylase enzymes (Figure 3a). The *Pluchea* infusion exhibited a good  
426  ~~$\alpha$ -amylase enzyme inhibition activity~~AA of more than 50-% and even almost 100-% in  
427 unstored *Pluchea* infusion steeped at 60, 70, and 80 °C, with the highest at 60 °C, and in  
428 stored *Pluchea* leaf infusion, which was steeped at 60 °C. The stored *Pluchea* leaf  
429 infusion steeped at 70, 80, and 95 °C for 5 min had lower enzyme inhibition activity of  
430 less than 50-%, with the lowest at 95 °C around 13-%. Widyawati *et al.* (2017) found that  
431 the ability to inhibit the  $\alpha$ -amylase enzyme in unstored *Pluchea* infusion steeped at 95 °C  
432 for 5 min was also low at 28.79-%. Increasing the steeping temperature and storage  
433 period reduced the ability of the phytochemicals in the *Pluchea* infusion to inhibit the  $\alpha$ -

Formatted: Highlight

Formatted: No underline

Formatted: No underline

Formatted: Highlight

Formatted: Highlight

Formatted: No underline

Formatted: Highlight

Formatted: No underline

Formatted: No underline

Formatted: No underline

Formatted: No underline

Formatted: Highlight

Formatted: No underline

Formatted: No underline

434 amylase enzyme activity period. **Table 2** further shows that the AA of *Pluchea* infusion  
435 was strongly and negatively significantly correlated with TPC, TTC, DPPH, and FRAP,  
436 but it was weakly and positively significantly correlated with TFC.

Formatted: Highlight

437 This inhibitory activity was thought to be contributed by other bioactive compounds,  
438 besides phenolics, which are sensitive to steeping temperature and storage period. **Li et**  
439 **al. (2018)** stated that there are flavonoid compounds that contribute to the ability to inhibit  
440 the  $\alpha$ -amylase enzyme. **Akah et al. (2011)** reported that phytochemical compounds, such  
441 as terpenoids, saponins, flavonoids, glycosides/carbohydrates, and alkaloids are good  
442 antidiabetic metabolites or  $\alpha$ -amylase enzyme activity inhibitors. **Sangeetha and**  
443 **Vedasree (2012)** explained that the ability of *Threspesia populnea* extract to inhibit the  $\alpha$ -  
444 amylase enzyme was determined ~~of-by~~ their phenolic compound content and protein.  
445 Moreover, the presence of the  $\alpha$ -amylase enzyme inhibitor in this extract may be  
446 proteinaceous or nonproteinaceous in nature. It was assumed that this enzyme inhibitory

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

447 activity in *Pluchea* infusion also was determined ~~with-by~~ their protein and polyphenolic  
448 content. **Alexandre et al. (2022)** also stated that phenolic acids have inhibition activity to  
449  $\alpha$ -amylase enzyme depending on their structures. There are C=C double bonds  
450 conjugated with a carbonyl group of phenolic structures that stabilize the binding forces  
451 to the active site of the  $\alpha$ -amylase. The hydroxyl groups can bind by non-covalent  
452 interaction (hydrogen bonding, cation-~~II#~~ interactions, salt bridge interactions, ionic  
453 interactions, or electrostatic forces) with amino acid residue at the active site in the  $\alpha$ -  
454 amylase enzyme. Elevated steeping temperatures and longer storage periods can easily  
455 cause the removal of the hydroxyl groups of phenolic compounds ~~that, which~~ can reduce

Formatted: No underline

Formatted: Highlight

456 their ability ~~of to~~ enzyme inhibition. The phenolic acids with a greater number of hydroxyl  
457 groups exhibits stronger capability to obstruct the  $\alpha$ -amylase enzyme.

458  ~~$\alpha$ -Alpha~~ glucosidase enzyme inhibition activity (GA).  ~~$\alpha$ -Alpha~~ glucosidase is  
459 an important enzyme in carbohydrate digestion, that catalysis the hydrolysis of 1,4- $\alpha$ -  
460 bonds of the unabsorbed oligo- and disaccharides, and converts them into  
461 monosaccharides (glucose), ~~thereby~~ resulting in hyperglycemia (Nurcholis *et al.* 2014;  
462 Proenca *et al.* 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -glucosidase  
463 enzyme is used to determine their antidiabetic activity. This is supported by Werdani and  
464 Widyawati (2018) stated that *Pluchea* infusion has the potential as an antidiabetic agent.  
465 Widyawati *et al.* (2020) found that the steeping of unstored *Pluchea* infusion at 95 °C for  
466 5 min has an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857%.

467 **Figure 3b** shows that the ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -  
468 glucosidase enzyme decreased with increasing steeping temperature and storage period.  
469 Steeping at 95 °C of the unstored *Pluchea* leaf infusion obtained the lowest inhibitory  
470 ability, *i.e.* 48.32  $\pm$  1.27%, and the highest inhibitory activity was at 70 °C at 95.11  $\pm$   
471 0.70%. The results of a paired t-test showed that GA of *Pluchea* infusion was significantly  
472 different between steeping temperature and long storage. **Figure 3** further shows that the  
473 ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher  
474 than the ability to inhibit the  $\alpha$ -amylase enzyme. Data analysis in **Table 2** showed that  
475 the TFC of the *Pluchea* leaf infusion was influenced weakly and positively by GA and AA,  
476 but the GA and AA were not affected by TPC, TTC, DPPH, and FRAP. Li *et al.* (2018)  
477 stated that flavonoid compounds can inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -  
478 glucosidase enzymes. Dias *et al.* (2021) stated that flavonoid compounds, such as rutin,

Formatted: Font: Not Bold

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: No underline

Formatted: Highlight

Formatted: No underline

Formatted: Highlight

Formatted: No underline

Formatted: No underline

Formatted: No underline

Formatted: Highlight

Formatted: No underline

Formatted: Highlight

Formatted: No underline

Formatted: Highlight

Formatted: Highlight

479 myricetin, kaempferol, and quercetin have antioxidant and antihyperglycemic activities.  
480 The ability to inhibit the action of enzymes from flavonoid compounds is determined by  
481 the position and number of hydroxyl groups, the number of double bonds in rings A and  
482 B, and the heterocyclic ring in ring C. Tadera *et al.* (2006) and Zhang *et al.* (2014) also  
483 explained that flavonoid compounds of samples significantly inhibit the  $\alpha$ -glucosidase  
484 enzyme activity.

485 The ability to inhibit the  $\alpha$ -glucosidase enzyme from *Pluchea* infusion was  
486 significantly affected by the steeping temperature and long storage. Figure 3 also showed  
487 that the capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater  
488 than the  $\alpha$ -amylase enzyme because the mechanism of the two enzymes was different,  
489 according to the opinion of McCue *et al.* (2005). The mechanism of the  $\alpha$ -glucosidase  
490 enzyme inhibitor includes making the sugar mimic structure, binding using ionic bonds  
491 with nucleophilic, making the transition state-like structure, binding hydrogen with catalytic  
492 acid residue, interacting ionic and hydrophobic with site other than the active site, and  
493 binding covalent with enzymes through an epoxy or aziridine group (Moorthy *et al.* 2012).  
494 Then, the mechanism of the  $\alpha$ -amylase enzyme inhibitor includes blocking carbohydrates,  
495 thereby limiting the digestibility and absorption of carbohydrates, and as well as blocking  
496 the active centers of several subsites of the enzyme (Gong *et al.* 2020).

497 Widyawati *et al.* (2017) stated that phenolic and non-phenolic compounds can  
498 inhibit of the  $\alpha$ -glucosidase enzyme activity. The ability of bound phenolic compounds to  
499 inhibit  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. The presence  
500 of polymerization and degradation reactions, that-which may be-occurred in *Pluchea*  
501 infusion during storage, affects the structure and profile of phenolic and non-phenolic

Formatted: Highlight

Formatted: Highlight

Formatted: No underline

Formatted: Highlight

Formatted: No underline

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

Formatted: No underline



502 compounds. **A. Siningtyas *et al.* (2014)** explained that the methyl-esterified quinic acid  
503 with the caffeic groups, such as 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid  
504 methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid,  
505 and 1,3,4,5-tetra-*O*-caffeoylquinic acid of *Pluchea* leaves inhibits the  $\alpha$ -glucosidase  
506 enzyme activity. The resulting analysis of caffeoylquinic acids (3,4-di-*O*-caffeoylquinic  
507 acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid in stored *Pluchea* leaf  
508 infusion higher concentration than in unstored *Pluchea* infusion, and the concentrations  
509 of the simple phenolic compounds were increased at higher steeping temperature, but  
510 the  ~~$\alpha$ -glucosidase inhibition activity~~GA of them was reduced. It means that the methyl-  
511 esterified quinic acid with the caffeic groups had more potential to inhibit  $\alpha$ -glucosidase  
512 enzyme than free caffeoylquinic acid.

513 This study showed that the increasing steeping temperature and storage period  
514 caused degradation of polyphenol compounds to produce simple phenolic compounds,  
515 such as gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,4-di-*O*-caffeoylquinic  
516 acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid that increased the ~~total~~  
517 ~~phenolic content~~TPC and ~~total tannin content~~TTC. The increase in the simple phenolic  
518 concentration of the *Pluchea* leaf infusion caused higher antioxidant activity and lower  
519 antidiabetic activity.

520

## 521 CONCLUSION

522 The ~~Total Phenol~~(TPC) of *Pluchea* infusion at different steeping temperatures and  
523 storage periods generally significantly increased with increasing steeping temperature  
524 and storage periods. Steeped and stored infusion had significantly higher amounts of

Formatted: Highlight

Formatted: No underline

Formatted: No underline

Formatted: No underline

Formatted: No underline

Formatted: No underline

525 phenolic compounds than the samples that were steeped and unsteeped. TPC was highest  
526 in the stored and steeped at 95 °C and lowest in the unsteeped and steeped at 60 °C.  
527 Unsteeped steeped samples exhibited significantly higher flavonoid content than the stored  
528 steeped samples. The highest ~~TFC~~total flavonoid content was exhibited by the unsteeped  
529 samples steeped at 95 °C. The ~~total tannin content~~TTC of *Pluchea* leaf infusion  
530 significantly increased with increasing steeping temperature and storage period. Among  
531 the unsteeped steeped samples, the tannin content was significantly the lowest in the  
532 samples steeped at 60 °C and the highest in the samples steeped at 95 °C.

Formatted: No underline

533 The ~~free radical scavenging property~~ (DPPH) of the stored and steeped *Pluchea*  
534 leaf infusion was significantly higher than the unsteeped steeped samples. The free radical  
535 scavenging property was highest in the stored samples steeped at 80 and 95 °C. The  
536 free radical scavenging activity of the samples was strongly and positively correlated with  
537 total phenolic and tannin contents, but inversely with total flavonoid levels. The ~~ferrie-~~  
538 ~~reducing antioxidant power~~ (FRAP) significantly increased with increasing steeping  
539 temperature and longer storage periods. The lowest FRAP was found in the unsteeped  
540 samples ~~which-that~~ were steeped at 60 °C, and the highest was exhibited in *Pluchea*  
541 ~~stored samples which-that~~ were stored for 5 yr and steeped at 95 °C. The FRAP of  
542 *Pluchea* leaf infusion was significantly strong and positively correlated with the free radical  
543 scavenging property, ~~total phenolic~~TPC, and ~~total tannin content~~TTC, but inversely with  
544 ~~TFC~~total flavonoid content. The ~~inhibition of the  $\alpha$ -amylase activity~~AA was generally found  
545 to be higher at lower steeping temperatures of the unsteeped *Pluchea* leaf infusion than at  
546 higher steeping temperatures of the stored sample. The  ~~$\alpha$ -amylase enzyme inhibition~~AA  
547 capacity of the *Pluchea* leaf infusion showed a significantly strong and negative

Formatted: No underline

Formatted: No underline

Formatted: No underline

Formatted: No underline

Formatted: No underline

548 correlation with TPC, TTC, DPPH, and FRAP, but it was weakly and positively correlated  
549 significantly with TFC.

550 The ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme  
551 decreased at high steeping temperatures and long storage periods. The highest inhibitory  
552 activity was obtained in the unsteeped *Pluchea* leaf infusion that was steeped at 70 °C,  
553 ~~while whereas~~ the lowest was obtained in the unsteeped sample that was steeped at 95  
554 °C. The ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be  
555 higher than the ability to inhibit the  $\alpha$ -amylase enzyme. The ~~inhibition of the  $\alpha$ -glucosidase  
556 enzyme activity~~GA was significantly strong and negative TPC, TTC, DPPH, and FRAP,  
557 and it was weakly and positively correlated significantly with TFC.

558 The simple phenolic compounds identified in *Pluchea* leaf infusion may affect the  
559 presence of the bioactive compounds, antioxidant potential, and antidiabetic properties  
560 at different steeping temperatures and storage periods ~~–~~ including gallic acids, (+)-  
561 catechins, myricetins, kaempferols, quercetins, 3,5-di-O-caffeoylquinic acids, 3,4-di-O-  
562 caffeoylquinic acids, ~~and~~ 4,5-di-O-caffeoylquinic acids.

563

#### 564 ACKNOWLEDGMENTS

565 The authors would like to thank the Ministry of Education and Culture of the  
566 Republic of Indonesia for a fundamental research grant to higher education institutions in  
567 2022.

568

#### 569 STATEMENT ON CONFLICT OF INTEREST

Formatted: No underline

Formatted: No underline

Formatted: No underline

Formatted: No underline

570 The authors declare no conflict of interest.

571

572 REFERENCES

573 ACAR A, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food  
574 wastes: evaluation of temperature and time effects on quality parameters. Applied  
575 Food Res 2(100087): 1–8.

Formatted: Highlight

Formatted: Highlight

576 ALEIXANDRE A *et al.* 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -  
577 glucosidase and influence of reaction conditions. Food Chem 372(131231): 1–9.

Formatted: Highlight

Formatted: Highlight

578 ALI *et al.* 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile.  
579 Food Chem 89: 27–36.

Formatted: Highlight

Formatted: Highlight

580 ALI A *et al.* 2018. Impact of storage conditions on the stability of predominant phenolic  
581 constituents and antioxidant activity of dried *Piper betle* extracts. Molecules 23(24):  
582 1–13.

Formatted: Highlight

Formatted: Highlight

583 AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and  
584 methanol extract and fractions of *Gongronema latifolium* (*Asclepidaceae*) leaves in  
585 alloxan diabetic rats. J Applied Pharm Sci 01(09): 99–102.

Formatted: Highlight

Formatted: Highlight

586 ARSININGTYAS IS *et al.* 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves  
587 of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural  
588 product use. Natural Product Res 28(17): 1350–1353.

Formatted: Highlight

Formatted: Highlight

589 ARYAL S *et al.* 2019. Total phenolic content, flavonoid content, and antioxidant of wild  
590 vegetables from Western Nepal. Plants 8(96): 1–12.

Formatted: Highlight

Formatted: Highlight

591 **AYELE** DT, **AKELE** ML, **MELESE** AT. **2022**. Analysis of total phenolic contents,  
592 flavonoids, antioxidant, and antibacterial activities of *Croton macrostachyus* root  
593 extracts. BMC Chem 16(30): 1–9.

Formatted: Highlight

Formatted: Highlight

594 **BALIYAN** S *et al.* **2022**. Determination of antioxidants by DPPH radical scavenging  
595 activity and quantitative phytochemical analysis of *Ficus religiosa*. Molecules  
596 27(1326): 1–19.

Formatted: Highlight

Formatted: Highlight

597 **CASTIGLIONI** S *et al.* **2015**. Influence of steeping conditions (time, temperature, and  
598 particle size) on antioxidant properties and sensory attributes of some white and  
599 green teas. Intern J Food Sci Nut Online. p. 1–7.

Formatted: Highlight

Formatted: Highlight

600 **CHAN** EWC *et al.* **2022**. *Pluchea indica*: an updated review of its botany, uses, bioactive  
601 compounds, and pharmacological properties. Pharmaceutical Sciences Asia 49(1):  
602 77–85.

Formatted: Highlight

Formatted: Highlight

603 **CHANDRA** S *et al.* **2014**. Assessment of total phenolic and flavonoid content, antioxidant  
604 properties, and yield of aeroponically and conventionally grown leafy vegetables and  
605 fruit crops: a comparative study. Hindawi Publishing Corporation Evidence-based  
606 Complementary Alter Med. p. 1–9.

Formatted: Highlight

Formatted: Highlight

607 **CHANDRAN** CI K, **INDIRA** G. **2016**. Quantitative estimation of total phenolic, flavonoids,  
608 tannin, and chlorophyll content of leaves of *Strobilanthes kunthiana* (neelakurinji). J  
609 Med Plants Studies 4(4): 282–286.

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

610 **DIAS** MC, **PINTO** DCGA, **SILVA** AMS. **2021**. Plant flavonoids: chemical characteristics  
611 and biological activity. Molecules 26(5377): 1–16.

Formatted: Highlight

Formatted: Highlight

612 DOBRINAS S, SOCEANU A, POPESCU V, POPOVICI IC, JITARIU D. 2021.

Formatted: Highlight

Formatted: Highlight

613 Relationship between total phenolic content, antioxidant capacity, and Fe and Cu  
614 content from tea plant samples at different brewing times. Processes 9(1311): 1–11.

615 FERRARIS Z. 2023. *Pluchea indica* Less. iNaturalist.org.

Formatted: Highlight

Formatted: Highlight

616 <https://www.gbif.org/occurrence/4022273660>

617 FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional

Formatted: Highlight

Formatted: Highlight

618 properties and visual appearance of Dampit Robusta coffee leaves tea. Advances in  
619 Food Sci Sustain Agric Agro-industrial Engineering 4(1): 25–32.

620 FU L *et al.* 2017. Total phenolic contents and antioxidant capacities of herbal and tea

Formatted: Highlight

Formatted: Highlight

621 infusions. Intern J Molecular Sci 12: 2112–2124.

622 GAO MR *et al.* 2019. A theoretical and experimental study: the influence of different

Formatted: Highlight

Formatted: Highlight

623 standards on the determination of total phenol content in the Folin-Ciocalteu assay.

624 J Food Measurement and Characteristic 13: 1349–1356.

625 GONG L, FENG D, WANG T, REN Y, LIU Y, WANG J. 2020. Inhibitors of  $\alpha$ -amylase and

Formatted: Highlight

Formatted: Highlight

626  $\alpha$ -glucosidase: potential linkage for whole cereal foods on prevention of  
627 hyperglycemia. Food Sci Nut 2020(8): 6320–6337.

628 HUANG D, OU B, PRIOR RL. 2005. The chemistry behind antioxidant capacity assays.

Formatted: Highlight

Formatted: Highlight

629 J Agric Food Chem 53: 1841–1856.

630 ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties

Formatted: Highlight

Formatted: Highlight

631 of rosehip tea beverage: study by response surface methodology. J Food Sci Tech

Formatted: Highlight

632 54(11): 3737–3743.

633 JAYANI NIE *et al.* 2022. Evaluation of stability and quality characteristics of moringa

Formatted: Highlight

Formatted: Highlight

634 (*Moringa oleifera*) herbal tea during storage. Food Res 6(4): 399–406.

635 **KIM JM et al. 2020**. Effect of storage temperature on the antioxidant activity and catechins  
636 stability of matcha (*Camellia sinensis*). Food Science and Biotechnology 29(9): 1261–  
637 1271.

Formatted: Highlight

Formatted: Highlight

638 **KILIC et al. 2017**. Antioxidant properties of some herbal teas (green tea, senna, corn silk,  
639 ~~rosemary~~[rosemary](#)) brewed at different temperatures. Intern J Secondary Metabolite  
640 4(3): 142–248.

Formatted: Highlight

Formatted: Highlight

641 **KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018**. Optimization of  
642 extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica*  
643 leaves from different provenances in Thailand. Revista Brasileira de Farmacognosia  
644 28: 145–150.

Formatted: Highlight

Formatted: Highlight

645 **KOWALSKA J et al. 2021**. Influence of tea brewing parameters on the antioxidant  
646 potential of infusions and extracts depending on the degree of processing of the  
647 leaves of *Camellia sinensis*. Molecules 26(4773): 1–17.

Formatted: Highlight

Formatted: Highlight

648 **KRUK J, ABOUL-ENAIN BH, DUCHNIK E, MARCHLEWICZ M. 2022**. Antioxidative  
649 properties of phenolic compounds and their effect on oxidative stress induced by  
650 severe physical exercise. J Physiol Sci 72(19): 1–24.

Formatted: Highlight

Formatted: Highlight

651 **RUMAN N, ROSA N. 2019**. Phenolic acids: natural versatile molecules with promising  
652 [therapeutic applications](#). Biotechnology Reports 24: e00370.

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

653 **L K et al. 2018**. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the flavonoids  
654 rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity  
655 relationship of its eight flavonoids by a refined assign score method. Chem Central J  
656 12(82): 1–11.

Formatted: Highlight

Formatted: Highlight

657 LIN YS. *et al.* 2020. Effects of storage time and temperature on antioxidants in juice from  
658 *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser. *Molecules*  
659 25(3614): 1–12.

Formatted: Highlight

Formatted: Highlight

660 MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content  
661 and antioxidant activity test of avocado seed extract (*Persea americana* Mill). *Jurnal*  
662 *MIPA Unsrat Online* 1(1): 5–10 [in Bahasa Indonesia].

Formatted: Highlight

Formatted: Highlight

663 MARTINEZ-SOLIS J *et al.* 2022. Antidiabetic and toxicological effect of the tea infusion  
664 of summer collection from *Annona cherimola* Miller leaves. *Plants* 11(3224): 1–17.

Formatted: Highlight

Formatted: Highlight

Formatted: Font: Not Italic

665 MATHUR R, VIJAYVERGIA R. 2017. Determination of total flavonoid and phenol content  
666 in *Mimusops elengi* Linn. *Intern J Pharm Sci Res* 8(12): 5282–5285.

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

667 MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of  
668 sprouted and solid-state bioprocessed soybean. *Asia Pacific J Clinical Nut* 14(2):  
669 145–152.

Formatted: Highlight

Formatted: Highlight

670 MOORTHY NS, RAMOS MJ, A FERNANDES P. 2012. Studies on  $\alpha$ -glucosidase  
671 inhibitors development: magic molecules for the treatment of carbohydrate mediated  
672 diseases. *Mini Rev Med Chem* 12: 713–720.

Formatted: Highlight

Formatted: Highlight

673 MORAES DE SOUZA RA, OLDONI TLC, REGITANO-D'ARCE MAB, ALENCAR SM.  
674 2008. Antioxidant activity and phenolic composition of herbal infusions consumed in  
675 Brazil. *Cienc Tecnol Aliment* 6(1): 41–47.

Formatted: Highlight

Formatted: Highlight

676 MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant  
677 activity: a Review. *Intern J Molecular Sci* 22(3380): 1–30.

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

678 NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus*  
679 *sabdariffa* L.): effect of dying temperature and brewing conditions on total soluble

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight



680 solid, phenolic content, antioxidant capacity, and sensory quality. *Beverages* 6(2): 1–  
681 11.

682 **NOREEN H *et al.* 2017.** Measurement of total phenolic content and antioxidant activity of  
683 aerial parts of medicinal plant *Coronopus didymus*. *Asian Pacific J Tropical Med*  
684 10(8): 792–801.

Formatted: Highlight

Formatted: Highlight

685 **NURCHOLIS GW *et al.* 2014.** Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity *in*  
686 *vitro*, and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum*  
687 *pictum* (L.). *Current Biochem* 1(2): 58–65.

Formatted: Highlight

Formatted: Highlight

688 **OH J *et al.* 2013.** Antioxidant and antimicrobial activities of various leafy herbal teas. *Food*  
689 *Control* 31: 403–409.

Formatted: Highlight

Formatted: Highlight

690 **PROENCA C *et al.* 2017.**  $\alpha$ -Glucosidase inhibition by flavonoids: an *in vitro* and *in silico*  
691 structure–activity relationship study. *J Enzyme Inhibition Med Chem* 32(1): 1216–  
692 1228.

Formatted: Highlight

Formatted: Highlight

693 **PUREWAL SS *et al.* 2022.** Unraveling the effect of storage duration on antioxidant  
694 properties, physicochemical and sensorial parameters of ready to serve Kinnow-Amla  
695 beverages. *Applied Food Res* 2(1): 1–8.

Formatted: Highlight

Formatted: Highlight

696 **RAHARJO D, HARYOTO. 2019.** Antioxidant activity of mangrove *Sonneratia caseolaris*  
697 L. using the FRAP method. *ISETH*. p. 623–629.

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

698 **RAMPHINWA ML *et al.* 2023.** Eco-physiological response of secondary metabolites of  
699 teas: review of quality attributes of herbal tea. *Frontiers in Sustain Food Systems*  
700 7(990334): 1–14.

Formatted: Highlight

Formatted: Highlight

701 **REBLOVA Z. 2012.** Effect of temperature on the antioxidant activity of phenolic acids.  
702 *Czech J Food Sci* 30(2): 171–177.

Formatted: Highlight

Formatted: Highlight

703 **RUAN J et al. 2019.** Comprehensive chemical profiling in the ethanol extract of *Pluchea*  
704 *indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica  
705 gel column chromatography fractions. *Molecules* 24(2784): 1–20.

Formatted: Highlight

Formatted: Highlight

706 **RUSITA YD et al. 2019.** Comparison of tannin concentration on fresh and dried tea leaf  
707 (*Camelia sinensis* L.) extract using UV-visible spectrophotometric method. 1st  
708 International Conference of Health, Science, & Technology (ICOHETECH) 2019 ;16  
709 Nov 2019; LPPM Universitas, Duta Bangsa Surakarta, Surakarta.

Formatted: Highlight

Formatted: Highlight

710 **SANGEETHA R, VEDASREE N. 2012.** *In vitro*  $\alpha$ -amylase inhibitory activity of the leaves  
711 of *Thespesia populnea*. Intern Scholarly Res Network. p. 1–4.

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

712 **SHRAIM AM et al. 2021.** Determination of total flavonoid content by aluminum chloride  
713 assay: a critical evaluation. *LWT Food Sci Tech* 150(111932): 1–11.

Formatted: Highlight

Formatted: Highlight

714 **SILVA-RAMIREZ KA et al. 2020.** Effect of drying and steeping temperature on the  
715 phenolic content, antioxidant activity, aromatic compounds, and sensory properties  
716 of *Culila polyantha* Benth. *Infusions Processes* 8(1378): 2–20.

Formatted: Highlight

Formatted: Highlight

717 **SRISOOK K et al. 2012.** Antioxidant and anti-inflammatory activities of hot water extract  
718 from *Pluchea indica* Less. herbal tea. *J Med Plants Res* 6(23): 4077–4081.

Formatted: Highlight

Formatted: Highlight

719 **SURIYAPHAN O. 2014.** Nutrition, health benefits, and applications of *Pluchea indica* (L)  
720 Less leaves. *Pharm Sci Asia* 41: 1–10.

Formatted: Highlight

Formatted: Highlight

721 **SU D et al. 2019.** Impact of thermal processing and storage temperature on phenolic  
722 profile and antioxidant activity of different varieties of lychee juice. *LWT–Food Tech*  
723 116(108578): 1–9.

Formatted: Highlight

Formatted: Highlight

724 **TADERA K, MINAMI Y, TAKAMATSU K, MATSUOKA T. 2006.** Inhibition of alpha-  
725 glucosidase and alpha-amylase by flavonoids. *J Nutr Sci Vitaminol* 52: 149–153.

Formatted: Highlight

Formatted: Highlight

726 **THANAJIRUSCHAYA P et al. 2010.** Effect of storage period and temperature on  
727 antioxidant components and properties of milled rice. *Khon Kaen Univ Res J* 15(9):  
728 843–851.

Formatted: Highlight

Formatted: Highlight

729 **VONGSAK B et al. 2018.** Comparison of active constituents, antioxidant capacity, and  
730 αglucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages.  
731 *Food Biosci* 25: 68–73.

Formatted: Highlight

Formatted: Highlight

732 **WANG SC et al. 2022.** Effect of brewing conditions on polyphenols in the dark tea  
733 (*Camellia sinensis* L.) infusions: content, composition, and antioxidant activities. *Food*  
734 *Sci Tech* 42(e36322): 1–9.

Formatted: Highlight

Formatted: Highlight

735 **WERDANI YDW, WIDYAWATI PS. 2018.** Antidiabetic effect on tea of *Pluchea indica*  
736 Less as functional beverage in diabetic patients. *ASSEHR* 98: 164–167.

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

737 **WIDYAWATI PS et al. 2014.** Potency of beluntas (*Pluchea indica* Less) leaves extract as  
738 antioxidant and anti-warmed-over flavor (WOF) of duck meat. International Congress  
739 on Challenges of Biotechnological Research in Food and Health; 15 Nov 2014;  
740 Agricultural Technology Faculty, Slamet Riyadi University, Surakarta, Indonesia.

Formatted: Highlight

Formatted: Highlight

741 **WIDYAWATI PS et al. 2016.** The physicochemical and antioxidant properties of *Pluchea*  
742 *indica* Less drink in tea bag packaging. *IJFNS* 5: 13–120.

Formatted: Highlight

Formatted: Highlight

743 **WIDYAWATI PS et al. 2017.** *In vitro* antioxidant capacities and antidiabetic properties of  
744 *Pluchea* leaves. *IJPPS* 9(8): 203–208.

Formatted: Highlight

Formatted: Highlight

745 **WIDYAWATI PS et al. 2020.** Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica*  
746 Less beverage. *CJFST* 12(4): 125–139.

Formatted: Highlight

Formatted: Highlight

747 **WIDYAWATI PS *et al.* 2022.** The effect of  $\kappa$ -carrageenan proportion and hot water  
748 extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of  
749 stink lily (*Amorphophallus muelleri*) wet noodles. *Molecules* 27(5062): 1–16.

Formatted: Highlight

Formatted: Highlight

750 **XU P, CHEN L, WANG Y. 2019.** Effect of storage period on antioxidant activity and  
751 inhibition on  $\alpha$ -amylase and  $\alpha$ -glucosidase of white tea. *Food Sci Nut* 7(2): 636–644.

Formatted: Highlight

Formatted: Highlight

752 **ZARWINDA, SARTIKA D. 2019.** Effect of temperature and extraction period on caffeine  
753 in coffee. *Lantanida J* 6(2): 103–202. [in Bahasa Indonesia].

Formatted: Highlight

Formatted: Highlight

Formatted: Highlight

754 **ZHANG H, WANG G, DONG J. 2014.** Inhibitory properties of aqueous ethanol extracts of  
755 propolis on alpha-glucosidase. *J Evid Based Complement Alternat Med.* p. 1–8.

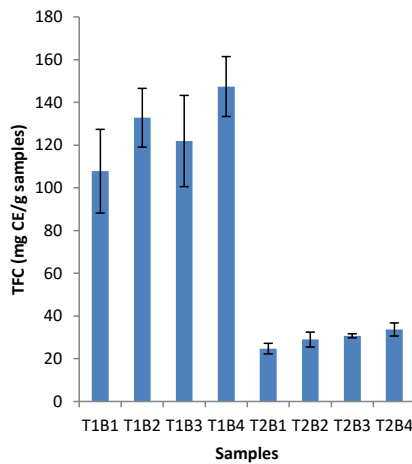
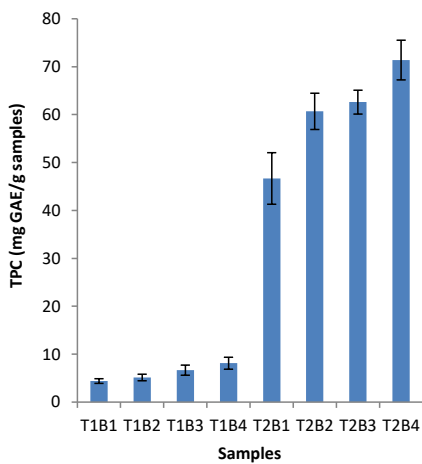
Formatted: Highlight

Formatted: Highlight

756 **ZHANG Y *et al.* 2021.** Effect of storage conditions and period on the polyphenol content  
757 of wheat flours. *Processes* 9(248): 1–11.

Formatted: Highlight

Formatted: Highlight

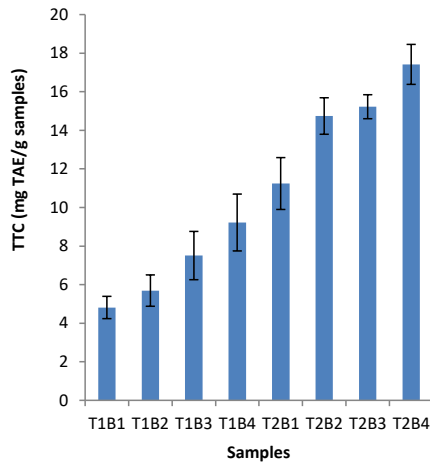


758

[a]

[b]

759



[c]

**Figure 1.** Bioactive compound contents of *Pluchea* infusion at different steeping temperatures and storage periods: [a] total phenolic content, [b] total flavonoid content, and [c] total tannin content. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  $\pm$  standard deviation ( $n = 6$ ). Samples: T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T2B2-steeped at 70 °C, stored for 5 yr; T2B3-steeped at 80 °C, stored for 5 yr; T2B4-steeped at 95 °C, stored for 5 yr.

Formatted: Highlight

**Table 1.** Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperatures and storage periods.

Formatted: Highlight

Phenolic compounds	Steeping temperature (°C)	Mean ± SD (unstored)	Mean ± SD (stored)	Mean difference ±SD	Sig. (two-tailed)
Gallic acid (µg/g samples)	60	0.2132 ± 0.0027	0.2364 ± 0.0015	0.0375 ± 0.0175	0.2030
	70	0.2157 ± 0.0013	0.2324 ± 0.0214	0.0167 ± 0.0227	0.4870
	80	0.2234 ± 0.0122	0.2347 ± 0.0078	0.0386 ± 0.0264	0.2870
	95	0.2316 ± 0.0104	0.2402 ± 0.0169	0.0086 ± 0.1990	0.8500
(+)-Catechin (µg/g samples)	60	0.3425 ± 0.0110	0.5085 ± 0.0111	-0.1576 ± 0.0885	0.241
	70	0.3260 ± 0.0265	0.5448 ± 0.0006	-0.2188 ± 0.0259	0.053
	80	0.3240 ± 0.0222	0.5023 ± 0.0773	-0.1451 ± 0.0248	0.077
	95	0.4039 ± 0.0320	0.5995 ± 0.0372	-0.2049 ± 0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756 ± 0.1234	1.4762 ± 0.0271	-1.2887 ± 0.3222	0.111
	70	0.2587 ± 0.0160	1.4245 ± 0.2526	-1.1657 ± 0.2695	0.103
	80	0.4175 ± 0.0104	1.4570 ± 0.0925	-1.0391 ± 0.0841	0.036*
	95	0.8786 ± 0.0434	2.6138 ± 0.0695	-1.1735 ± 0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220 ± 0.0268	0.6220 ± 0.0706	-0.5999 ± 0.9733	0.544
	70	0.1530 ± 0.0511	1.0708 ± 0.0289	-0.9177 ± 0.0222	0.011*
	80	0.3666 ± 0.0103	0.8629 ± 0.0815	-0.1082 ± 0.4462	0.790
	95	0.6559 ± 0.0570	2.0230 ± 0.0573	-1.4123 ± 0.3203	0.101

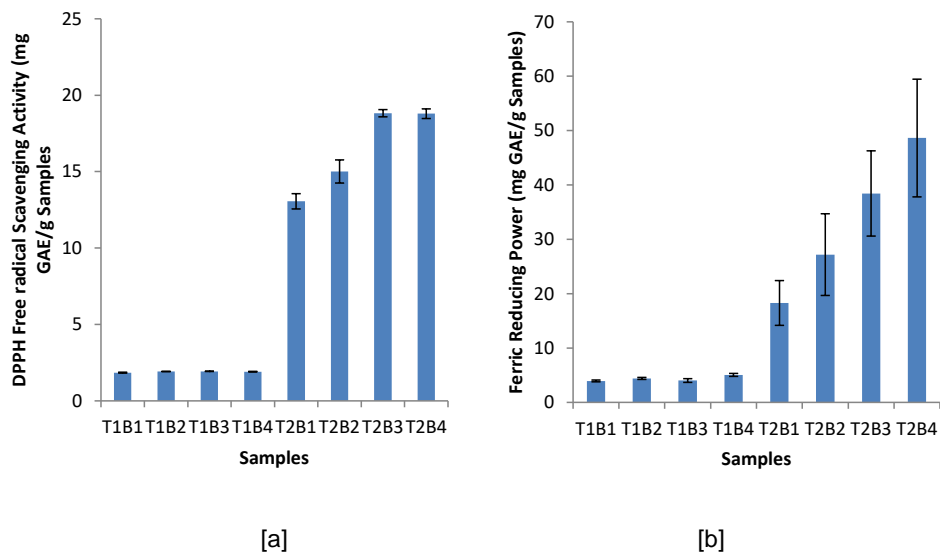
\*Corresponding author: paini@ukwms.ac.id

Widyawati *et al.*: Effect of Steeping Temperature and Storage Period from Powdered *Pluchea indica* Less  
 Date Received: 05 May 2023

Kaempferol (µg/g samples)	60	0.1394 ± 0.0202	0.3675 ± 0.0183	-0.3207 ± 0.1122	0.154
	70	0.0514 ± 0.0037	0.3726 ± 0.0944	0.3213 ± 0.0907	0.125
	80	0.3699 ± 0.0924	0.7966 ± 0.0366	-0.4267 ± 0.2727	0.271
	95	0.5913 ± 0.0239	0.9478 ± 0.0287	-0.3565 ± 0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103 ± 0.0628	2.4863 ± 0.0270	-1.8760 ± 0.2074	0.050*
	70	0.6271 ± 0.0099	2.3403 ± 0.0325	-1.7131 ± 0.3152	0.082
	80	0.7967 ± 0.03060	2.6278 ± 0.0211	-1.8311 ± 0.0095	0.002*
	95	1.5386 ± 0.0668	4.0211 ± 0.0851	-2.4825 ± 0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635 ± 0.0628	0.9449 ± 0.0501	-0.2814 ± 0.4458	0.536
	70	0.6162 ± 0.0099	0.9485 ± 0.0794	-0.3323 ± 0.0301	0.041*
	80	0.6601 ± 0.0306	0.9099 ± 0.0387	-0.2498 ± 0.3127	0.461
	95	0.6642 ± 0.0668	1.3156 ± 0.0166	-0.6514 ± 0.2666	0.179
4,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.4906 ± 0.0060	1.1842 ± 0.0120	-0.6886 ± 0.2723	0.018*
	70	0.4807 ± 0.0034	1.0089 ± 0.0736	-0.5281 ± 0.0702	0.060
	80	0.5299 ± 0.0053	1.2382 ± 0.1435	-0.7082 ± 0.1489	0.094
	95	1.0018 ± 0.0526	1.3797 ± 0.2170	-0.3086 ± 0.3086	0.333

772 Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean ± standard deviation (n  
 773 = 6). Samples: T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C,  
 774 unstored; T2B1-steeped at 60 °C, stored for 5 yr; T3B2-steeped at 70 °C, stored for 5 yr; T3B3-steeped at 80 °C, stored for 5 yr; T3B4-steeped at  
 775 95 °C, stored for 5 yr.

\*Corresponding author: paini@ukwms.ac.id



776

777

778

779

780

781

782

783

784

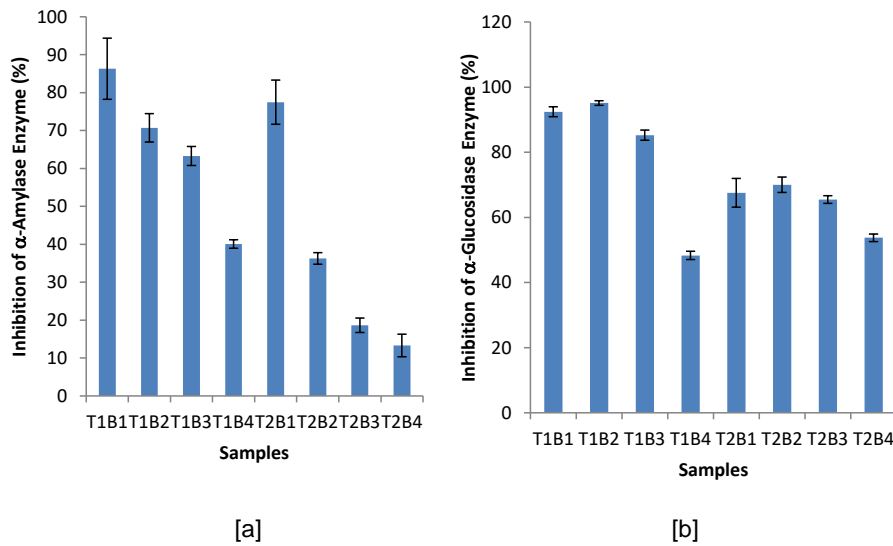
785

**Figure 2.** Antioxidant activity of *Pluchea* tea at different steeping temperatures and storage periods: [a] DPPH; [b] FRAP. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  $\pm$  standard deviation ( $n = 6$ ). Samples : T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T2B2-steeped at 70 °C, stored for 5 yr; T2B3-steeped at 80 °C, stored for 5 yr; T2B4-steeped at 95 °C, stored for 5 yr.

Formatted: Highlight

Formatted: Font: Italic





786

787

788 **Figure 3.** Antidiabetic activity of pluchea tea at different steeping temperatures and

789 storage periods: [a]  $\alpha$ -amylase; [b]  $\alpha$ -glucosidase. Data analysis using ANOVA

790 at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were

791 expressed as mean  $\pm$  standard deviation ( $n = 6$ ). Samples: T1B1-steeped at 60

792  $^{\circ}\text{C}$ , unstored; T1B2-steeped at 70  $^{\circ}\text{C}$ , unstored; T1B3-steeped at 80  $^{\circ}\text{C}$ ,

793 unstored; T1B4-steeped at 95  $^{\circ}\text{C}$ , unstored; T2B1-steeped at 60  $^{\circ}\text{C}$ , stored for

794 5 yr; T2B2-steeped at 70  $^{\circ}\text{C}$ , stored for 5 yr; T2B3-steeped at 80  $^{\circ}\text{C}$ , stored for

795 5 yr; T2B4-steeped at 95  $^{\circ}\text{C}$ , stored for 5 yr.

Formatted: Highlight

796 **Table 2.** Pearson correlation coefficients between bioactive contents (TPC, TFC, and TAC), antioxidant activity (DPPH and  
797 FRAP), and antidiabetic activity (AA and GA).

Formatted: Highlight

	TPC	TFC	TTC	DPPH	FRAP	$\alpha$ -Glucosidase	$\alpha$ -Amylase
TPC	1						
TFC	-0.93589	1					
TTC	0.960028	-0.81321	1				
DPPH	0.992776	-0.93992	0.942273	1			
FRAP	0.953366	-0.82636	0.947778	0.956242	1		
$\alpha$ -Glucosidase	-0.55512	0.349873	-0.71534	-0.5272	-0.55947	1	
$\alpha$ -Amylase	-0.70842	0.429393	-0.8569	-0.69579	-0.80548	0.725161631	1

798 Significant at the 0.05 level (two-tailed)

7. Gallery Proofreading (19-6-2024)
  - Correspondence
  - Document



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**First Draft of PJS Article Ms 23-158**

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Wed, Jun 19, 2024 at 7:10 AM

Dear Dr. Widyawati,

Greetings! Attached below is the first draft of your article titled "Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea indica* Less Tea" [Ms 23-158] accepted for publication in the Philippine Journal of Science.

Kindly review this copy and, should you have no further corrections, provide us with your approval for publication.

We hope to hear from you on the matter within 48 hours of receipt of this letter. We shall consider this version of your paper as the galley proof for final editorial processing in the absence of a response within the cut-off time.

Thank you very much!

Sincerely,  
Mr. ALLYSTER A. ENDOZO  
Managing Editor



**Philippine Journal of Science**  
**Science and Technology Information Institute**  
**Department of Science and Technology**  
**DOST Complex, Gen. Santos Ave., Bicutan 1631**  
**Taguig City, Metro Manila, Philippines**  
Telephone no. : 837 - 2191  
Email: [pjs@stii.dost.gov.ph](mailto:pjs@stii.dost.gov.ph); [philjournsci@gmail.com](mailto:philjournsci@gmail.com)  
Website: <https://philjournalsci.dost.gov.ph>  
Scopus: <https://www.scopus.com/sourceid/19700175735>

---

 **[PRO] 23-158 - Widyawati and Wilianto - Article (1st Draft) (18 Jun 2024).pdf**  
1714K

## Effect of Steeping Temperature and Storage Period on the Bioactive Compounds plus Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea indica* Less

Paini Sri Widyawati<sup>1\*</sup> and Yufita Ratnasari Wilianto<sup>2</sup>

<sup>1</sup>Food Technology Study Program, Agricultural Technology Faculty,  
Widya Mandala Surabaya Catholic University, Surabaya 60265 Indonesia

<sup>2</sup>Pharmacy Study Program, Pharmacy Faculty,  
Widya Mandala Surabaya Catholic University, Surabaya 60272 Indonesia

This study was done to determine the effects of steeping temperature and storage period on the bioactive contents plus antioxidant and antidiabetic activities of *Pluchea* leaf infusion. The research used a randomized block design with two factors, *i.e.* steeping temperature (T) and storage period (B). The *Pluchea* leaf blades were exposed to four steeping temperatures of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) °C with the storage period of 0 (B1) and 5 (B2) yr – resulting in eight treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). Statistical analysis using a paired t-test at  $\alpha \leq 0.05$  showed that treatments significantly affected the bioactive contents [total phenol (TPC), total tannin (TTC), and total flavonoid (TFC)], antioxidant [DPPH scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)] potential and antidiabetic [ $\alpha$ -amylase (AA) and  $\alpha$ -glucosidase (GA) inhibition] properties of the *Pluchea* leaf infusion. TPC, TTC, DPPH, and FRAP significantly increased for the storage period and the steeping temperatures. Then, TFC decreased during the storage period but significantly increased at higher steeping temperatures. The AA and GA of *Pluchea* leaf infusion increased until 70 °C of the steeping temperature but decreased until 95 °C. The DPPH and FRAP of the *Pluchea* leaf infusion were strongly and positively correlated with TPC and TTC. The GA and AA of *Pluchea* leaf infusion were not influenced by the TPC and TTC but were weakly and positively correlated with TFC. The antioxidant activity of the *Pluchea* leaf infusion was inversely proportional to the antidiabetic activity. The simple phenolic compounds derived from *Pluchea* leaf infusion at different steeping temperatures and storage included gallic acid, kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid.

Keywords: antioxidant, antidiabetic, bioactive compound, *Pluchea indica* Less, steeping temperature, storage period

\*Corresponding author: paini@ukwms.ac.id

## INTRODUCTION

*Pluchea* herbal tea is a product of dried *Pluchea* leaf processing introduced by world people (Srisook *et al.* 2012; Widyawati *et al.* 2016) because of the efficacy of the active components in *Pluchea* leaves, as a herbal plant that has been widely used for traditional medicine and food (Chan *et al.* 2022). *Pluchea* leaves are composed of many nutrients and bioactive compounds useful to body health. The nutrient compositions in the *Pluchea* leaves include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates, calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive compounds are comprised, *i.e.* chlorogenic acid, caffeic acid, 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, quercetin, myricetin, kaempferol, total anthocyanin,  $\beta$ -carotene, and total carotenoid (Suriyaphan 2014; Vongsak *et al.* 2018; Ruan *et al.* 2019; Widyawati *et al.* 2022; Chan *et al.* 2022).

The steeping process of *Pluchea* leaves can be performed with fresh or dry leaves in hot or boiling water for a few min (Suriyaphan 2014; Silva-Ramirez *et al.* 2020; Jayani *et al.* 2022). In Asia, especially in Indonesia, people usually consume the *Pluchea* infusion by steeping 2 g of powdered *Pluchea* leaves in a tea bag in 100 mL of hot or boiling water. Widyawati *et al.* (2016) claimed that steeping 2 g of *Pluchea* leaf powder at 95 °C for 5 min exhibits total phenolic and flavonoid contents, the ability to scavenge DPPH free radicals, and the capability to reduce ferric ions at 9.3, 22.0, 27.2, and 10.2 mg gallic acid equivalent (GAE)/ g sample, respectively. Werdani and Widyawati (2018) reported that drinking *Pluchea* leaf powder infusion in the morning and evening regularly (2 g/ 100 mL) can decline blood sugar levels.

The steeping of *Pluchea* herbal tea with hot water at 95 °C for 5 min certainly determines the stability and amount of extracted bioactive compounds that influence the biological activity especially antioxidant and antidiabetic activities. Silva-Ramirez *et al.* (2020) reported that the infusion process can influence the content and composition of the bioactive compounds and antioxidant activity of tea. Acar *et al.* (2022) stated that the infusion quality of herbal tea extract depends on a number of factors, *i.e.* storage and temperature. The polyphenol profile and antioxidant properties of herbal tea infusion decline with an increase in steeping or brewing and storage temperatures, as well as longer exposure periods.

Several studies have mentioned the effect of steeping temperature on the bioactive compound contents and antioxidant activity, as some white and green teas are effective with hot water at 90 °C for 7 min (Castiglioni *et al.* 2015), on rosehip tea is effective at infusion period

around 6–8 min at temperatures of 84–86 °C (Ilyasoglu and Arpa 2017), on the caffeine content extracted at the brewing temperature of coffee (Zarwinda and Sartika 2018), and the high total phenol content and antioxidant activity of dark tea at 92 °C for 27 min (Wang *et al.* 2022). The study of the effect of steeping temperature on *Pluchea* infusion was carried out to afford information about the most efficient preparation of powdered *Pluchea* leaves to get higher bioactive compounds, antioxidant, and antidiabetic activities.

Storage period tea usually for several months to yr *Pluchea* herbal tea also affects the levels of the bioactive compounds and biological activity (Jayani *et al.* 2022). Tea or herbal tea is generally stored at ambient temperature and packed in a tea bag or aluminum foil standing pouch or a combination of both. Many researchers reported that the storage period decreases the bioactive compounds plus antioxidant and antidiabetic activities, *i.e.* juice from *Momordica charantia* L. (Lin *et al.* 2020), dried *Piper betle* extracts (Ali *et al.* 2018), white tea (Xu *et al.* 2019), Kinnow-Amla beverages (Purewal *et al.* 2022), and whole-wheat flour (Zhang *et al.* 2021).

Therefore, this research studied the effect of steeping temperature and storage period on the bioactive compounds [total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC)], antioxidant [DPPH free radical scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)], and antidiabetic activities [ $\alpha$ -amylase (AA) and  $\alpha$ -glycosidase (GA) inhibition] of the infusion from powdered *Pluchea* leaves and on the phenolic compound profile.

## MATERIALS AND METHODS

### Raw Materials and Preparation

The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya, East Java, Indonesia. The *Pluchea* plants were included in the Asteraceae family with specifications according to the GBIF taxon ID number database:3132728 (Ferraris 2023). *Pluchea* leaves at 1–6 levels of each branch from the shoot were collected, sorted, washed, and dried to get a moisture content of around  $11.16 \pm 0.09\%$  dry basis (Widyawati *et al.* 2022). The dried *Pluchea* leaves were pulverized to a 45-mesh size powder. The *Pluchea* leaf powder was dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 °C for 10 min to reduce microbial organisms. Then, 2 g of the powder was packed into a paper filter infusion bag. Packed samples were stored for 0 (unstored) and 5 (stored) yr in a standing pouch before analysis.

In the research, one tea bag of *Pluchea* herbal tea that was stored for 0 (B1) and 5 (B2) yr was steeped with 100-mL hot water at various temperatures – including 60 (T1), 70 (T2), 80 (T3), and 95 (T4) °C for 5 min – with infusion method obtaining eight treatment combinations – namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, and T4B2. After the temperature of *Pluchea* infusion similar to ambient temperature was analyzed further.

### Reagents

The reagents used in the analysis include 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid,  $\alpha$ -amylase,  $\alpha$ -glucosidase, p-nitrophenyl- $\alpha$ -glucopyranoside (pNPG), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and (+)-catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, Folin-Ciocalteu's phenol, sodium nitric, aluminum chloride, ferric chloride, sodium dihydrogen phosphate, sodium phosphate, potassium ferricyanide, starch, acetic acid, and sodium hydroxide were purchased from Merck (Kenilworth, NJ, USA). All reagents used were of analytical grade except for distilled water which was purchased from PT Aqua Industry Surabaya.

### Analysis of the Bioactive Compounds

**Total phenolic content (TPC) analysis.** The TPC of treated *Pluchea* infusion was carried out using the technique by Gao *et al.* (2019). About 10  $\mu$ L *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10% were mixed in 10-mL volumetric flask and incubated for 5 min. Then, 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5% was added and filled up to 10 mL volume with distilled water. The blue color intensity of the solution was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at  $\lambda = 760$  nm, with gallic acid as the reference standard. The TPC was calculated using the following formula:  $y = 0.00009x + 0.008$ , with  $R^2 = 0.9941$ . The results were expressed as mg GAE/g samples.

**Total flavonoid content (TFC) assay.** The TFC of the samples was measured based on the reaction between AlCl<sub>3</sub> and NaNO<sub>2</sub> with the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim *et al.* 2021). The reaction between AlCl<sub>3</sub> and flavonoid compounds resulted in a yellow solution. About 30- $\mu$ L *Pluchea* infusion was mixed with 0.3 mL NaNO<sub>2</sub> 5% in 10-mL volumetric flask and incubated for 5 min. The mixture was added with 0.3 mL AlCl<sub>3</sub> 10% for 5 min. Then, 2-mL NaOH 1 M and distilled water were added to a 10-mL volume. Then, the red solution was produced after NaOH solution addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda = 510$  nm, with (+)-catechin as

the reference standard compound, and the results were expressed as mg catechin equivalents (CE)/ g samples using the following formula:  $y = 0.00008x - 0.0023$ , with  $R^2 = 0.9980$ .

**Total tannin content (TTC) analysis.** The TTC of the samples was analyzed using the Folin-Ciocalteu method (Chandran and Indira 2016). Approximately 10- $\mu$ L *Pluchea* infusion was added with 1-mL Folin-Ciocalteu's phenol reagent 10% in 10-mL volumetric flask and incubated for 5 min. Then, the mixture was added with 2-mL Na<sub>2</sub>CO<sub>3</sub> 7.5% and filled up to 10-mL volume with distilled water. The blue dark color solution was measured in UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at  $\lambda = 760$  nm, with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/ g samples using the following formula:  $y = 0.00009x + 0.0021$ , with  $R^2 = 0.9993$

### Analysis of the Antioxidant Potential

**DPPH free radical scavenging activity assay.** The DPPH free radical scavenging activity (DPPH) was measured by the spectrophotometric method (Widyawati *et al.* 2017) to determine the ability of the phytochemicals in the *Pluchea* leaf infusion to donate hydrogen atoms to the nitrogen atom in DPPH, resulting in the formation of DPPH-H compound exhibiting a yellow-colored solution. About 25  $\mu$ L *Pluchea* leaf infusion was poured into the reaction tube, into which 3-mL DPPH solution (4 mg/ 100 mL) was added. After incubation for 15 min in a dark room, the absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda = 517$  nm. The reference standard compound was gallic acid, and the results of the analysis were expressed as mg GAE/g samples calculated using the following formula:  $y = 0.146x + 1.7896$ , with  $R^2 = 0.9975$ .

**Ferric-reducing power (FRAP) analysis.** FRAP was determined following the method used by Widyawati *et al.* (2014) method. Approximately 10  $\mu$ L of samples were added to 2.5 mL phosphate buffer pH 6.6 and 2.5 mL of 1% potassium ferricyanide in the reaction tube. Then, the mixture was shaken and incubated for 20 min at 50 °C. Finally, 2.5 mL chloroacetic acid 10% (w/v) was added. Into the 2.5-mL supernatant, 2.5 mL distilled water and 0.5 mL ferric chloride 0.1% w/v were added, and the mixture was incubated for 10 min. The potency of the samples reducing iron (III) to iron (II) ion was indicated by the intensity of blue color formed that was measured using UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda = 700$  nm. The intensity of the blue color indicated a higher reducing capacity. The reducing power, expressed as mg GAE/g samples, was calculated using the following formula:  $y = 0.0002x + 0.0256$ , with  $R^2 = 0.9906$ .

### Analysis of the Antidiabetic Properties

**$\alpha$ -amylase enzyme inhibition (AA) capacity assay.** *In vitro* AA followed the procedure, as described by Widyawati *et al.* (2020). Each 500  $\mu$ L of the samples was mixed with starch 1% (w/v) and sodium acetate buffer pH 5. Into 250  $\mu$ L of the mixture, an  $\alpha$ -amylase solution (0.1 g of this enzyme 12.5 unit/mL) was added and then dissolved in 50 mL of 0.2 M sodium acetate pH 5. The mixture was shaken, into which 2-mL sodium hydroxide 1M was added. Before the analysis, this mixture was incubated at 37 °C for 10 min. Then, the capacity of the  $\alpha$ -amylase enzyme to hydrolyze the starch to release glucose was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda = 540$  nm. The inhibition percentage of  $\alpha$ -amylase was assessed using the formula  $(ACb - ACa) - (As - Ab) / (ACb - ACa) \times 100\%$  – where ACb is the absorbance of 100% enzyme activity (solvent with the enzyme), ACa is the absorbance of 0 % enzyme activity (solvent without the enzyme), As is the absorbance of the test sample with enzyme, and Ab is the absorbance of test sample without enzyme.

**$\alpha$ -glucosidase enzyme inhibition (GA) capacity assay.** The analysis of the  $\alpha$ -glycosidase inhibitor activity (GA) was done using the method of Widyawati *et al.* (2020) with slight modifications. About 150- $\mu$ L samples containing 100- $\mu$ L *Pluchea* infusion and 50  $\mu$ L pNPG (0.0150 g in 100-mL sodium phosphate 0.2 M at pH 7) were reacted with 50- $\mu$ L  $\alpha$ -glycosidase 2 mM (0.0833 unit/mL); then, the mixture was incubated at 37 °C for 15 min. The reaction was stopped with the addition of 1000- $\mu$ L sodium carbonate 0.2 M. The amount of these enzymes that did not react with bioactive compounds of *Pluchea* infusion hydrolyzed pNPG as a substrate to result in p-nitrophenol. The inhibition activity of the *Pluchea* infusion was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at  $\lambda = 405$  nm. The inhibition percentage of  $\alpha$ -glycosidase was calculated using the formula  $(ACb - ACa) - (As - Ab) / (ACb - ACa) \times 100\%$  – where ACb is the absorbance of 100% enzyme activity (solvent with enzyme), ACa is the absorbance of 0% enzyme activity (solvent without enzyme), As is the absorbance of test sample with enzyme, and Ab is the absorbance of test sample without enzyme.

### Analysis of Phenolics

The phenolic compounds of the samples were analyzed using high-performance liquid chromatography (HPLC) based on the method of Kongkiatpaiboon *et al.* (2018) with modifications. Each *Pluchea* infusion was sonicated for 15 min (Branson 1510); then, the sample was filtered using a filter syringe (Whatmann, 0.2  $\mu$ m, NYL). About 20  $\mu$ L of the sample was injected in an HPLC (LC20AD series, Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC LC-20AD pump, CTO-30A

column oven, CBM-20A/20 Alite system controller, and SPD-20A/20 AV UV-Vis detector. Separation of phenolic compounds in samples was carried out using a Shim-pack VP-ODS C18 column (ID 5  $\mu$ m  $\times$  50 mm  $\times$  4.6 mm) with a GVP-ODS Cartridge guard column (two pieces) (ID 10 mm  $\times$  4.6 mm). The mobile phase used consisted of a solution of [A] 0.5% acetic acid in water and [B] absolute methanol. Analysis was carried out using a gradient system in the following order: initial conditions of 10% B in A to 50% B in A were maintained for 40 min; then, 100% B was maintained for 20 min. Next, the column was re-equilibrated with 10% B in A and maintained for 10 min before analysis of the next sample. The sample flow rate was set at 1.0 mL/min with a controlled temperature of 40 °C. Detection was used at a wavelength of 280 nm. The reference standard used were gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid. All of the reference standard was dissolved in distilled water and prepared similarly to the samples before being injected in HPLC.

**Experiment design and statistical analysis.** The research design used a randomized block design with two factors, *i.e.* the steeping temperature (T) and the storage period. *Pluchea* leaf blades were subjected to 4 steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95 °C (T4), and the storage period of 0 year /unstored (B1), and 5 year /stored (B2) resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic was repeated for six periods. The data analysis of samples was repeated for six periods. The data were analyzed using a paired t-test at  $\alpha \leq 0.05$ , treatment means of specific phenolic compounds that were identified were expressed as the mean  $\pm$  SD. The analysis used SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

## RESULTS AND DISCUSSIONS

### Bioactive Compounds

**Phenolic compounds.** Bioactive compounds are active compounds in plants that are essential to protect body health (Nguyen and Chuyen 2020). These compounds usually have many biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer, antimicrobial, antibacterial, anti-cholesterol, and so on (Suriyaphan 2014; Acar *et al.* 2022). Phenolic compounds have potential redox properties that can scavenge free radicals that can cause a number of chronic diseases (Noreen *et al.* 2017; Aryal *et al.* 2019; Acar *et al.* 2022).

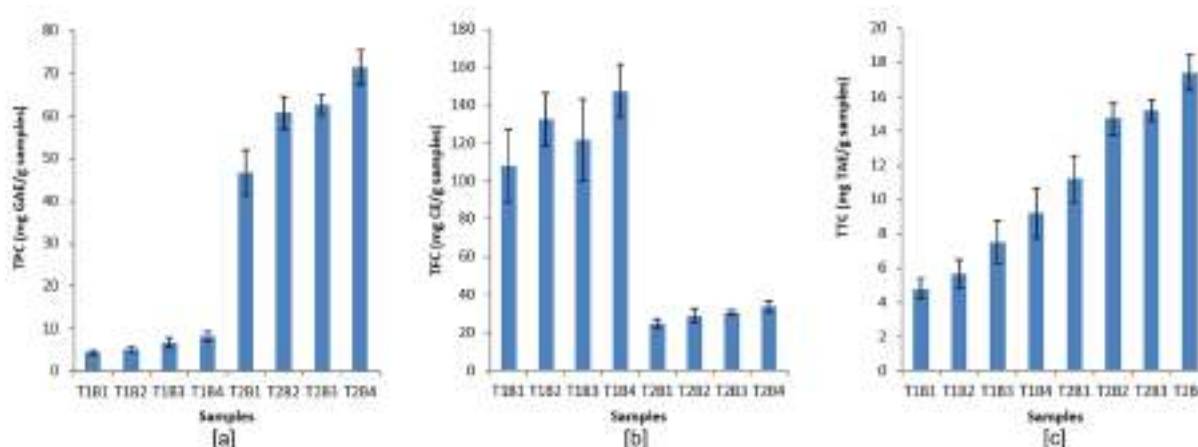


The TPC of *Pluchea* infusion at different steeping temperatures and storage periods generally significantly increased with increasing steeping temperature and storage period based on paired t-test at  $\alpha \leq 0.05$  (Figure 1a). Steeped and stored infusion had significantly higher amounts of phenolic compounds than the samples that were steeped and unstored. Further, the highest TPC was observed in samples infused at 95 °C and stored for 5 yr (at  $71.38 \pm 4.14$  mg GAE/g sample), whereas the lowest was measured in the unstored samples and infused at 60 °C (at  $4.39 \pm 0.49$  mg GAE/g sample). The phenolic content of stored samples that were steeped only at 60 and 95 °C showed a significant increase in their phenolic content. This implies that the steeping temperature and the storage periods significantly resulted in the high amounts of phenolic compounds in the infusions. Results also indicated that phenolic compounds were generally greater in the infusion at high steeping temperatures and long storage periods. This could have been due to the fact that the steeping temperature and storage period could cause the process of degradation, oxidation, and leaching or release of phenolic compounds. Phenolic compounds are water-soluble and, thus, soaking in hot water for a certain period, as steeping causes the migration process of more phenolic compounds to the water because of longer exposure of phenolic compounds to water (Castiglioni *et al.* 2015; Kilic *et al.* 2017; Acar *et al.* 2022). Su *et al.* (2019) reported that temperature treatment can stimulate the release of phenolic compounds and increase the antioxidant activity of lychee juice stored at different temperatures of 4 and 45 °C and different long storage (fresh and 72 h).

Temperature treatment degrades (or hydrolyzes) the hydrogen bond between phenolic compounds and proteins, resulting in an increase of phenolic compounds

when exposed to higher temperatures (Ali *et al.* 2018; Jayani *et al.* 2022; Ramphinwa *et al.* 2023). Zhang *et al.* (2021) reported that phenolic compounds present in plants are not completely stable but are easily degraded during storage after harvest. Reblova (2012) claimed that antioxidant compounds can be slowly degraded with increasing temperature. Fibrianto *et al.* (2021) also stated that the brewing temperature has an effect on the extracted antioxidant compounds such as alkaloids, catechins, and tannins. Thus, there is an assumption that temperature and storage caused the degradation, oxidation, and hydrolysis of the phenolic compounds period, resulting in the increased amount of the phenolic compounds at higher steeping temperatures and longer storage periods.

Simple phenolic compounds are identified in steeped and stored. *Pluchea* leaf infusion included gallic acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids is shown in Table 1. The treatment effects using the t-test at  $\alpha \leq 0.05$  showed that gallic acid and kaempferol content were insignificantly different at various steeping temperatures and storage periods. The concentration of quercetin and 3,5-di-*O*-caffeoylquinic acid of the unstored and stored *Pluchea* infusion was significantly different from the rest of the samples between 70 °C, whereas (+)-catechin concentration of *Pluchea* infusion was only significantly different at 95 °C. The myricetin content was significantly different at 80 and 95 °C. The 3,4-di-*O*-caffeoylquinic acid content showed significant difference at 60, 80, and 95 °C, whereas 4,5-di-*O*-caffeoylquinic acid content was only significantly different at 60 °C.



**Figure 1.** Bioactive compound contents of *Pluchea* infusion at different steeping temperatures and storage periods: [a] total phenolic content, [b] total flavonoid content, and [c] total tannin content. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  $\pm$  standard deviation (n = 6). Samples: T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T2B2-steeped at 70 °C, stored for 5 yr; T2B3-steeped at 80 °C, stored for 5 yr; T2B4-steeped at 95 °C, stored for 5 yr.

**Table 1.** Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperatures and storage periods.

Phenolic compounds	Steeping temperature (°C)	Mean ± SD (unstored)	Mean ± SD (stored)	Mean difference ±SD	Sig. (two-tailed)
Gallic acid (µg/g samples)	60	0.2132 ± 0.0027	0.2364 ± 0.0015	0.0375 ± 0.0175	0.2030
	70	0.2157 ± 0.0013	0.2324 ± 0.0214	0.0167 ± 0.0227	0.4870
	80	0.2234 ± 0.0122	0.2347 ± 0.0078	0.0386 ± 0.0264	0.2870
	95	0.2316 ± 0.0104	0.2402 ± 0.0169	0.0086 ± 0.1990	0.8500
(+) -Catechin (µg/g samples)	60	0.3425 ± 0.0110	0.5085 ± 0.0111	-0.1576 ± 0.0885	0.241
	70	0.3260 ± 0.0265	0.5448 ± 0.0006	-0.2188 ± 0.0259	0.053
	80	0.3240 ± 0.0222	0.5023 ± 0.0773	-0.1451 ± 0.0248	0.077
	95	0.4039 ± 0.0320	0.5995 ± 0.0372	-0.2049 ± 0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756 ± 0.1234	1.4762 ± 0.0271	-1.2887 ± 0.3222	0.111
	70	0.2587 ± 0.0160	1.4245 ± 0.2526	-1.1657 ± 0.2695	0.103
	80	0.4175 ± 0.0104	1.4570 ± 0.0925	-1.0391 ± 0.0841	0.036*
	95	0.8786 ± 0.0434	2.6138 ± 0.0695	-1.1735 ± 0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220 ± 0.0268	0.6220 ± 0.0706	-0.5999 ± 0.9733	0.544
	70	0.1530 ± 0.0511	1.0708 ± 0.0289	-0.9177 ± 0.0222	0.011*
	80	0.3666 ± 0.0103	0.8629 ± 0.0815	-0.1082 ± 0.4462	0.790
	95	0.6559 ± 0.0570	2.0230 ± 0.0573	-1.4123 ± 0.3203	0.101
Kaempferol (µg/g samples)	60	0.1394 ± 0.0202	0.3675 ± 0.0183	-0.3207 ± 0.1122	0.154
	70	0.0514 ± 0.0037	0.3726 ± 0.0944	0.3213 ± 0.0907	0.125
	80	0.3699 ± 0.0924	0.7966 ± 0.0366	-0.4267 ± 0.2727	0.271
	95	0.5913 ± 0.0239	0.9478 ± 0.0287	-0.3565 ± 0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103 ± 0.0628	2.4863 ± 0.0270	-1.8760 ± 0.2074	0.050*
	70	0.6271 ± 0.0099	2.3403 ± 0.0325	-1.7131 ± 0.3152	0.082
	80	0.7967 ± 0.03060	2.6278 ± 0.0211	-1.8311 ± 0.0095	0.002*
	95	1.5386 ± 0.0668	4.0211 ± 0.0851	-2.4825 ± 0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635 ± 0.0628	0.9449 ± 0.0501	-0.2814 ± 0.4458	0.536
	70	0.6162 ± 0.0099	0.9485 ± 0.0794	-0.3323 ± 0.0301	0.041*
	80	0.6601 ± 0.0306	0.9099 ± 0.0387	-0.2498 ± 0.3127	0.461
	95	0.6642 ± 0.0668	1.3156 ± 0.0166	-0.6514 ± 0.2666	0.179
4,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.4906 ± 0.0060	1.1842 ± 0.0120	-0.6886 ± 0.2723	0.018*
	70	0.4807 ± 0.0034	1.0089 ± 0.0736	-0.5281 ± 0.0702	0.060
	80	0.5299 ± 0.0053	1.2382 ± 0.1435	-0.7082 ± 0.1489	0.094
	95	1.0018 ± 0.0526	1.3797 ± 0.2170	-0.3086 ± 0.3086	0.333

Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean ± standard deviation (n = 6). Samples: T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T3B2-steeped at 70 °C, stored for 5 yr; T3B3-steeped at 80 °C, stored for 5 yr; T3B4-steeped at 95 °C, stored for 5 yr.

Results further showed that gallic acids and kaempferol were relatively stable, as reflected by the insignificant changes when exposed to the different steeping temperatures and storage periods. Myricetin, (+)-catechin, and 3,4-di-O-caffeoylquinic acid showed a drastic increase

at higher steeping temperatures and longer storage periods, implying that these compounds tended to be relatively labile. Quercetin, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid underwent moderate changes. Therefore, myricetin, (+)-catechin, and 3,4-di-

*O*-caffeoylquinic acid were easier to dissolve or degrade to form simple phenolic acids at higher temperatures and storage period (Su *et al.* 2019; Ali *et al.* 2018; Jayani *et al.* 2022; Ramphinwa *et al.* 2023; Zhang *et al.* (2021). Degradable polyphenol compounds have a simple structure and free hydroxyl groups that can react with Folin-Ciocalteu's phenol reagent, resulting in a complex blue solution that can be detected as TPC.

**Flavonoid content (TFC).** Flavonoids are the major phenolic compounds that have potential chemical and biological activities such as radical scavenging and antimicrobial activities (Ayele *et al.* 2022; Chandra *et al.* 2014) that can protect the human body from the oxidative stress caused by many degenerative diseases – especially cancer, cardiovascular problems, and aging (Mathur and Vijayvergia 2017). The TFC of steeped *Pluchea* infusion decreased with a longer storage period. Unstored samples exhibited higher flavonoid content than the stored samples. The statistical analysis using a paired t-test at  $\alpha \leq 0.05$  showed that the TFC of *Pluchea* infusion was significantly different between the steeped unstored and steeped stored samples (Figure 1b). The highest TFC was exhibited by the unstored samples steeped at 95 °C at about  $147.42 \pm 14.03$  mg CE/g sample. The TFC was significantly lower in the stored samples than those of the unstored samples, implying that the increase in the flavonoid content of the infusion was affected primarily by the steeping temperature.

**Tannin content (TTC).** Tannins are bioactive compounds that provide properties, such as astringent, anti-diarrheal, antibacterial, and antioxidant (Malangngi *et al.* 2012). Generally, results indicated that the TTC of *Pluchea* infusion significantly increased with increasing steeping temperature and storage period (Figure 1c). Among the unstored steeped samples, the tannin content was significantly lowest in the samples infused at 60 °C at about  $4.81 \pm 0.58$  to  $17.42 \pm 1.04$  mg TAE/g samples, which was significantly different lower from that of the lowest tannin content of the stored samples. Among the stored and steeped samples, the highest tannin content was observed at samples steeped at 95 °C about  $17.42 \pm 1.04$  mg TAE/g samples and was significantly different from that of the highest tannin content of the unstored steeped samples at 95 °C about  $9.22 \pm 1.48$  mg TAE/g samples. Indicating that the tannin content was primarily affected by a longer storage period than high steeping temperature. The condensation of catechins to tannins is a dominant process occurring in tea leaves that is accelerated during the maceration of raw tea leaves (Kowalska *et al.* 2021) and could have contributed to the observed increase in the tannin content in the treated samples.

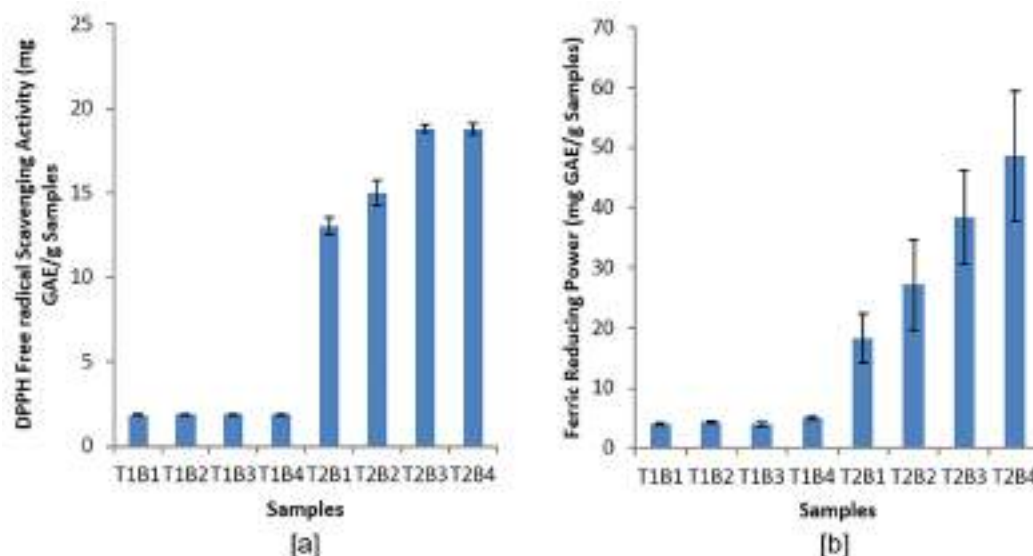
Nonetheless, high temperatures and long storage periods can cause the degradation of tannins to catechins. Rusita *et al.* (2019) emphasized that tannins are polar

thermostable complex compounds that are resistant to heating, indicating that even with the exposure to high temperature, the tannins still remained high in the treated samples period.

**Antioxidant activity.** Antioxidant activity is the capability of compounds to inhibit the oxidation of macromolecules from biological targets that are involved in oxidative chain reactions (Ali *et al.* 2005; Oh *et al.* 2013). The antioxidant activity assay was done in this research using DPPH and FRAP methods. The phenolic compounds are active antioxidants with antioxidant capability that depends on their redox properties. The structure of phenolic compounds determines the effectivity to donate hydrogen atoms, which is negatively correlated with the O-H phenolic bond strength. The antioxidant power of phenolic compounds is due to the weak hydrogen bonds in the OH group of the phenolic compound, so that it is easier to donate hydrogen atoms (Kruk *et al.* 2022). The mechanism of phenolic compounds as antioxidants depends on their ability to donate hydrogen atoms and transfer electrons, as well as reducing agents and singlet oxygen quenchers (Ali *et al.* 2005; Huang *et al.* 2005).

**DPPH free radical scavenging activity (DPPH).** DPPH is a free radical that is often used to evaluate antioxidant activity because this method is simple and is suitable for measuring the donating hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of DPPH to change to a yellow color (Munteanu and Apetrei 2021; Baliyan *et al.* 2022). Figure 2a shows that the free radical scavenging properties of the stored and steeped samples were significantly higher than the unstored steeped samples. It can also be observed that the free radical scavenging property was significantly different among the stored and steeped samples but insignificant among the unstored and steeped sample period. *Pluchea* infusion stored at room temperature for 5 yr resulted in high free radical scavenging activity by more than 10%. Steeping at higher temperatures significantly increased the DPPH free radical scavenging activity in stored *Pluchea* infusion by around 15–25%. This implies that the higher free radical scavenging property was primarily affected by the storage period than the steeping temperature. During the storage process, it is possible to form complex phenolic compounds that provide a high ability to scavenge free radicals (Thanajiruschaya *et al.* 2010).

The scavenging activity of the samples was strongly and positively correlated with total phenolic and tannin contents but inversely with total flavonoid levels (Table 2). The antioxidant activity was strongly and negatively correlated with flavonoid content. The storage period could be reduced flavonoid content. The study also demonstrated that longer storage period and higher infusion temperatures produced many simple phenolic compounds with free



**Figure 2.** Antioxidant activity of *Pluchea* tea at different steeping temperatures and storage periods: [a] DPPH; [b] FRAP. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  $\pm$  standard deviation ( $n = 6$ ). Samples : T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T2B2-steeped at 70 °C, stored for 5 yr; T2B3-steeped at 80 °C, stored for 5 yr; T2B4-steeped at 95 °C, stored for 5 yr.

**Table 2.** Pearson correlation coefficients between bioactive contents (TPC, TFC, and TTC), antioxidant activity (DPPH and FRAP), and antidiabetic activity (AA and GA).

	TPC	TFC	TTC	DPPH	FRAP	$\alpha$ -glucosidase	$\alpha$ -amylase
TPC	1						
TFC	-0.93589	1					
TTC	0.960028	-0.81321	1				
DPPH	0.992776	-0.93992	0.942273	1			
FRAP	0.953366	-0.82636	0.947778	0.956242	1		
$\alpha$ -glucosidase	-0.55512	0.349873	-0.71534	-0.5272	-0.55947	1	
$\alpha$ -amylase	-0.70842	0.429393	-0.8569	-0.69579	-0.80548	0.725161631	1

Significant at the 0.05 level (two-tailed)

hydroxyl groups capable to donate hydrogen atoms to DPPH free radicals. Many phenolic acids such as gallic acids, (+)-catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids have established potential antioxidant activity (Kumar and Goel 2019) (Table 1). Kruk *et al.* (2022) informed that the capability of phenolic compounds to donate hydrogen atom depends on the chemical structure, number, and position of hydroxyl groups attached to a benzene ring, a double bond between C2 and C3 rings, and a carbonyl group (C=O) on the C ring at C4. The effectivity of antioxidant compounds to donate hydrogen atoms is determined by O-H bond dissociation energy.

The free radical scavenging property observed in the

study was not consistent with the results of the study by Moraes-de Souza *et al.* (2008). The research shows that the TPC of herbal infusion is lowly correlated with free radical scavenging activity. However, Dobrinis *et al.* (2021) informed that TPC is positively and significantly correlated with the free radical scavenging property of tea infusion.

**Ferric reducing antioxidant power (FRAP).** FRAP is an analysis of the antioxidant power of the phytochemical compounds that is based on the ability of antioxidant compounds to reduce iron ions of potassium ferricyanide ( $Fe^{3+}$ ) to potassium ferrocyanide ( $Fe^{2+}$ ). Potassium ferrocyanide reacts with ferric chloride to form a ferric-ferrous complex and results green color solution (Widyawati *et al.* 2017; Raharjo and Haryoto 2019).

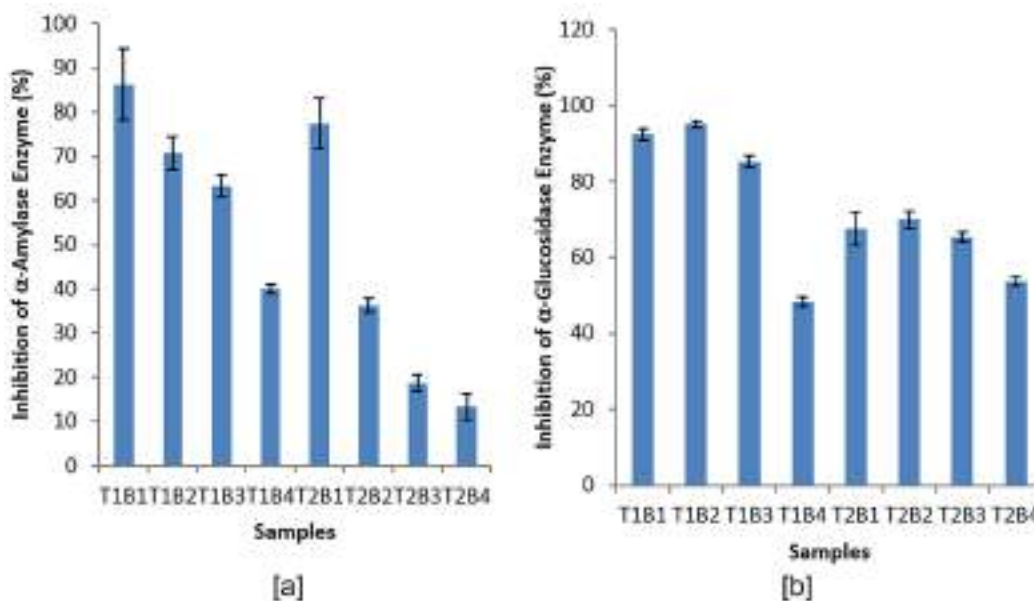
The results showed that the FRAP increased at higher steeping temperatures and longer storage periods. The lowest FRAP was observed in the unstored samples, which were steeped at 60 °C at  $3.95 \pm 0.17$  mg GAE/g samples, and the highest was exhibited in *Pluchea* infusion which was stored for 5 yr at 95 °C at  $48.63 \pm 10.83$  mg GAE/g samples (Figure 2b). FRAP increased significantly as the steeping temperature was increased. FRAP of the samples stored for 5 yr was also significantly higher than the unstored samples at  $\alpha \leq 0.05$ .

This is in contrast with the study on the antioxidant activity of DPPH and FRAP of matcha. The longer storage period reduces the levels of catechin content due to the catechins such as epigallocatechin gallate, epicatechin gallate, epigallocatechin, and epicatechin, which are bioactive compounds that have high antioxidant activity (Kim *et al.* 2020). The ferric-reducing capability of *Pluchea* could have been due to the presence of simple phenolic acid that can transfer electrons from their free hydroxyl groups of samples. The FRAP of *Pluchea* infusion was strongly and positively significantly correlated with the DPPH, TPC, and TTC but inversely to TFC.

### Antidiabetic Activity

**$\alpha$ -amylase enzyme inhibition activity (AA).** Antidiabetic activity is a measure of the potency of phenolic compounds to regulate the uptake of glucose by the cells from the

blood through the mediation of two digestive enzymes, *i.e.*  $\alpha$ -amylase and  $\alpha$ -glucosidase, which are involved in the control of dietary carbohydrate digestion and release in the postprandial blood glucose in human body (Fu *et al.* 2017). The phenolic compounds have the capability to bind with the protein component of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Martinez-Solis *et al.* 2022), resulting in the reduced activity of the enzymes. The results showed that lower steeping *Pluchea* leaf infusion was able to inhibit the action of the  $\alpha$ -amylase enzymes (Figure 3a). The *Pluchea* infusion exhibited a good AA of more than 50% and even almost 100% in unstored *Pluchea* infusion steeped at 60, 70, and 80 °C, with the highest at 60 °C and in stored *Pluchea* leaf infusion, which was steeped at 60 °C. The stored *Pluchea* leaf infusion steeped at 70, 80, and 95 °C for 5 min had lower enzyme inhibition activity of less than 50%, with the lowest at 95 °C around 13%. Widyawati *et al.* (2017) found that the ability to inhibit the  $\alpha$ -amylase enzyme in unstored *Pluchea* infusion steeped at 95 °C for 5 min was also low at 28.79%. Increasing the steeping temperature and storage period reduced the ability of the phytochemicals in the *Pluchea* infusion to inhibit the  $\alpha$ -amylase enzyme activity period. Table 2 further shows that the AA of *Pluchea* infusion was strongly and negatively significantly correlated with TPC, TTC, DPPH, and FRAP, but it was weakly and positively significantly correlated with TFC.



**Figure 3.** Antidiabetic activity of pluchea tea at different steeping temperatures and storage periods: [a]  $\alpha$ -amylase; [b]  $\alpha$ -glucosidase. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  $\pm$  standard deviation ( $n = 6$ ). Samples: T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T2B2-steeped at 70 °C, stored for 5 yr; T2B3-steeped at 80 °C, stored for 5 yr; T2B4-steeped at 95 °C, stored for 5 yr.

This inhibitory activity was thought to be contributed by other bioactive compounds besides phenolics, which are sensitive to steeping temperature and storage period. Li *et al.* (2018) stated that there are flavonoid compounds that contribute to the ability to inhibit the  $\alpha$ -amylase enzyme. Akah *et al.* (2011) reported that phytochemical compounds such as terpenoids, saponins, flavonoids, glycosides/carbohydrates, and alkaloids are good antidiabetic metabolites or  $\alpha$ -amylase enzyme activity inhibitors. Sangeetha and Vedesree (2012) explained that the ability of *Threspesia populnea* extract to inhibit the  $\alpha$ -amylase enzyme was determined by their phenolic compound content and protein. Moreover, the presence of the  $\alpha$ -amylase enzyme inhibitor in this extract may be proteinaceous or nonproteinaceous in nature. It was assumed that this enzyme inhibitory activity in *Pluchea* infusion also was determined by their protein and polyphenolic content. Aleixandre *et al.* (2022) also stated that phenolic acids have inhibition activity to  $\alpha$ -amylase enzyme depending on their structures. There are C=C double bonds conjugated with a carbonyl group of phenolic structures that stabilize the binding forces to the active site of the  $\alpha$ -amylase. The hydroxyl groups can bind by non-covalent interaction (hydrogen bonding, cation- $\pi$  interactions, salt bridge interactions, ionic interactions, or electrostatic forces) with amino acid residue at the active site in the  $\alpha$ -amylase enzyme. Elevated steeping temperatures and longer storage periods can easily cause the removal of the hydroxyl groups of phenolic compounds, which can reduce their ability to enzyme inhibition. The phenolic acids with a greater number of hydroxyl groups exhibits stronger capability to obstruct the  $\alpha$ -amylase enzyme.

**$\alpha$ -glucosidase enzyme inhibition activity (GA).**  $\alpha$ -glucosidase is an important enzyme in carbohydrate digestion, that catalysis the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and converts them into monosaccharides (glucose), thereby resulting in hyperglycemia (Nurcholis *et al.* 2014; Proenca *et al.* 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -glucosidase enzyme is used to determine their antidiabetic activity. This is supported by Werdani and Widyawati (2018) stated that *Pluchea* infusion has the potential as an antidiabetic agent. Widyawati *et al.* (2020) found that the steeping of unstored *Pluchea* infusion at 95 °C for 5 min has an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857%.

Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme decreased with increasing steeping temperature and storage period. Steeping at 95 °C of the unstored *Pluchea* leaf infusion obtained the lowest inhibitory ability, *i.e.*  $48.32 \pm 1.27\%$ , and the highest inhibitory activity was at 70 °C at  $95.11 \pm 0.70\%$ . The results of a paired t-test showed that GA

of *Pluchea* infusion was significantly different between steeping temperature and long storage. Figure 3 further shows that the ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher than the ability to inhibit the  $\alpha$ -amylase enzyme. Data analysis in Table 2 showed that the TFC of the *Pluchea* leaf infusion was influenced weakly and positively by GA and AA, but the GA and AA were not affected by TPC, TTC, DPPH, and FRAP. Li *et al.* (2018) stated that flavonoid compounds can inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. Dias *et al.* (2021) stated that flavonoid compounds such as rutin, myricetin, kaempferol, and quercetin have antioxidant and antihyperglycemic activities. The ability to inhibit the action of enzymes from flavonoid compounds is determined by the position and number of hydroxyl groups, the number of double bonds in rings A and B, and the heterocyclic ring in ring C. Tadera *et al.* (2006) and Zhang *et al.* (2014) also explained that flavonoid compounds of samples significantly inhibit the  $\alpha$ -glucosidase enzyme activity.

The ability to inhibit the  $\alpha$ -glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping temperature and long storage. Figure 3 also showed that the capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater than the  $\alpha$ -amylase enzyme because the mechanism of the two enzymes was different, according to the opinion of McCue *et al.* (2005). The mechanism of the  $\alpha$ -glucosidase enzyme inhibitor includes making the sugar mimic structure, binding using ionic bonds with nucleophilic, making the transition state-like structure, binding hydrogen with catalytic acid residue, interacting ionic and hydrophobic with site other than the active site, and binding covalent with enzymes through an epoxy or aziridine group (Moorthy *et al.* 2012). Then, the mechanism of the  $\alpha$ -amylase enzyme inhibitor includes blocking carbohydrates, thereby limiting the digestibility and absorption of carbohydrates, as well as blocking the active centers of several subsites of the enzyme (Gong *et al.* 2020).

Widyawati *et al.* (2017) stated that phenolic and non-phenolic compounds can inhibit the  $\alpha$ -glucosidase enzyme activity. The ability of bound phenolic compounds to inhibit  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, which may occur in *Pluchea* infusion during storage, affects the structure and profile of phenolic and non-phenolic compounds. Arsiningtyas *et al.* (2014) explained that the methyl-esterified quinic acid with the caffeic groups such as 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid, and 1,3,4,5-tetra-*O*-caffeoylquinic acid of *Pluchea* leaves inhibits the

$\alpha$ -glucosidase enzyme activity. The resulting analysis of caffeoylquinic acids (3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid) in stored *Pluchea* leaf infusion had higher concentrations than in unsteeped *Pluchea* infusion, and the concentrations of the simple phenolic compounds were increased at higher steeping temperatures, but the GA of them was reduced. It means that the methyl-esterified quinic acid with the caffeic groups had more potential to inhibit  $\alpha$ -glucosidase enzyme than free caffeoylquinic acid.

This study showed that the increasing steeping temperature and storage period caused degradation of polyphenolic compounds to produce simple phenolic compounds such as gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid that increased the TPC and TTC. The increase in the simple phenolic concentration of the *Pluchea* leaf infusion caused higher antioxidant activity and lower antidiabetic activity.

## CONCLUSION

The TPC of *Pluchea* infusion at different steeping temperatures and storage periods generally significantly increased with increasing steeping temperature and storage periods. Steeped and stored infusion had significantly higher amounts of phenolic compounds than the samples that were steeped and unsteeped. TPC was highest in the stored and steeped at 95 °C and lowest in the unsteeped and steeped at 60 °C. Unsteeped steeped samples exhibited significantly higher flavonoid content than the stored steeped samples. The highest TFC was exhibited by the unsteeped samples steeped at 95 °C. The TTC of *Pluchea* leaf infusion significantly increased with increasing steeping temperature and storage period. Among the unsteeped steeped samples, the tannin content was significantly the lowest in the samples steeped at 60 °C and the highest in the samples steeped at 95 °C.

The DPPH of the stored and steeped *Pluchea* leaf infusion was significantly higher than the unsteeped steeped samples. The free radical scavenging property was highest in the stored samples steeped at 80 and 95 °C. The free radical scavenging activity of the samples was strongly and positively correlated with total phenolic and tannin contents but inversely with total flavonoid levels. The FRAP significantly increased with increasing steeping temperature and longer storage periods. The lowest FRAP was found in the unsteeped samples that were steeped at 60 °C, and the highest was exhibited in *Pluchea* samples that were stored for 5 yr and steeped at 95 °C. The FRAP of *Pluchea* leaf infusion was significantly strong and positively correlated with the free radical scavenging

property, TPC, and TTC but inversely with TFC. The AA was generally found to be higher at lower steeping temperatures of the unsteeped *Pluchea* leaf infusion than at higher steeping temperatures of the stored sample. The AA capacity of the *Pluchea* leaf infusion showed a significantly strong and negative correlation with TPC, TTC, DPPH, and FRAP, but it was weakly and positively correlated significantly with TFC.

The ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme decreased at high steeping temperatures and long storage periods. The highest inhibitory activity was obtained in the unsteeped *Pluchea* leaf infusion that was steeped at 70 °C, whereas the lowest was obtained in the unsteeped sample that was steeped at 95 °C. The ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher than the ability to inhibit the  $\alpha$ -amylase enzyme. The GA was significantly strong and negative TPC, TTC, DPPH, and FRAP, and it was weakly and positively correlated significantly with TFC.

The simple phenolic compounds identified in *Pluchea* leaf infusion may affect the presence of the bioactive compounds, antioxidant potential, and antidiabetic properties at different steeping temperatures and storage periods – including gallic acids, (+)-catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids.

## ACKNOWLEDGMENTS

The authors would like to thank the Ministry of Education and Culture of the Republic of Indonesia for a fundamental research grant to higher education institutions in 2022.

## STATEMENT ON CONFLICT OF INTEREST

The authors declare no conflict of interest.

## REFERENCES

- ACARA, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food wastes: evaluation of temperature and time effects on quality parameters. *Applied Food Res* 2(100087): 1–8.
- ALEIXANDRE A *et al.* 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase and influence of reaction conditions. *Food Chem* 372(131231): 1–9.

- ALI *et al.* 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile. *Food Chem* 89: 27–36.
- ALI A *et al.* 2018. Impact of storage conditions on the stability of predominant phenolic constituents and antioxidant activity of dried *Piper betle* extracts. *Molecules* 23(24): 1–13.
- AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and methanol extract and fractions of *Gongronema latifolium* (*Asclepidaceae*) leaves in alloxan diabetic rats. *J Applied Pharm Sci* 01(09): 99–102.
- ARSININGTYAS IS *et al.* 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product use. *Natural Product Res* 28(17): 1350–1353.
- ARYAL S *et al.* 2019. Total phenolic content, flavonoid content, and antioxidant of wild vegetables from Western Nepal. *Plants* 8(96): 1–12.
- AYELE DT, AKELE ML, MELESE AT. 2022. Analysis of total phenolic contents, flavonoids, antioxidant, and antibacterial activities of *Croton macrostachyus* root extracts. *BMC Chem* 16(30): 1–9.
- BALIYAN S *et al.* 2022. Determination of antioxidants by DPPH radical scavenging activity and quantitative phytochemical analysis of *Ficus religiosa*. *Molecules* 27(1326): 1–19.
- CASTIGLIONI S *et al.* 2015. Influence of steeping conditions (time, temperature, and particle size) on antioxidant properties and sensory attributes of some white and green teas. *Intern J Food Sci Nut Online*. p. 1–7.
- CHAN EWC *et al.* 2022. *Pluchea indica*: an updated review of its botany, uses, bioactive compounds, and pharmacological properties. *Pharmaceutical Sciences Asia* 49(1): 77–85.
- CHANDRA S *et al.* 2014. Assessment of total phenolic and flavonoid content, antioxidant properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit crops: a comparative study. *Hindawi Publishing Corporation Evidence-based Complementary Alter Med*. p. 1–9.
- CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic, flavonoids, tannin, and chlorophyll content of leaves of *Strobilanthes kunthiana* (neelakurinji). *J Med Plants Studies* 4(4): 282–286.
- DIAS MC, PINTO DCGA, SILVA AMS. 2021. Plant flavonoids: chemical characteristics and biological activity. *Molecules* 26(5377): 1–16.
- DOBRINAS S, SOCEANU A, POPESCU V, POPOVICI IC, JITARIU D. 2021. Relationship between total phenolic content, antioxidant capacity, and Fe and Cu content from tea plant samples at different brewing times. *Processes* 9(1311): 1–11.
- FERRARIS Z. 2023. *Pluchea indica* Less. iNaturalist.org. <https://www.gbif.org/occurrence/4022273660>
- FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional properties and visual appearance of Dampit Robusta coffee leaves tea. *Advances in Food Sci Sustain Agric Agro-industrial Engineering* 4(1): 25–32.
- FU L *et al.* 2017. Total phenolic contents and antioxidant capacities of herbal and tea infusions. *Intern J Molecular Sci* 12: 2112–2124.
- GAO MR *et al.* 2019. A theoretical and experimental study: the influence of different standards on the determination of total phenol content in the Folin-Ciocalteu assay. *J Food Measurement and Characteristic* 13: 1349–1356.
- GONG L, FENG D, WANG T, REN Y, LIU Y, WANG J. 2020. Inhibitors of  $\alpha$ -amylase and  $\alpha$ -glucosidase: potential linkage for whole cereal foods on prevention of hyperglycemia. *Food Sci Nut* 2020(8): 6320–6337.
- HUANG D, OU B, PRIOR RL. 2005. The chemistry behind antioxidant capacity assays. *J Agric Food Chem* 53: 1841–1856.
- ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties of rosehip tea beverage: study by response surface methodology. *J Food Sci Tech* 54(11): 3737–3743.
- JAYANI NIE *et al.* 2022. Evaluation of stability and quality characteristics of moringa (*Moringa oleifera*) herbal tea during storage. *Food Res* 6(4): 399–406.
- KIM JM *et al.* 2020. Effect of storage temperature on the antioxidant activity and catechins stability of matcha (*Camellia sinensis*). *Food Science and Biotechnology* 29(9): 1261–1271.
- KILIC *et al.* 2017. Antioxidant properties of some herbal teas (green tea, senna, corn silk, rosemary) brewed at different temperatures. *Intern J Secondary Metabolite* 4(3): 142–248.
- KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves from different provenances in Thailand. *Revista Brasileira de Farmacognosia* 28: 145–150.
- KOWALSKA J *et al.* 2021. Influence of tea brewing parameters on the antioxidant potential of infusions and



- extracts depending on the degree of processing of the leaves of *Camellia sinensis*. *Molecules* 26(4773): 1–17.
- KRUK J, ABOUL-ENAIN BH, DUCHNIK E, MARCHLEWICZ M. 2022. Antioxidative properties of phenolic compounds and their effect on oxidative stress induced by severe physical exercise. *J Physiol Sci* 72(19): 1–24.
- KUMARN, GOEL N. 2019. Phenolic acids: natural versatile molecules with promising therapeutic applications. *Biotechnology Reports* 24: e00370.
- LI K *et al.* 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the flavonoids rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity relationship of its eight flavonoids by a refined assign score method. *Chem Central J* 12(82): 1–11.
- LIN YS. *et al.* 2020. Effects of storage time and temperature on antioxidants in juice from *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser. *Molecules* 25(3614): 1–12.
- MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content and antioxidant activity test of avocado seed extract (*Persea americana* Mill). *Jurnal MIPA Unsrat Online* 1(1): 5–10 [in Bahasa Indonesia].
- MARTINEZ-SOLIS J *et al.* 2022. Antidiabetic and toxicological effect of the tea infusion of summer collection from *Annona cherimola* Miller leaves. *Plants* 11(3224): 1–17.
- MATHUR R, VIJAYVERGIAR. 2017. Determination of total flavonoid and phenol content in *Mimusops elengi* Linn. *Intern J Pharm Sci Res* 8(12): 5282–5285.
- MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of sprouted and solid-state bioprocessed soybean. *Asia Pacific J Clinical Nut* 14(2): 145–152.
- MOORTHY NS, RAMOS MJ, A FERNANDES P. 2012. Studies on  $\alpha$ -glucosidase inhibitors development: magic molecules for the treatment of carbohydrate mediated diseases. *Mini Rev Med Chem* 12: 713–720.
- MORAES-DE SOUZA RA, OLDONI TLC, REGITANO-D'ARCE MAB, ALENCAR SM. 2008. Antioxidant activity and phenolic composition of herbal infusions consumed in Brazil. *Cienc Tecnol Aliment* 6(1): 41–47.
- MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant activity: a Review. *Intern J Molecular Sci* 22(3380): 1–30.
- NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus sabdariffa* L.): effect of dyeing temperature and brewing conditions on total soluble solid, phenolic content, antioxidant capacity, and sensory quality. *Beverages* 6(2): 1–11.
- NOREEN H *et al.* 2017. Measurement of total phenolic content and antioxidant activity of aerial parts of medicinal plant *Coronopus didymus*. *Asian Pacific J Tropical Med* 10(8): 792–801.
- NURCHOLIS GW *et al.* 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity *in vitro*, and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum pictum* (L.). *Current Biochem* 1(2): 58–65.
- OH J *et al.* 2013. Antioxidant and antimicrobial activities of various leafy herbal teas. *Food Control* 31: 403–409.
- PROENCA C *et al.* 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an *in vitro* and *in silico* structure-activity relationship study. *J Enzyme Inhibition Med Chem* 32(1): 1216–1228.
- PUREWAL SS *et al.* 2022. Unraveling the effect of storage duration on antioxidant properties, physico-chemical and sensorial parameters of ready to serve Kinnow-Amla beverages. *Applied Food Res* 2(1): 1–8.
- RAHARJO D, HARYOTO. 2019. Antioxidant activity of mangrove *Sonnenatia caseolaris* L. using the FRAP method. *ISETH*. p. 623–629.
- RAMPHINWA ML *et al.* 2023. Eco-physiological response of secondary metabolites of teas: review of quality attributes of herbal tea. *Frontiers in Sustain Food Systems* 7(990334): 1–14.
- REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids. *Czech J Food Sci* 30(2): 171–177.
- RUAN J *et al.* 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel column chromatography fractions. *Molecules* 24(2784): 1–20.
- RUSITA YD *et al.* 2019. Comparison of tannin concentration on fresh and dried tea leaf (*Camellia sinensis* L.) extract using UV-visible spectrophotometric method. 1st International Conference of Health, Science, & Technology (ICOHETECH) 2019 ;16 Nov 2019; LPPM Universitas, Duta Bangsa Surakarta, Surakarta.
- SANGEETHA R, VEDASREE N. 2012. *In vitro*  $\alpha$ -amylase inhibitory activity of the leaves of *Thespesia populnea*. *Intern Scholarly Res Network*. p. 1–4.

- SHRAIM AM *et al.* 2021. Determination of total flavonoid content by aluminum chloride assay: a critical evaluation. *LWT Food Sci Tech* 150(111932): 1–11.
- SILVA-RAMIREZ KA *et al.* 2020. Effect of drying and steeping temperature on the phenolic content, antioxidant activity, aromatic compounds, and sensory properties of *Culila polyantha* Benth. *Infusions Processes* 8(1378): 2–20.
- SRISOOK K *et al.* 2012. Antioxidant and anti-inflammatory activities of hot water extract from *Pluchea indica* Less. herbal tea. *J Med Plants Res* 6(23): 4077–4081.
- SURIYAPHAN O. 2014. Nutrition, health benefits, and applications of *Pluchea indica* (L) Less leaves. *Pharm Sci Asia* 41: 1–10.
- SUD *et al.* 2019. Impact of thermal processing and storage temperature on phenolic profile and antioxidant activity of different varieties of lychee juice. *LWT–Food Tech* 116(108578): 1–9.
- TADERA K, MINAMI Y, TAKAMATSU K, MATSUOKA T. 2006. Inhibition of alpha-glucosidase and alpha-amylase by flavonoids. *J Nutr Sci Vitaminol* 52: 149–153.
- THANAJIRUSCHAYA P *et al.* 2010. Effect of storage period and temperature on antioxidant components and properties of milled rice. *Khon Kaen Univ Res J* 15(9): 843–851.
- VONGSAK B *et al.* 2018. Comparison of active constituents, antioxidant capacity, and  $\alpha$ glucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. *Food Biosci* 25: 68–73.
- WANG SC *et al.* 2022. Effect of brewing conditions on polyphenols in the dark tea (*Camellia sinensis* L.) infusions: content, composition, and antioxidant activities. *Food Sci Tech* 42(e36322): 1–9.
- WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica* Less as functional beverage in diabetic patients. *ASSEHR* 98: 164–167.
- WIDYAWATI PS *et al.* 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract as antioxidant and anti-warmed-over flavor (WOF) of duck meat. International Congress on Challenges of Biotechnological Research in Food and Health; 15 Nov 2014; Agricultural Technology Faculty, Slamet Riyadi University, Surakarta, Indonesia.
- WIDYAWATI PS *et al.* 2016. The physicochemical and antioxidant properties of *Pluchea indica* Less drink in tea bag packaging. *IJFNS* 5: 13–120.
- WIDYAWATI PS *et al.* 2017. *In vitro* antioxidant capacities and antidiabetic properties of *Pluchea* leaves. *IJPPS* 9(8): 203–208.
- WIDYAWATI PS *et al.* 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica* Less beverage. *CJFST* 12(4): 125–139.
- WIDYAWATI PS *et al.* 2022. The effect of  $\kappa$ -carrageenan proportion and hot water extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of stink lily (*Amorphophallus muelleri*) wet noodles. *Molecules* 27(5062): 1–16.
- XU P, CHEN L, WANG Y. 2019. Effect of storage period on antioxidant activity and inhibition on  $\alpha$ -amylase and  $\alpha$ -glucosidase of white tea. *Food Sci Nut* 7(2): 636–644.
- ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction period on caffeine in coffee. *Lantanida J* 6(2): 103–202. [in Bahasa Indonesia].
- ZHANG H, WANG G, DONG J. 2014. Inhibitory properties of aqueous ethanol extracts of propolis on alpha-glucosidase. *J Evid Based Complement Alternat Med.* p. 1–8.
- ZHANG Y *et al.* 2021. Effect of storage conditions and period on the polyphenol content of wheat flours. *Processes* 9(248): 1–11.



Paini Sri Widyawati <paini@ukwms.ac.id>

---

## First Draft of PJS Article Ms 23-158

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Wed, Jun 19, 2024 at 10:28 AM

Dear Mr. ALLYSTER A. ENDOZO  
Managing Editor

Greetings,

I have looked at the manuscript and basically I agree, but there is one word yr that I marked in the manuscript, should it still be written in full? and please add contract number of this research.

Thank you for your cooperation

Regards

Paini SW

[Quoted text hidden]



**[PRO] 23-158 - Widyawati and Wilianto - Article (1st Draft) (18 Jun 2024).pdf**  
1713K

## Effect of Steeping Temperature and Storage Period on the Bioactive Compounds plus Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea indica* Less

Paini Sri Widyawati<sup>1\*</sup> and Yufita Ratnasari Wilianto<sup>2</sup>

<sup>1</sup>Food Technology Study Program, Agricultural Technology Faculty,  
Widya Mandala Surabaya Catholic University, Surabaya 60265 Indonesia

<sup>2</sup>Pharmacy Study Program, Pharmacy Faculty,  
Widya Mandala Surabaya Catholic University, Surabaya 60272 Indonesia

This study was done to determine the effects of steeping temperature and storage period on the bioactive contents plus antioxidant and antidiabetic activities of *Pluchea* leaf infusion. The research used a randomized block design with two factors, *i.e.* steeping temperature (T) and storage period (B). The *Pluchea* leaf blades were exposed to four steeping temperatures of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) °C with the storage period of 0 (B1) and 5 (B2) yr – resulting in eight treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). Statistical analysis using a paired t-test at  $\alpha \leq 0.05$  showed that treatments significantly affected the bioactive contents [total phenol (TPC), total tannin (TTC), and total flavonoid (TFC)], antioxidant [DPPH scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)] potential and antidiabetic [ $\alpha$ -amylase (AA) and  $\alpha$ -glucosidase (GA) inhibition] properties of the *Pluchea* leaf infusion. TPC, TTC, DPPH, and FRAP significantly increased for the storage period and the steeping temperatures. Then, TFC decreased during the storage period but significantly increased at higher steeping temperatures. The AA and GA of *Pluchea* leaf infusion increased until 70 °C of the steeping temperature but decreased until 95 °C. The DPPH and FRAP of the *Pluchea* leaf infusion were strongly and positively correlated with TPC and TTC. The GA and AA of *Pluchea* leaf infusion were not influenced by the TPC and TTC but were weakly and positively correlated with TFC. The antioxidant activity of the *Pluchea* leaf infusion was inversely proportional to the antidiabetic activity. The simple phenolic compounds derived from *Pluchea* leaf infusion at different steeping temperatures and storage included gallic acid, kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-caffeoylquinic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid.

Keywords: antioxidant, antidiabetic, bioactive compound, *Pluchea indica* Less, steeping temperature, storage period

\*Corresponding author: paini@ukwms.ac.id

## INTRODUCTION

*Pluchea* herbal tea is a product of dried *Pluchea* leaf processing introduced by world people (Srisook *et al.* 2012; Widyawati *et al.* 2016) because of the efficacy of the active components in *Pluchea* leaves, as a herbal plant that has been widely used for traditional medicine and food (Chan *et al.* 2022). *Pluchea* leaves are composed of many nutrients and bioactive compounds useful to body health. The nutrient compositions in the *Pluchea* leaves include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates, calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive compounds are comprised, *i.e.* chlorogenic acid, caffeic acid, 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, quercetin, myricetin, kaempferol, total anthocyanin,  $\beta$ -carotene, and total carotenoid (Suriyaphan 2014; Vongsak *et al.* 2018; Ruan *et al.* 2019; Widyawati *et al.* 2022; Chan *et al.* 2022).

The steeping process of *Pluchea* leaves can be performed with fresh or dry leaves in hot or boiling water for a few min (Suriyaphan 2014; Silva-Ramirez *et al.* 2020; Jayani *et al.* 2022). In Asia, especially in Indonesia, people usually consume the *Pluchea* infusion by steeping 2 g of powdered *Pluchea* leaves in a tea bag in 100 mL of hot or boiling water. Widyawati *et al.* (2016) claimed that steeping 2 g of *Pluchea* leaf powder at 95 °C for 5 min exhibits total phenolic and flavonoid contents, the ability to scavenge DPPH free radicals, and the capability to reduce ferric ions at 9.3, 22.0, 27.2, and 10.2 mg gallic acid equivalent (GAE)/ g sample, respectively. Werdani and Widyawati (2018) reported that drinking *Pluchea* leaf powder infusion in the morning and evening regularly (2 g/ 100 mL) can decline blood sugar levels.

The steeping of *Pluchea* herbal tea with hot water at 95 °C for 5 min certainly determines the stability and amount of extracted bioactive compounds that influence the biological activity especially antioxidant and antidiabetic activities. Silva-Ramirez *et al.* (2020) reported that the infusion process can influence the content and composition of the bioactive compounds and antioxidant activity of tea. Acar *et al.* (2022) stated that the infusion quality of herbal tea extract depends on a number of factors, *i.e.* storage and temperature. The polyphenol profile and antioxidant properties of herbal tea infusion decline with an increase in steeping or brewing and storage temperatures, as well as longer exposure periods.

Several studies have mentioned the effect of steeping temperature on the bioactive compound contents and antioxidant activity, as some white and green teas are effective with hot water at 90 °C for 7 min (Castiglioni *et al.* 2015), on rosehip tea is effective at infusion period

around 6–8 min at temperatures of 84–86 °C (Ilyasoglu and Arpa 2017), on the caffeine content extracted at the brewing temperature of coffee (Zarwinda and Sartika 2018), and the high total phenol content and antioxidant activity of dark tea at 92 °C for 27 min (Wang *et al.* 2022). The study of the effect of steeping temperature on *Pluchea* infusion was carried out to afford information about the most efficient preparation of powdered *Pluchea* leaves to get higher bioactive compounds, antioxidant, and antidiabetic activities.

Storage period tea usually for several months to yr *Pluchea* herbal tea also affects the levels of the bioactive compounds and biological activity (Jayani *et al.* 2022). Tea or herbal tea is generally stored at ambient temperature and packed in a tea bag or aluminum foil standing pouch or a combination of both. Many researchers reported that the storage period decreases the bioactive compounds plus antioxidant and antidiabetic activities, *i.e.* juice from *Momordica charantia* L. (Lin *et al.* 2020), dried *Piper betle* extracts (Ali *et al.* 2018), white tea (Xu *et al.* 2019), Kinnow-Amla beverages (Purewal *et al.* 2022), and whole-wheat flour (Zhang *et al.* 2021).

Therefore, this research studied the effect of steeping temperature and storage period on the bioactive compounds [total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC)], antioxidant [DPPH free radical scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)], and antidiabetic activities [ $\alpha$ -amylase (AA) and  $\alpha$ -glycosidase (GA) inhibition] of the infusion from powdered *Pluchea* leaves and on the phenolic compound profile.

## MATERIALS AND METHODS

### Raw Materials and Preparation

The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya, East Java, Indonesia. The *Pluchea* plants were included in the Asteraceae family with specifications according to the GBIF taxon ID number database:3132728 (Ferraris 2023). *Pluchea* leaves at 1–6 levels of each branch from the shoot were collected, sorted, washed, and dried to get a moisture content of around  $11.16 \pm 0.09\%$  dry basis (Widyawati *et al.* 2022). The dried *Pluchea* leaves were pulverized to a 45-mesh size powder. The *Pluchea* leaf powder was dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 °C for 10 min to reduce microbial organisms. Then, 2 g of the powder was packed into a paper filter infusion bag. Packed samples were stored for 0 (unstored) and 5 (stored) yr in a standing pouch before analysis.

In the research, one tea bag of *Pluchea* herbal tea that was stored for 0 (B1) and 5 (B2) yr was steeped with 100-mL hot water at various temperatures – including 60 (T1), 70 (T2), 80 (T3), and 95 (T4) °C for 5 min – with infusion method obtaining eight treatment combinations – namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, and T4B2. After the temperature of *Pluchea* infusion similar to ambient temperature was analyzed further.

### Reagents

The reagents used in the analysis include 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid,  $\alpha$ -amylase,  $\alpha$ -glucosidase, p-nitrophenyl- $\alpha$ -glucopyranoside (pNPG), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and (+)-catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, Folin-Ciocalteu's phenol, sodium nitric, aluminum chloride, ferric chloride, sodium dihydrogen phosphate, sodium phosphate, potassium ferricyanide, starch, acetic acid, and sodium hydroxide were purchased from Merck (Kenilworth, NJ, USA). All reagents used were of analytical grade except for distilled water which was purchased from PT Aqua Industry Surabaya.

### Analysis of the Bioactive Compounds

**Total phenolic content (TPC) analysis.** The TPC of treated *Pluchea* infusion was carried out using the technique by Gao *et al.* (2019). About 10  $\mu$ L *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10% were mixed in 10-mL volumetric flask and incubated for 5 min. Then, 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5% was added and filled up to 10 mL volume with distilled water. The blue color intensity of the solution was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at  $\lambda = 760$  nm, with gallic acid as the reference standard. The TPC was calculated using the following formula:  $y = 0.00009x + 0.008$ , with  $R^2 = 0.9941$ . The results were expressed as mg GAE/g samples.

**Total flavonoid content (TFC) assay.** The TFC of the samples was measured based on the reaction between AlCl<sub>3</sub> and NaNO<sub>2</sub> with the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim *et al.* 2021). The reaction between AlCl<sub>3</sub> and flavonoid compounds resulted in a yellow solution. About 30- $\mu$ L *Pluchea* infusion was mixed with 0.3 mL NaNO<sub>2</sub> 5% in 10-mL volumetric flask and incubated for 5 min. The mixture was added with 0.3 mL AlCl<sub>3</sub> 10% for 5 min. Then, 2-mL NaOH 1 M and distilled water were added to a 10-mL volume. Then, the red solution was produced after NaOH solution addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda = 510$  nm, with (+)-catechin as

the reference standard compound, and the results were expressed as mg catechin equivalents (CE)/ g samples using the following formula:  $y = 0.00008x - 0.0023$ , with  $R^2 = 0.9980$ .

**Total tannin content (TTC) analysis.** The TTC of the samples was analyzed using the Folin-Ciocalteu method (Chandran and Indira 2016). Approximately 10- $\mu$ L *Pluchea* infusion was added with 1-mL Folin-Ciocalteu's phenol reagent 10% in 10-mL volumetric flask and incubated for 5 min. Then, the mixture was added with 2-mL Na<sub>2</sub>CO<sub>3</sub> 7.5% and filled up to 10-mL volume with distilled water. The blue dark color solution was measured in UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at  $\lambda = 760$  nm, with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/ g samples using the following formula:  $y = 0.00009x + 0.0021$ , with  $R^2 = 0.9993$

### Analysis of the Antioxidant Potential

**DPPH free radical scavenging activity assay.** The DPPH free radical scavenging activity (DPPH) was measured by the spectrophotometric method (Widyawati *et al.* 2017) to determine the ability of the phytochemicals in the *Pluchea* leaf infusion to donate hydrogen atoms to the nitrogen atom in DPPH, resulting in the formation of DPPH-H compound exhibiting a yellow-colored solution. About 25  $\mu$ L *Pluchea* leaf infusion was poured into the reaction tube, into which 3-mL DPPH solution (4 mg/ 100 mL) was added. After incubation for 15 min in a dark room, the absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda = 517$  nm. The reference standard compound was gallic acid, and the results of the analysis were expressed as mg GAE/g samples calculated using the following formula:  $y = 0.146x + 1.7896$ , with  $R^2 = 0.9975$ .

**Ferric-reducing power (FRAP) analysis.** FRAP was determined following the method used by Widyawati *et al.* (2014) method. Approximately 10  $\mu$ L of samples were added to 2.5 mL phosphate buffer pH 6.6 and 2.5 mL of 1% potassium ferricyanide in the reaction tube. Then, the mixture was shaken and incubated for 20 min at 50 °C. Finally, 2.5 mL chloroacetic acid 10% (w/v) was added. Into the 2.5-mL supernatant, 2.5 mL distilled water and 0.5 mL ferric chloride 0.1% w/v were added, and the mixture was incubated for 10 min. The potency of the samples reducing iron (III) to iron (II) ion was indicated by the intensity of blue color formed that was measured using UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda = 700$  nm. The intensity of the blue color indicated a higher reducing capacity. The reducing power, expressed as mg GAE/g samples, was calculated using the following formula:  $y = 0.0002x + 0.0256$ , with  $R^2 = 0.9906$ .

### Analysis of the Antidiabetic Properties

**$\alpha$ -amylase enzyme inhibition (AA) capacity assay.** *In vitro* AA followed the procedure, as described by Widyawati *et al.* (2020). Each 500  $\mu$ L of the samples was mixed with starch 1% (w/v) and sodium acetate buffer pH 5. Into 250  $\mu$ L of the mixture, an  $\alpha$ -amylase solution (0.1 g of this enzyme 12.5 unit/mL) was added and then dissolved in 50 mL of 0.2 M sodium acetate pH 5. The mixture was shaken, into which 2-mL sodium hydroxide 1M was added. Before the analysis, this mixture was incubated at 37 °C for 10 min. Then, the capacity of the  $\alpha$ -amylase enzyme to hydrolyze the starch to release glucose was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda = 540$  nm. The inhibition percentage of  $\alpha$ -amylase was assessed using the formula  $(ACb - ACa) - (As - Ab) / (ACb - ACa) \times 100\%$  – where ACb is the absorbance of 100% enzyme activity (solvent with the enzyme), ACa is the absorbance of 0 % enzyme activity (solvent without the enzyme), As is the absorbance of the test sample with enzyme, and Ab is the absorbance of test sample without enzyme.

**$\alpha$ -glucosidase enzyme inhibition (GA) capacity assay.** The analysis of the  $\alpha$ -glycosidase inhibitor activity (GA) was done using the method of Widyawati *et al.* (2020) with slight modifications. About 150- $\mu$ L samples containing 100- $\mu$ L *Pluchea* infusion and 50  $\mu$ L pNPG (0.0150 g in 100-mL sodium phosphate 0.2 M at pH 7) were reacted with 50- $\mu$ L  $\alpha$ -glycosidase 2 mM (0.0833 unit/mL); then, the mixture was incubated at 37 °C for 15 min. The reaction was stopped with the addition of 1000- $\mu$ L sodium carbonate 0.2 M. The amount of these enzymes that did not react with bioactive compounds of *Pluchea* infusion hydrolyzed pNPG as a substrate to result in p-nitrophenol. The inhibition activity of the *Pluchea* infusion was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at  $\lambda = 405$  nm. The inhibition percentage of  $\alpha$ -glycosidase was calculated using the formula  $(ACb - ACa) - (As - Ab) / (ACb - ACa) \times 100\%$  – where ACb is the absorbance of 100% enzyme activity (solvent with enzyme), ACa is the absorbance of 0% enzyme activity (solvent without enzyme), As is the absorbance of test sample with enzyme, and Ab is the absorbance of test sample without enzyme.

### Analysis of Phenolics

The phenolic compounds of the samples were analyzed using high-performance liquid chromatography (HPLC) based on the method of Kongkiatpaiboon *et al.* (2018) with modifications. Each *Pluchea* infusion was sonicated for 15 min (Branson 1510); then, the sample was filtered using a filter syringe (Whatmann, 0.2  $\mu$ m, NYL). About 20  $\mu$ L of the sample was injected in an HPLC (LC20AD series, Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC LC-20AD pump, CTO-30A

column oven, CBM-20A/20 Alite system controller, and SPD-20A/20 AV UV-Vis detector. Separation of phenolic compounds in samples was carried out using a Shim-pack VP-ODS C18 column (ID 5  $\mu$ m  $\times$  50 mm  $\times$  4.6 mm) with a GVP-ODS Cartridge guard column (two pieces) (ID 10 mm  $\times$  4.6 mm). The mobile phase used consisted of a solution of [A] 0.5% acetic acid in water and [B] absolute methanol. Analysis was carried out using a gradient system in the following order: initial conditions of 10% B in A to 50% B in A were maintained for 40 min; then, 100% B was maintained for 20 min. Next, the column was re-equilibrated with 10% B in A and maintained for 10 min before analysis of the next sample. The sample flow rate was set at 1.0 mL/min with a controlled temperature of 40 °C. Detection was used at a wavelength of 280 nm. The reference standard used were gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid. All of the reference standard was dissolved in distilled water and prepared similarly to the samples before being injected in HPLC.

**Experiment design and statistical analysis.** The research design used a randomized block design with two factors, *i.e.* the steeping temperature (T) and the storage period. *Pluchea* leaf blades were subjected to 4 steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95 °C (T4), and the storage period of 0 year /unstored (B1), and 5 year /stored (B2) resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic was repeated for six periods. The data analysis of samples was repeated for six periods. The data were analyzed using a paired t-test at  $\alpha \leq 0.05$ , treatment means of specific phenolic compounds that were identified were expressed as the mean  $\pm$  SD. The analysis used SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

## RESULTS AND DISCUSSIONS

### Bioactive Compounds

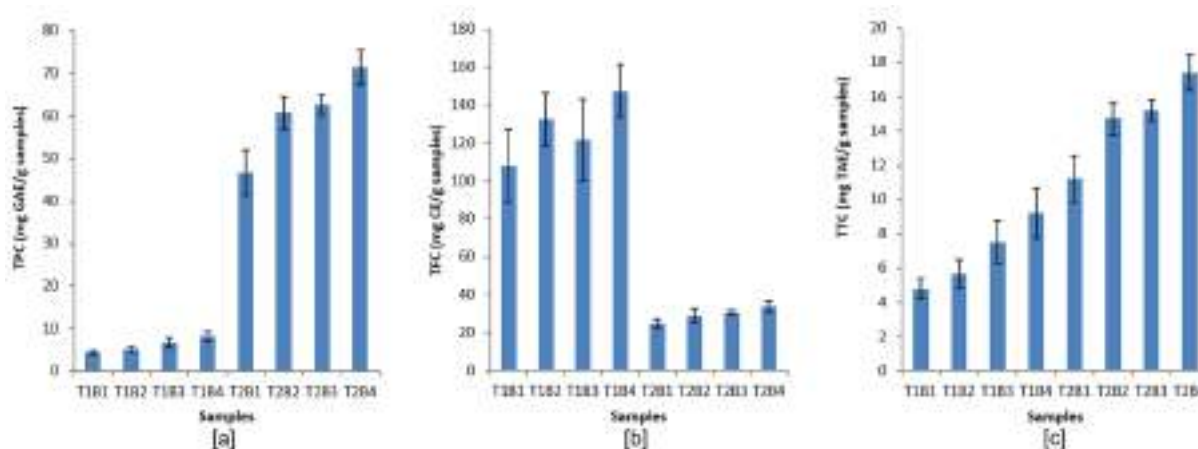
**Phenolic compounds.** Bioactive compounds are active compounds in plants that are essential to protect body health (Nguyen and Chuyen 2020). These compounds usually have many biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer, antimicrobial, antibacterial, anti-cholesterol, and so on (Suriyaphan 2014; Acar *et al.* 2022). Phenolic compounds have potential redox properties that can scavenge free radicals that can cause a number of chronic diseases (Noreen *et al.* 2017; Aryal *et al.* 2019; Acar *et al.* 2022).

The TPC of *Pluchea* infusion at different steeping temperatures and storage periods generally significantly increased with increasing steeping temperature and storage period based on paired t-test at  $\alpha \leq 0.05$  (Figure 1a). Steeped and stored infusion had significantly higher amounts of phenolic compounds than the samples that were steeped and unstored. Further, the highest TPC was observed in samples infused at 95 °C and stored for 5 yr (at  $71.38 \pm 4.14$  mg GAE/g sample), whereas the lowest was measured in the unstored samples and infused at 60 °C (at  $4.39 \pm 0.49$  mg GAE/g sample). The phenolic content of stored samples that were steeped only at 60 and 95 °C showed a significant increase in their phenolic content. This implies that the steeping temperature and the storage periods significantly resulted in the high amounts of phenolic compounds in the infusions. Results also indicated that phenolic compounds were generally greater in the infusion at high steeping temperatures and long storage periods. This could have been due to the fact that the steeping temperature and storage period could cause the process of degradation, oxidation, and leaching or release of phenolic compounds. Phenolic compounds are water-soluble and, thus, soaking in hot water for a certain period, as steeping causes the migration process of more phenolic compounds to the water because of longer exposure of phenolic compounds to water (Castiglioni *et al.* 2015; Kilic *et al.* 2017; Acar *et al.* 2022). Su *et al.* (2019) reported that temperature treatment can stimulate the release of phenolic compounds and increase the antioxidant activity of lychee juice stored at different temperatures of 4 and 45 °C and different long storage (fresh and 72 h).

Temperature treatment degrades (or hydrolyzes) the hydrogen bond between phenolic compounds and proteins, resulting in an increase of phenolic compounds

when exposed to higher temperatures (Ali *et al.* 2018; Jayani *et al.* 2022; Ramphinwa *et al.* 2023). Zhang *et al.* (2021) reported that phenolic compounds present in plants are not completely stable but are easily degraded during storage after harvest. Reblova (2012) claimed that antioxidant compounds can be slowly degraded with increasing temperature. Fibrianto *et al.* (2021) also stated that the brewing temperature has an effect on the extracted antioxidant compounds such as alkaloids, catechins, and tannins. Thus, there is an assumption that temperature and storage caused the degradation, oxidation, and hydrolysis of the phenolic compounds period, resulting in the increased amount of the phenolic compounds at higher steeping temperatures and longer storage periods.

Simple phenolic compounds are identified in steeped and stored. *Pluchea* leaf infusion included gallic acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids is shown in Table 1. The treatment effects using the t-test at  $\alpha \leq 0.05$  showed that gallic acid and kaempferol content were insignificantly different at various steeping temperatures and storage periods. The concentration of quercetin and 3,5-di-*O*-caffeoylquinic acid of the unstored and stored *Pluchea* infusion was significantly different from the rest of the samples between 70 °C, whereas (+)-catechin concentration of *Pluchea* infusion was only significantly different at 95 °C. The myricetin content was significantly different at 80 and 95 °C. The 3,4-di-*O*-caffeoylquinic acid content showed significant difference at 60, 80, and 95 °C, whereas 4,5-di-*O*-caffeoylquinic acid content was only significantly different at 60 °C.



**Figure 1.** Bioactive compound contents of *Pluchea* infusion at different steeping temperatures and storage periods: [a] total phenolic content, [b] total flavonoid content, and [c] total tannin content. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  $\pm$  standard deviation (n = 6). Samples: T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T2B2-steeped at 70 °C, stored for 5 yr; T2B3-steeped at 80 °C, stored for 5 yr; T2B4-steeped at 95 °C, stored for 5 yr.



**Table 1.** Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperatures and storage periods.

Phenolic compounds	Steeping temperature (°C)	Mean ± SD (unstored)	Mean ± SD (stored)	Mean difference ±SD	Sig. (two-tailed)
Gallic acid (µg/g samples)	60	0.2132 ± 0.0027	0.2364 ± 0.0015	0.0375 ± 0.0175	0.2030
	70	0.2157 ± 0.0013	0.2324 ± 0.0214	0.0167 ± 0.0227	0.4870
	80	0.2234 ± 0.0122	0.2347 ± 0.0078	0.0386 ± 0.0264	0.2870
	95	0.2316 ± 0.0104	0.2402 ± 0.0169	0.0086 ± 0.1990	0.8500
(+) -Catechin (µg/g samples)	60	0.3425 ± 0.0110	0.5085 ± 0.0111	-0.1576 ± 0.0885	0.241
	70	0.3260 ± 0.0265	0.5448 ± 0.0006	-0.2188 ± 0.0259	0.053
	80	0.3240 ± 0.0222	0.5023 ± 0.0773	-0.1451 ± 0.0248	0.077
	95	0.4039 ± 0.0320	0.5995 ± 0.0372	-0.2049 ± 0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756 ± 0.1234	1.4762 ± 0.0271	-1.2887 ± 0.3222	0.111
	70	0.2587 ± 0.0160	1.4245 ± 0.2526	-1.1657 ± 0.2695	0.103
	80	0.4175 ± 0.0104	1.4570 ± 0.0925	-1.0391 ± 0.0841	0.036*
	95	0.8786 ± 0.0434	2.6138 ± 0.0695	-1.1735 ± 0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220 ± 0.0268	0.6220 ± 0.0706	-0.5999 ± 0.9733	0.544
	70	0.1530 ± 0.0511	1.0708 ± 0.0289	-0.9177 ± 0.0222	0.011*
	80	0.3666 ± 0.0103	0.8629 ± 0.0815	-0.1082 ± 0.4462	0.790
	95	0.6559 ± 0.0570	2.0230 ± 0.0573	-1.4123 ± 0.3203	0.101
Kaempferol (µg/g samples)	60	0.1394 ± 0.0202	0.3675 ± 0.0183	-0.3207 ± 0.1122	0.154
	70	0.0514 ± 0.0037	0.3726 ± 0.0944	0.3213 ± 0.0907	0.125
	80	0.3699 ± 0.0924	0.7966 ± 0.0366	-0.4267 ± 0.2727	0.271
	95	0.5913 ± 0.0239	0.9478 ± 0.0287	-0.3565 ± 0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103 ± 0.0628	2.4863 ± 0.0270	-1.8760 ± 0.2074	0.050*
	70	0.6271 ± 0.0099	2.3403 ± 0.0325	-1.7131 ± 0.3152	0.082
	80	0.7967 ± 0.03060	2.6278 ± 0.0211	-1.8311 ± 0.0095	0.002*
	95	1.5386 ± 0.0668	4.0211 ± 0.0851	-2.4825 ± 0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635 ± 0.0628	0.9449 ± 0.0501	-0.2814 ± 0.4458	0.536
	70	0.6162 ± 0.0099	0.9485 ± 0.0794	-0.3323 ± 0.0301	0.041*
	80	0.6601 ± 0.0306	0.9099 ± 0.0387	-0.2498 ± 0.3127	0.461
	95	0.6642 ± 0.0668	1.3156 ± 0.0166	-0.6514 ± 0.2666	0.179
4,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.4906 ± 0.0060	1.1842 ± 0.0120	-0.6886 ± 0.2723	0.018*
	70	0.4807 ± 0.0034	1.0089 ± 0.0736	-0.5281 ± 0.0702	0.060
	80	0.5299 ± 0.0053	1.2382 ± 0.1435	-0.7082 ± 0.1489	0.094
	95	1.0018 ± 0.0526	1.3797 ± 0.2170	-0.3086 ± 0.3086	0.333

Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean ± standard deviation (n = 6). Samples: T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T3B2-steeped at 70 °C, stored for 5 yr; T3B3-steeped at 80 °C, stored for 5 yr; T3B4-steeped at 95 °C, stored for 5 yr.

Results further showed that gallic acids and kaempferol were relatively stable, as reflected by the insignificant changes when exposed to the different steeping temperatures and storage periods. Myricetin, (+)-catechin, and 3,4-di-O-caffeoylquinic acid showed a drastic increase

at higher steeping temperatures and longer storage periods, implying that these compounds tended to be relatively labile. Quercetin, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid underwent moderate changes. Therefore, myricetin, (+)-catechin, and 3,4-di-

*O*-caffeoylquinic acid were easier to dissolve or degrade to form simple phenolic acids at higher temperatures and storage period (Su *et al.* 2019; Ali *et al.* 2018; Jayani *et al.* 2022; Ramphinwa *et al.* 2023; Zhang *et al.* (2021). Degradable polyphenol compounds have a simple structure and free hydroxyl groups that can react with Folin-Ciocalteu's phenol reagent, resulting in a complex blue solution that can be detected as TPC.

**Flavonoid content (TFC).** Flavonoids are the major phenolic compounds that have potential chemical and biological activities such as radical scavenging and antimicrobial activities (Ayele *et al.* 2022; Chandra *et al.* 2014) that can protect the human body from the oxidative stress caused by many degenerative diseases – especially cancer, cardiovascular problems, and aging (Mathur and Vijayvergia 2017). The TFC of steeped *Pluchea* infusion decreased with a longer storage period. Unstored samples exhibited higher flavonoid content than the stored samples. The statistical analysis using a paired t-test at  $\alpha \leq 0.05$  showed that the TFC of *Pluchea* infusion was significantly different between the steeped unstored and steeped stored samples (Figure 1b). The highest TFC was exhibited by the unstored samples steeped at 95 °C at about  $147.42 \pm 14.03$  mg CE/g sample. The TFC was significantly lower in the stored samples than those of the unstored samples, implying that the increase in the flavonoid content of the infusion was affected primarily by the steeping temperature.

**Tannin content (TTC).** Tannins are bioactive compounds that provide properties, such as astringent, anti-diarrheal, antibacterial, and antioxidant (Malangngi *et al.* 2012). Generally, results indicated that the TTC of *Pluchea* infusion significantly increased with increasing steeping temperature and storage period (Figure 1c). Among the unstored steeped samples, the tannin content was significantly lowest in the samples infused at 60 °C at about  $4.81 \pm 0.58$  to  $17.42 \pm 1.04$  mg TAE/g samples, which was significantly different lower from that of the lowest tannin content of the stored samples. Among the stored and steeped samples, the highest tannin content was observed at samples steeped at 95 °C about  $17.42 \pm 1.04$  mg TAE/g samples and was significantly different from that of the highest tannin content of the unstored steeped samples at 95 °C about  $9.22 \pm 1.48$  mg TAE/g samples. Indicating that the tannin content was primarily affected by a longer storage period than high steeping temperature. The condensation of catechins to tannins is a dominant process occurring in tea leaves that is accelerated during the maceration of raw tea leaves (Kowalska *et al.* 2021) and could have contributed to the observed increase in the tannin content in the treated samples.

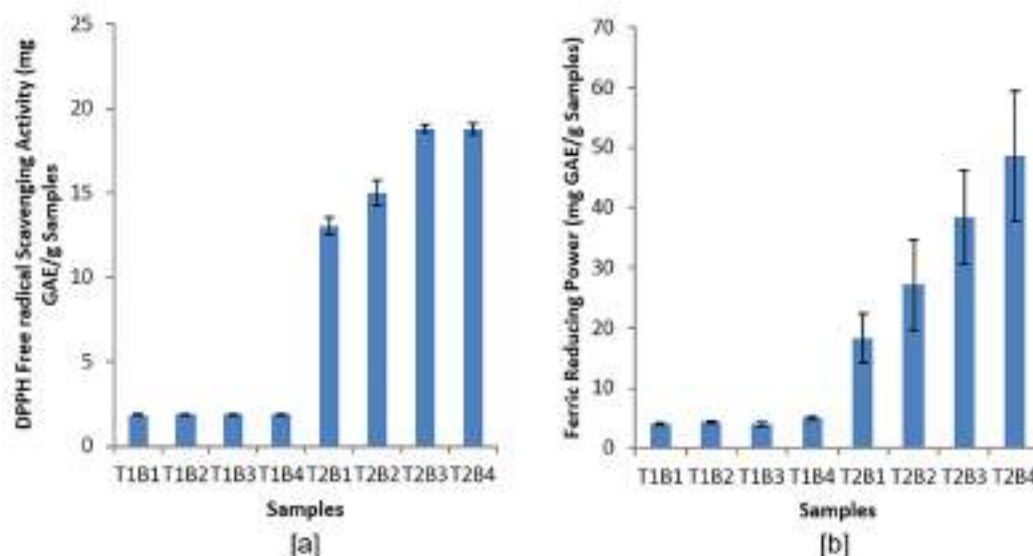
Nonetheless, high temperatures and long storage periods can cause the degradation of tannins to catechins. Rusita *et al.* (2019) emphasized that tannins are polar

thermostable complex compounds that are resistant to heating, indicating that even with the exposure to high temperature, the tannins still remained high in the treated samples period.

**Antioxidant activity.** Antioxidant activity is the capability of compounds to inhibit the oxidation of macromolecules from biological targets that are involved in oxidative chain reactions (Ali *et al.* 2005; Oh *et al.* 2013). The antioxidant activity assay was done in this research using DPPH and FRAP methods. The phenolic compounds are active antioxidants with antioxidant capability that depends on their redox properties. The structure of phenolic compounds determines the effectivity to donate hydrogen atoms, which is negatively correlated with the O-H phenolic bond strength. The antioxidant power of phenolic compounds is due to the weak hydrogen bonds in the OH group of the phenolic compound, so that it is easier to donate hydrogen atoms (Kruk *et al.* 2022). The mechanism of phenolic compounds as antioxidants depends on their ability to donate hydrogen atoms and transfer electrons, as well as reducing agents and singlet oxygen quenchers (Ali *et al.* 2005; Huang *et al.* 2005).

**DPPH free radical scavenging activity (DPPH).** DPPH is a free radical that is often used to evaluate antioxidant activity because this method is simple and is suitable for measuring the donating hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of DPPH to change to a yellow color (Munteanu and Apetrei 2021; Baliyan *et al.* 2022). Figure 2a shows that the free radical scavenging properties of the stored and steeped samples were significantly higher than the unstored steeped samples. It can also be observed that the free radical scavenging property was significantly different among the stored and steeped samples but insignificant among the unstored and steeped sample period. *Pluchea* infusion stored at room temperature for 5 yr resulted in high free radical scavenging activity by more than 10%. Steeping at higher temperatures significantly increased the DPPH free radical scavenging activity in stored *Pluchea* infusion by around 15–25%. This implies that the higher free radical scavenging property was primarily affected by the storage period than the steeping temperature. During the storage process, it is possible to form complex phenolic compounds that provide a high ability to scavenge free radicals (Thanajiruschaya *et al.* 2010).

The scavenging activity of the samples was strongly and positively correlated with total phenolic and tannin contents but inversely with total flavonoid levels (Table 2). The antioxidant activity was strongly and negatively correlated with flavonoid content. The storage period could be reduced flavonoid content. The study also demonstrated that longer storage period and higher infusion temperatures produced many simple phenolic compounds with free



**Figure 2.** Antioxidant activity of *Pluchea* tea at different steeping temperatures and storage periods: [a] DPPH; [b] FRAP. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  $\pm$  standard deviation ( $n = 6$ ). Samples : T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T2B2-steeped at 70 °C, stored for 5 yr; T2B3-steeped at 80 °C, stored for 5 yr; T2B4-steeped at 95 °C, stored for 5 yr.

**Table 2.** Pearson correlation coefficients between bioactive contents (TPC, TFC, and TAC), antioxidant activity (DPPH and FRAP), and antidiabetic activity (AA and GA).

	TPC	TFC	TTC	DPPH	FRAP	$\alpha$ -glucosidase	$\alpha$ -amylase
TPC	1						
TFC	-0.93589	1					
TTC	0.960028	-0.81321	1				
DPPH	0.992776	-0.93992	0.942273	1			
FRAP	0.953366	-0.82636	0.947778	0.956242	1		
$\alpha$ -glucosidase	-0.55512	0.349873	-0.71534	-0.5272	-0.55947	1	
$\alpha$ -amylase	-0.70842	0.429393	-0.8569	-0.69579	-0.80548	0.725161631	1

Significant at the 0.05 level (two-tailed)

hydroxyl groups capable to donate hydrogen atoms to DPPH free radicals. Many phenolic acids such as gallic acids, (+)-catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids have established potential antioxidant activity (Kumar and Goel 2019) (Table 1). Kruk *et al.* (2022) informed that the capability of phenolic compounds to donate hydrogen atom depends on the chemical structure, number, and position of hydroxyl groups attached to a benzene ring, a double bond between C2 and C3 rings, and a carbonyl group (C=O) on the C ring at C4. The effectivity of antioxidant compounds to donate hydrogen atoms is determined by O-H bond dissociation energy.

The free radical scavenging property observed in the

study was not consistent with the results of the study by Moraes-de Souza *et al.* (2008). The research shows that the TPC of herbal infusion is lowly correlated with free radical scavenging activity. However, Dobrinas *et al.* (2021) informed that TPC is positively and significantly correlated with the free radical scavenging property of tea infusion.

**Ferric reducing antioxidant power (FRAP).** FRAP is an analysis of the antioxidant power of the phytochemical compounds that is based on the ability of antioxidant compounds to reduce iron ions of potassium ferricyanide ( $Fe^{3+}$ ) to potassium ferrocyanide ( $Fe^{2+}$ ). Potassium ferrocyanide reacts with ferric chloride to form a ferric-ferrous complex and results green color solution (Widyawati *et al.* 2017; Raharjo and Haryoto 2019).

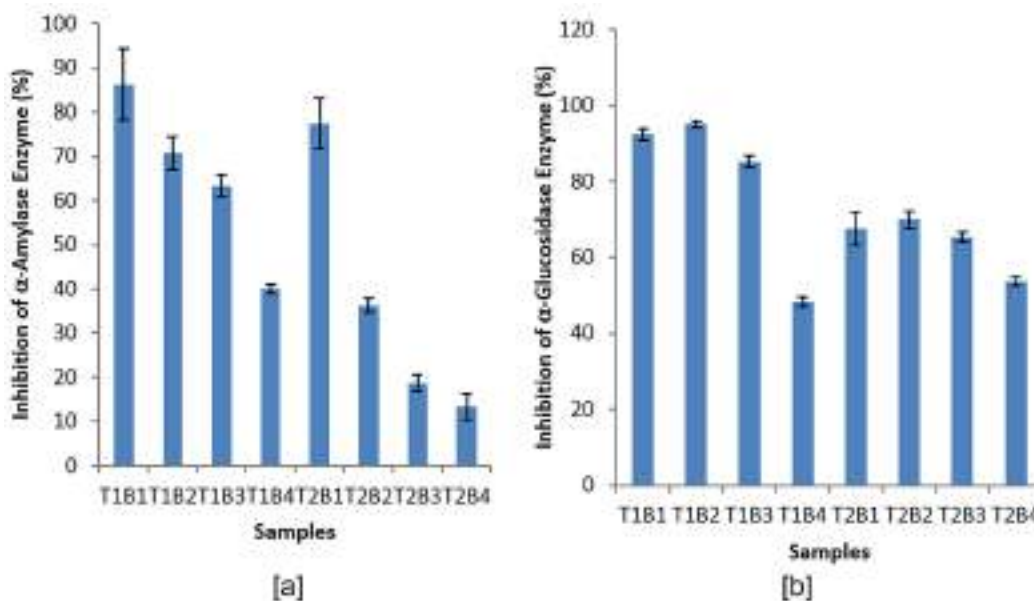
The results showed that the FRAP increased at higher steeping temperatures and longer storage periods. The lowest FRAP was observed in the unstored samples, which were steeped at 60 °C at  $3.95 \pm 0.17$  mg GAE/g samples, and the highest was exhibited in *Pluchea* infusion which was stored for 5 yr at 95 °C at  $48.63 \pm 10.83$  mg GAE/g samples (Figure 2b). FRAP increased significantly as the steeping temperature was increased. FRAP of the samples stored for 5 yr was also significantly higher than the unstored samples at  $\alpha \leq 0.05$ .

This is in contrast with the study on the antioxidant activity of DPPH and FRAP of matcha. The longer storage period reduces the levels of catechin content due to the catechins such as epigallocatechin gallate, epicatechin gallate, epigallocatechin, and epicatechin, which are bioactive compounds that have high antioxidant activity (Kim *et al.* 2020). The ferric-reducing capability of *Pluchea* could have been due to the presence of simple phenolic acid that can transfer electrons from their free hydroxyl groups of samples. The FRAP of *Pluchea* infusion was strongly and positively significantly correlated with the DPPH, TPC, and TTC but inversely to TFC.

### Antidiabetic Activity

**$\alpha$ -amylase enzyme inhibition activity (AA).** Antidiabetic activity is a measure of the potency of phenolic compounds to regulate the uptake of glucose by the cells from the

blood through the mediation of two digestive enzymes, *i.e.*  $\alpha$ -amylase and  $\alpha$ -glucosidase, which are involved in the control of dietary carbohydrate digestion and release in the postprandial blood glucose in human body (Fu *et al.* 2017). The phenolic compounds have the capability to bind with the protein component of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Martinez-Solis *et al.* 2022), resulting in the reduced activity of the enzymes. The results showed that lower steeping *Pluchea* leaf infusion was able to inhibit the action of the  $\alpha$ -amylase enzymes (Figure 3a). The *Pluchea* infusion exhibited a good AA of more than 50% and even almost 100% in unstored *Pluchea* infusion steeped at 60, 70, and 80 °C, with the highest at 60 °C and in stored *Pluchea* leaf infusion, which was steeped at 60 °C. The stored *Pluchea* leaf infusion steeped at 70, 80, and 95 °C for 5 min had lower enzyme inhibition activity of less than 50%, with the lowest at 95 °C around 13%. Widyawati *et al.* (2017) found that the ability to inhibit the  $\alpha$ -amylase enzyme in unstored *Pluchea* infusion steeped at 95 °C for 5 min was also low at 28.79%. Increasing the steeping temperature and storage period reduced the ability of the phytochemicals in the *Pluchea* infusion to inhibit the  $\alpha$ -amylase enzyme activity period. Table 2 further shows that the AA of *Pluchea* infusion was strongly and negatively significantly correlated with TPC, TTC, DPPH, and FRAP, but it was weakly and positively significantly correlated with TFC.



**Figure 3.** Antidiabetic activity of pluchea tea at different steeping temperatures and storage periods: [a]  $\alpha$ -amylase; [b]  $\alpha$ -glucosidase. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  $\pm$  standard deviation ( $n = 6$ ). Samples: T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T2B2-steeped at 70 °C, stored for 5 yr; T2B3-steeped at 80 °C, stored for 5 yr; T2B4-steeped at 95 °C, stored for 5 yr.

This inhibitory activity was thought to be contributed by other bioactive compounds besides phenolics, which are sensitive to steeping temperature and storage period. Li *et al.* (2018) stated that there are flavonoid compounds that contribute to the ability to inhibit the  $\alpha$ -amylase enzyme. Akah *et al.* (2011) reported that phytochemical compounds such as terpenoids, saponins, flavonoids, glycosides/carbohydrates, and alkaloids are good antidiabetic metabolites or  $\alpha$ -amylase enzyme activity inhibitors. Sangeetha and Vedesree (2012) explained that the ability of *Threspesia populnea* extract to inhibit the  $\alpha$ -amylase enzyme was determined by their phenolic compound content and protein. Moreover, the presence of the  $\alpha$ -amylase enzyme inhibitor in this extract may be proteinaceous or nonproteinaceous in nature. It was assumed that this enzyme inhibitory activity in *Pluchea* infusion also was determined by their protein and polyphenolic content. Aleixandre *et al.* (2022) also stated that phenolic acids have inhibition activity to  $\alpha$ -amylase enzyme depending on their structures. There are C=C double bonds conjugated with a carbonyl group of phenolic structures that stabilize the binding forces to the active site of the  $\alpha$ -amylase. The hydroxyl groups can bind by non-covalent interaction (hydrogen bonding, cation- $\pi$  interactions, salt bridge interactions, ionic interactions, or electrostatic forces) with amino acid residue at the active site in the  $\alpha$ -amylase enzyme. Elevated steeping temperatures and longer storage periods can easily cause the removal of the hydroxyl groups of phenolic compounds, which can reduce their ability to enzyme inhibition. The phenolic acids with a greater number of hydroxyl groups exhibits stronger capability to obstruct the  $\alpha$ -amylase enzyme.

**$\alpha$ -glucosidase enzyme inhibition activity (GA).**  $\alpha$ -glucosidase is an important enzyme in carbohydrate digestion, that catalysis the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and converts them into monosaccharides (glucose), thereby resulting in hyperglycemia (Nurcholis *et al.* 2014; Proenca *et al.* 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -glucosidase enzyme is used to determine their antidiabetic activity. This is supported by Werdani and Widyawati (2018) stated that *Pluchea* infusion has the potential as an antidiabetic agent. Widyawati *et al.* (2020) found that the steeping of unstored *Pluchea* infusion at 95 °C for 5 min has an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857%.

Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme decreased with increasing steeping temperature and storage period. Steeping at 95 °C of the unstored *Pluchea* leaf infusion obtained the lowest inhibitory ability, *i.e.*  $48.32 \pm 1.27\%$ , and the highest inhibitory activity was at 70 °C at  $95.11 \pm 0.70\%$ . The results of a paired t-test showed that GA

of *Pluchea* infusion was significantly different between steeping temperature and long storage. Figure 3 further shows that the ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher than the ability to inhibit the  $\alpha$ -amylase enzyme. Data analysis in Table 2 showed that the TFC of the *Pluchea* leaf infusion was influenced weakly and positively by GA and AA, but the GA and AA were not affected by TPC, TTC, DPPH, and FRAP. Li *et al.* (2018) stated that flavonoid compounds can inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. Dias *et al.* (2021) stated that flavonoid compounds such as rutin, myricetin, kaempferol, and quercetin have antioxidant and antihyperglycemic activities. The ability to inhibit the action of enzymes from flavonoid compounds is determined by the position and number of hydroxyl groups, the number of double bonds in rings A and B, and the heterocyclic ring in ring C. Tadera *et al.* (2006) and Zhang *et al.* (2014) also explained that flavonoid compounds of samples significantly inhibit the  $\alpha$ -glucosidase enzyme activity.

The ability to inhibit the  $\alpha$ -glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping temperature and long storage. Figure 3 also showed that the capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater than the  $\alpha$ -amylase enzyme because the mechanism of the two enzymes was different, according to the opinion of McCue *et al.* (2005). The mechanism of the  $\alpha$ -glucosidase enzyme inhibitor includes making the sugar mimic structure, binding using ionic bonds with nucleophilic, making the transition state-like structure, binding hydrogen with catalytic acid residue, interacting ionic and hydrophobic with site other than the active site, and binding covalent with enzymes through an epoxy or aziridine group (Moorthy *et al.* 2012). Then, the mechanism of the  $\alpha$ -amylase enzyme inhibitor includes blocking carbohydrates, thereby limiting the digestibility and absorption of carbohydrates, as well as blocking the active centers of several subsites of the enzyme (Gong *et al.* 2020).

Widyawati *et al.* (2017) stated that phenolic and non-phenolic compounds can inhibit the  $\alpha$ -glucosidase enzyme activity. The ability of bound phenolic compounds to inhibit  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, which may occur in *Pluchea* infusion during storage, affects the structure and profile of phenolic and non-phenolic compounds. Arsiningtyas *et al.* (2014) explained that the methyl-esterified quinic acid with the caffeic groups such as 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid, and 1,3,4,5-tetra-*O*-caffeoylquinic acid of *Pluchea* leaves inhibits the

$\alpha$ -glucosidase enzyme activity. The resulting analysis of caffeoylquinic acids (3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid) in stored *Pluchea* leaf infusion had higher concentrations than in unsteeped *Pluchea* infusion, and the concentrations of the simple phenolic compounds were increased at higher steeping temperatures, but the GA of them was reduced. It means that the methyl-esterified quinic acid with the caffeic groups had more potential to inhibit  $\alpha$ -glucosidase enzyme than free caffeoylquinic acid.

This study showed that the increasing steeping temperature and storage period caused degradation of polyphenolic compounds to produce simple phenolic compounds such as gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid that increased the TPC and TTC. The increase in the simple phenolic concentration of the *Pluchea* leaf infusion caused higher antioxidant activity and lower antidiabetic activity.

## CONCLUSION

The TPC of *Pluchea* infusion at different steeping temperatures and storage periods generally significantly increased with increasing steeping temperature and storage periods. Steeped and stored infusion had significantly higher amounts of phenolic compounds than the samples that were steeped and unsteeped. TPC was highest in the stored and steeped at 95 °C and lowest in the unsteeped and steeped at 60 °C. Unsteeped steeped samples exhibited significantly higher flavonoid content than the stored steeped samples. The highest TFC was exhibited by the unsteeped samples steeped at 95 °C. The TTC of *Pluchea* leaf infusion significantly increased with increasing steeping temperature and storage period. Among the unsteeped steeped samples, the tannin content was significantly the lowest in the samples steeped at 60 °C and the highest in the samples steeped at 95 °C.

The DPPH of the stored and steeped *Pluchea* leaf infusion was significantly higher than the unsteeped steeped samples. The free radical scavenging property was highest in the stored samples steeped at 80 and 95 °C. The free radical scavenging activity of the samples was strongly and positively correlated with total phenolic and tannin contents but inversely with total flavonoid levels. The FRAP significantly increased with increasing steeping temperature and longer storage periods. The lowest FRAP was found in the unsteeped samples that were steeped at 60 °C, and the highest was exhibited in *Pluchea* samples that were stored for 5 yr and steeped at 95 °C. The FRAP of *Pluchea* leaf infusion was significantly strong and positively correlated with the free radical scavenging

property, TPC, and TTC but inversely with TFC. The AA was generally found to be higher at lower steeping temperatures of the unsteeped *Pluchea* leaf infusion than at higher steeping temperatures of the stored sample. The AA capacity of the *Pluchea* leaf infusion showed a significantly strong and negative correlation with TPC, TTC, DPPH, and FRAP, but it was weakly and positively correlated significantly with TFC.

The ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme decreased at high steeping temperatures and long storage periods. The highest inhibitory activity was obtained in the unsteeped *Pluchea* leaf infusion that was steeped at 70 °C, whereas the lowest was obtained in the unsteeped sample that was steeped at 95 °C. The ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher than the ability to inhibit the  $\alpha$ -amylase enzyme. The GA was significantly strong and negative TPC, TTC, DPPH, and FRAP, and it was weakly and positively correlated significantly with TFC.

The simple phenolic compounds identified in *Pluchea* leaf infusion may affect the presence of the bioactive compounds, antioxidant potential, and antidiabetic properties at different steeping temperatures and storage periods – including gallic acids, (+)-catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids.

## ACKNOWLEDGMENTS

The authors would like to thank the Ministry of Education and Culture of the Republic of Indonesia for a fundamental research grant to higher education institutions in 2022.



## STATEMENT ON CONFLICT OF INTEREST

The authors declare no conflict of interest.

## REFERENCES

- ACARA, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food wastes: evaluation of temperature and time effects on quality parameters. *Applied Food Res* 2(100087): 1–8.
- ALEIXANDRE A *et al.* 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase and influence of reaction conditions. *Food Chem* 372(131231): 1–9.

- ALI *et al.* 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile. *Food Chem* 89: 27–36.
- ALI A *et al.* 2018. Impact of storage conditions on the stability of predominant phenolic constituents and antioxidant activity of dried *Piper betle* extracts. *Molecules* 23(24): 1–13.
- AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and methanol extract and fractions of *Gongronema latifolium* (*Asclepidaceae*) leaves in alloxan diabetic rats. *J Applied Pharm Sci* 01(09): 99–102.
- ARSININGTYAS IS *et al.* 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product use. *Natural Product Res* 28(17): 1350–1353.
- ARYAL S *et al.* 2019. Total phenolic content, flavonoid content, and antioxidant of wild vegetables from Western Nepal. *Plants* 8(96): 1–12.
- AYELE DT, AKELE ML, MELESE AT. 2022. Analysis of total phenolic contents, flavonoids, antioxidant, and antibacterial activities of *Croton macrostachyus* root extracts. *BMC Chem* 16(30): 1–9.
- BALIYAN S *et al.* 2022. Determination of antioxidants by DPPH radical scavenging activity and quantitative phytochemical analysis of *Ficus religiosa*. *Molecules* 27(1326): 1–19.
- CASTIGLIONI S *et al.* 2015. Influence of steeping conditions (time, temperature, and particle size) on antioxidant properties and sensory attributes of some white and green teas. *Intern J Food Sci Nut Online*. p. 1–7.
- CHAN EWC *et al.* 2022. *Pluchea indica*: an updated review of its botany, uses, bioactive compounds, and pharmacological properties. *Pharmaceutical Sciences Asia* 49(1): 77–85.
- CHANDRA S *et al.* 2014. Assessment of total phenolic and flavonoid content, antioxidant properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit crops: a comparative study. *Hindawi Publishing Corporation Evidence-based Complementary Alter Med*. p. 1–9.
- CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic, flavonoids, tannin, and chlorophyll content of leaves of *Strobilanthes kunthiana* (neelakurinji). *J Med Plants Studies* 4(4): 282–286.
- DIAS MC, PINTO DCGA, SILVA AMS. 2021. Plant flavonoids: chemical characteristics and biological activity. *Molecules* 26(5377): 1–16.
- DOBRINAS S, SOCEANU A, POPESCU V, POPOVICI IC, JITARIU D. 2021. Relationship between total phenolic content, antioxidant capacity, and Fe and Cu content from tea plant samples at different brewing times. *Processes* 9(1311): 1–11.
- FERRARIS Z. 2023. *Pluchea indica* Less. iNaturalist.org. <https://www.gbif.org/occurrence/4022273660>
- FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional properties and visual appearance of Dampit Robusta coffee leaves tea. *Advances in Food Sci Sustain Agric Agro-industrial Engineering* 4(1): 25–32.
- FU L *et al.* 2017. Total phenolic contents and antioxidant capacities of herbal and tea infusions. *Intern J Molecular Sci* 12: 2112–2124.
- GAO MR *et al.* 2019. A theoretical and experimental study: the influence of different standards on the determination of total phenol content in the Folin-Ciocalteu assay. *J Food Measurement and Characteristic* 13: 1349–1356.
- GONG L, FENG D, WANG T, REN Y, LIU Y, WANG J. 2020. Inhibitors of  $\alpha$ -amylase and  $\alpha$ -glucosidase: potential linkage for whole cereal foods on prevention of hyperglycemia. *Food Sci Nut* 2020(8): 6320–6337.
- HUANG D, OU B, PRIOR RL. 2005. The chemistry behind antioxidant capacity assays. *J Agric Food Chem* 53: 1841–1856.
- ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties of rosehip tea beverage: study by response surface methodology. *J Food Sci Tech* 54(11): 3737–3743.
- JAYANI NIE *et al.* 2022. Evaluation of stability and quality characteristics of moringa (*Moringa oleifera*) herbal tea during storage. *Food Res* 6(4): 399–406.
- KIM JM *et al.* 2020. Effect of storage temperature on the antioxidant activity and catechins stability of matcha (*Camellia sinensis*). *Food Science and Biotechnology* 29(9): 1261–1271.
- KILIC *et al.* 2017. Antioxidant properties of some herbal teas (green tea, senna, corn silk, rosemary) brewed at different temperatures. *Intern J Secondary Metabolite* 4(3): 142–248.
- KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves from different provenances in Thailand. *Revista Brasileira de Farmacognosia* 28: 145–150.
- KOWALSKA J *et al.* 2021. Influence of tea brewing parameters on the antioxidant potential of infusions and

- extracts depending on the degree of processing of the leaves of *Camellia sinensis*. *Molecules* 26(4773): 1–17.
- KRUK J, ABOUL-ENAIN BH, DUCHNIK E, MARCHLEWICZ M. 2022. Antioxidative properties of phenolic compounds and their effect on oxidative stress induced by severe physical exercise. *J Physiol Sci* 72(19): 1–24.
- KUMARN, GOEL N. 2019. Phenolic acids: natural versatile molecules with promising therapeutic applications. *Biotechnology Reports* 24: e00370.
- LI K *et al.* 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the flavonoids rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity relationship of its eight flavonoids by a refined assign score method. *Chem Central J* 12(82): 1–11.
- LIN YS. *et al.* 2020. Effects of storage time and temperature on antioxidants in juice from *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser. *Molecules* 25(3614): 1–12.
- MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content and antioxidant activity test of avocado seed extract (*Persea americana* Mill). *Jurnal MIPA Unsrat Online* 1(1): 5–10 [in Bahasa Indonesia].
- MARTINEZ-SOLIS J *et al.* 2022. Antidiabetic and toxicological effect of the tea infusion of summer collection from *Annona cherimola* Miller leaves. *Plants* 11(3224): 1–17.
- MATHUR R, VIJAYVERGIAR. 2017. Determination of total flavonoid and phenol content in *Mimusops elengi* Linn. *Intern J Pharm Sci Res* 8(12): 5282–5285.
- MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of sprouted and solid-state bioprocessed soybean. *Asia Pacific J Clinical Nut* 14(2): 145–152.
- MOORTHY NS, RAMOS MJ, A FERNANDES P. 2012. Studies on  $\alpha$ -glucosidase inhibitors development: magic molecules for the treatment of carbohydrate mediated diseases. *Mini Rev Med Chem* 12: 713–720.
- MORAES-DE SOUZA RA, OLDONI TLC, REGITANO-D'ARCE MAB, ALENCAR SM. 2008. Antioxidant activity and phenolic composition of herbal infusions consumed in Brazil. *Cienc Tecnol Aliment* 6(1): 41–47.
- MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant activity: a Review. *Intern J Molecular Sci* 22(3380): 1–30.
- NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus sabdariffa* L.): effect of dyeing temperature and brewing conditions on total soluble solid, phenolic content, antioxidant capacity, and sensory quality. *Beverages* 6(2): 1–11.
- NOREEN H *et al.* 2017. Measurement of total phenolic content and antioxidant activity of aerial parts of medicinal plant *Coronopus didymus*. *Asian Pacific J Tropical Med* 10(8): 792–801.
- NURCHOLIS GW *et al.* 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity *in vitro*, and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum pictum* (L.). *Current Biochem* 1(2): 58–65.
- OH J *et al.* 2013. Antioxidant and antimicrobial activities of various leafy herbal teas. *Food Control* 31: 403–409.
- PROENCA C *et al.* 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an *in vitro* and *in silico* structure-activity relationship study. *J Enzyme Inhibition Med Chem* 32(1): 1216–1228.
- PUREWAL SS *et al.* 2022. Unraveling the effect of storage duration on antioxidant properties, physico-chemical and sensorial parameters of ready to serve Kinnow-Amla beverages. *Applied Food Res* 2(1): 1–8.
- RAHARJO D, HARYOTO. 2019. Antioxidant activity of mangrove *Sonnenatia caseolaris* L. using the FRAP method. *ISETH*. p. 623–629.
- RAMPHINWA ML *et al.* 2023. Eco-physiological response of secondary metabolites of teas: review of quality attributes of herbal tea. *Frontiers in Sustain Food Systems* 7(990334): 1–14.
- REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids. *Czech J Food Sci* 30(2): 171–177.
- RUAN J *et al.* 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel column chromatography fractions. *Molecules* 24(2784): 1–20.
- RUSITA YD *et al.* 2019. Comparison of tannin concentration on fresh and dried tea leaf (*Camellia sinensis* L.) extract using UV-visible spectrophotometric method. 1st International Conference of Health, Science, & Technology (ICOHETECH) 2019 ;16 Nov 2019; LPPM Universitas, Duta Bangsa Surakarta, Surakarta.
- SANGEETHA R, VEDASREE N. 2012. *In vitro*  $\alpha$ -amylase inhibitory activity of the leaves of *Thespesia populnea*. *Intern Scholarly Res Network*. p. 1–4.



- SHRAIM AM *et al.* 2021. Determination of total flavonoid content by aluminum chloride assay: a critical evaluation. *LWT Food Sci Tech* 150(111932): 1–11.
- SILVA-RAMIREZ KA *et al.* 2020. Effect of drying and steeping temperature on the phenolic content, antioxidant activity, aromatic compounds, and sensory properties of *Culila polyantha* Benth. *Infusions Processes* 8(1378): 2–20.
- SRISOOK K *et al.* 2012. Antioxidant and anti-inflammatory activities of hot water extract from *Pluchea indica* Less. herbal tea. *J Med Plants Res* 6(23): 4077–4081.
- SURIYAPHAN O. 2014. Nutrition, health benefits, and applications of *Pluchea indica* (L) Less leaves. *Pharm Sci Asia* 41: 1–10.
- SUD *et al.* 2019. Impact of thermal processing and storage temperature on phenolic profile and antioxidant activity of different varieties of lychee juice. *LWT–Food Tech* 116(108578): 1–9.
- TADERA K, MINAMI Y, TAKAMATSU K, MATSUOKA T. 2006. Inhibition of alpha-glucosidase and alpha-amylase by flavonoids. *J Nutr Sci Vitaminol* 52: 149–153.
- THANAJIRUSCHAYA P *et al.* 2010. Effect of storage period and temperature on antioxidant components and properties of milled rice. *Khon Kaen Univ Res J* 15(9): 843–851.
- VONGSAK B *et al.* 2018. Comparison of active constituents, antioxidant capacity, and  $\alpha$ glucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. *Food Biosci* 25: 68–73.
- WANG SC *et al.* 2022. Effect of brewing conditions on polyphenols in the dark tea (*Camellia sinensis* L.) infusions: content, composition, and antioxidant activities. *Food Sci Tech* 42(e36322): 1–9.
- WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica* Less as functional beverage in diabetic patients. *ASSEHR* 98: 164–167.
- WIDYAWATI PS *et al.* 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract as antioxidant and anti-warmed-over flavor (WOF) of duck meat. International Congress on Challenges of Biotechnological Research in Food and Health; 15 Nov 2014; Agricultural Technology Faculty, Slamet Riyadi University, Surakarta, Indonesia.
- WIDYAWATI PS *et al.* 2016. The physicochemical and antioxidant properties of *Pluchea indica* Less drink in tea bag packaging. *IJFNS* 5: 13–120.
- WIDYAWATI PS *et al.* 2017. *In vitro* antioxidant capacities and antidiabetic properties of *Pluchea* leaves. *IJPPS* 9(8): 203–208.
- WIDYAWATI PS *et al.* 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica* Less beverage. *CJFST* 12(4): 125–139.
- WIDYAWATI PS *et al.* 2022. The effect of  $\kappa$ -carrageenan proportion and hot water extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of stink lily (*Amorphophallus muelleri*) wet noodles. *Molecules* 27(5062): 1–16.
- XU P, CHEN L, WANG Y. 2019. Effect of storage period on antioxidant activity and inhibition on  $\alpha$ -amylase and  $\alpha$ -glucosidase of white tea. *Food Sci Nut* 7(2): 636–644.
- ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction period on caffeine in coffee. *Lantanida J* 6(2): 103–202. [in Bahasa Indonesia].
- ZHANG H, WANG G, DONG J. 2014. Inhibitory properties of aqueous ethanol extracts of propolis on alpha-glucosidase. *J Evid Based Complement Alternat Med.* p. 1–8.
- ZHANG Y *et al.* 2021. Effect of storage conditions and period on the polyphenol content of wheat flours. *Processes* 9(248): 1–11.



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**First Draft of PJS Article Ms 23-158**

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Wed, Jun 19, 2024 at 3:00 PM

Dear Dr. Widyawati,

Greetings! Attached below is the second draft of your article titled "Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea indica* Less Tea" [Ms 23-158] accepted for publication in the Philippine Journal of Science.

Kindly review this copy and, should you have no further corrections, provide us with your approval for publication.

We hope to hear from you on the matter within 48 hours of receipt of this letter. We shall consider this version of your paper as the galley proof for final editorial processing in the absence of a response within the cut-off time.

Thank you very much!

Sincerely,  
Mr. ALLYSTER A. ENDOZO  
Managing Editor  
[Quoted text hidden]

---

 **[PRO] 23-158 - Widyawati and Wilianto - Article (2nd Draft) (19 Jun 2024).pdf**  
1717K

## Effect of Steeping Temperature and Storage Period on the Bioactive Compounds plus Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea indica* Less

Paini Sri Widyawati<sup>1\*</sup> and Yufita Ratnasari Wilianto<sup>2</sup>

<sup>1</sup>Food Technology Study Program, Agricultural Technology Faculty,  
Widya Mandala Surabaya Catholic University, Surabaya 60265 Indonesia

<sup>2</sup>Pharmacy Study Program, Pharmacy Faculty,  
Widya Mandala Surabaya Catholic University, Surabaya 60272 Indonesia

This study was done to determine the effects of steeping temperature and storage period on the bioactive contents plus antioxidant and antidiabetic activities of *Pluchea* leaf infusion. The research used a randomized block design with two factors, *i.e.* steeping temperature (T) and storage period (B). The *Pluchea* leaf blades were exposed to four steeping temperatures of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) °C with the storage period of 0 (B1) and 5 (B2) yr – resulting in eight treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). Statistical analysis using a paired t-test at  $\alpha \leq 0.05$  showed that treatments significantly affected the bioactive contents [total phenol (TPC), total tannin (TTC), and total flavonoid (TFC)], antioxidant [DPPH scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)] potential and antidiabetic [ $\alpha$ -amylase (AA) and  $\alpha$ -glucosidase (GA) inhibition] properties of the *Pluchea* leaf infusion. TPC, TTC, DPPH, and FRAP significantly increased for the storage period and the steeping temperatures. Then, TFC decreased during the storage period but significantly increased at higher steeping temperatures. The AA and GA of *Pluchea* leaf infusion increased until 70 °C of the steeping temperature but decreased until 95 °C. The DPPH and FRAP of the *Pluchea* leaf infusion were strongly and positively correlated with TPC and TTC. The GA and AA of *Pluchea* leaf infusion were not influenced by the TPC and TTC but were weakly and positively correlated with TFC. The antioxidant activity of the *Pluchea* leaf infusion was inversely proportional to the antidiabetic activity. The simple phenolic compounds derived from *Pluchea* leaf infusion at different steeping temperatures and storage included gallic acid, kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-caffeoylquinic acid, 3,5- di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid.

Keywords: antioxidant, antidiabetic, bioactive compound, *Pluchea indica* Less, steeping temperature, storage period

\*Corresponding author: paini@ukwms.ac.id

## INTRODUCTION

*Pluchea* herbal tea is a product of dried *Pluchea* leaf processing introduced by world people (Srisook *et al.* 2012; Widyawati *et al.* 2016) because of the efficacy of the active components in *Pluchea* leaves, as a herbal plant that has been widely used for traditional medicine and food (Chan *et al.* 2022). *Pluchea* leaves are composed of many nutrients and bioactive compounds useful to body health. The nutrient compositions in the *Pluchea* leaves include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates, calcium,  $\beta$ -carotene, and vitamin C, whereas bioactive compounds are comprised, *i.e.* chlorogenic acid, caffeic acid, 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, quercetin, myricetin, kaempferol, total anthocyanin,  $\beta$ -carotene, and total carotenoid (Suriyaphan 2014; Vongsak *et al.* 2018; Ruan *et al.* 2019; Widyawati *et al.* 2022; Chan *et al.* 2022).

The steeping process of *Pluchea* leaves can be performed with fresh or dry leaves in hot or boiling water for a few min (Suriyaphan 2014; Silva-Ramirez *et al.* 2020; Jayani *et al.* 2022). In Asia, especially in Indonesia, people usually consume the *Pluchea* infusion by steeping 2 g of powdered *Pluchea* leaves in a tea bag in 100 mL of hot or boiling water. Widyawati *et al.* (2016) claimed that steeping 2 g of *Pluchea* leaf powder at 95 °C for 5 min exhibits total phenolic and flavonoid contents, the ability to scavenge DPPH free radicals, and the capability to reduce ferric ions at 9.3, 22.0, 27.2, and 10.2 mg gallic acid equivalent (GAE)/ g sample, respectively. Werdani and Widyawati (2018) reported that drinking *Pluchea* leaf powder infusion in the morning and evening regularly (2 g/ 100 mL) can decline blood sugar levels.

The steeping of *Pluchea* herbal tea with hot water at 95 °C for 5 min certainly determines the stability and amount of extracted bioactive compounds that influence the biological activity especially antioxidant and antidiabetic activities. Silva-Ramirez *et al.* (2020) reported that the infusion process can influence the content and composition of the bioactive compounds and antioxidant activity of tea. Acar *et al.* (2022) stated that the infusion quality of herbal tea extract depends on a number of factors, *i.e.* storage and temperature. The polyphenol profile and antioxidant properties of herbal tea infusion decline with an increase in steeping or brewing and storage temperatures, as well as longer exposure periods.

Several studies have mentioned the effect of steeping temperature on the bioactive compound contents and antioxidant activity, as some white and green teas are effective with hot water at 90 °C for 7 min (Castiglioni *et al.* 2015), on rosehip tea is effective at infusion period

around 6–8 min at temperatures of 84–86 °C (Ilyasoglu and Arpa 2017), on the caffeine content extracted at the brewing temperature of coffee (Zarwinda and Sartika 2018), and the high total phenol content and antioxidant activity of dark tea at 92 °C for 27 min (Wang *et al.* 2022). The study of the effect of steeping temperature on *Pluchea* infusion was carried out to afford information about the most efficient preparation of powdered *Pluchea* leaves to get higher bioactive compounds, antioxidant, and antidiabetic activities.

Storage period tea usually for several months to years *Pluchea* herbal tea also affects the levels of the bioactive compounds and biological activity (Jayani *et al.* 2022). Tea or herbal tea is generally stored at ambient temperature and packed in a tea bag or aluminum foil standing pouch or a combination of both. Many researchers reported that the storage period decreases the bioactive compounds plus antioxidant and antidiabetic activities, *i.e.* juice from *Momordica charantia* L. (Lin *et al.* 2020), dried *Piper betle* extracts (Ali *et al.* 2018), white tea (Xu *et al.* 2019), Kinnow-Amla beverages (Purewal *et al.* 2022), and whole-wheat flour (Zhang *et al.* 2021).

Therefore, this research studied the effect of steeping temperature and storage period on the bioactive compounds [total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC)], antioxidant [DPPH free radical scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)], and antidiabetic activities [ $\alpha$ -amylase (AA) and  $\alpha$ -glycosidase (GA) inhibition] of the infusion from powdered *Pluchea* leaves and on the phenolic compound profile.

## MATERIALS AND METHODS

### Raw Materials and Preparation

The *Pluchea* leaves were collected from Mangrove areas in Wonorejo, Surabaya, East Java, Indonesia. The *Pluchea* plants were included in the Asteraceae family with specifications according to the GBIF taxon ID number database:3132728 (Ferraris 2023). *Pluchea* leaves at 1–6 levels of each branch from the shoot were collected, sorted, washed, and dried to get a moisture content of around  $11.16 \pm 0.09\%$  dry basis (Widyawati *et al.* 2022). The dried *Pluchea* leaves were pulverized to a 45-mesh size powder. The *Pluchea* leaf powder was dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 °C for 10 min to reduce microbial organisms. Then, 2 g of the powder was packed into a paper filter infusion bag. Packed samples were stored for 0 (unstored) and 5 (stored) yr in a standing pouch before analysis.

In the research, one tea bag of *Pluchea* herbal tea that was stored for 0 (B1) and 5 (B2) yr was steeped with 100-mL hot water at various temperatures – including 60 (T1), 70 (T2), 80 (T3), and 95 (T4) °C for 5 min – with infusion method obtaining eight treatment combinations – namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, and T4B2. After the temperature of *Pluchea* infusion similar to ambient temperature was analyzed further.

### Reagents

The reagents used in the analysis include 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid,  $\alpha$ -amylase,  $\alpha$ -glucosidase, p-nitrophenyl- $\alpha$ -glucopyranoside (pNPG), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and (+)-catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, Folin-Ciocalteu's phenol, sodium nitric, aluminum chloride, ferric chloride, sodium dihydrogen phosphate, sodium phosphate, potassium ferricyanide, starch, acetic acid, and sodium hydroxide were purchased from Merck (Kenilworth, NJ, USA). All reagents used were of analytical grade except for distilled water which was purchased from PT Aqua Industry Surabaya.

### Analysis of the Bioactive Compounds

**Total phenolic content (TPC) analysis.** The TPC of treated *Pluchea* infusion was carried out using the technique by Gao *et al.* (2019). About 10  $\mu$ L *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10% were mixed in 10-mL volumetric flask and incubated for 5 min. Then, 2 mL Na<sub>2</sub>CO<sub>3</sub> 7.5% was added and filled up to 10 mL volume with distilled water. The blue color intensity of the solution was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at  $\lambda = 760$  nm, with gallic acid as the reference standard. The TPC was calculated using the following formula:  $y = 0.00009x + 0.008$ , with  $R^2 = 0.9941$ . The results were expressed as mg GAE/g samples.

**Total flavonoid content (TFC) assay.** The TFC of the samples was measured based on the reaction between AlCl<sub>3</sub> and NaNO<sub>2</sub> with the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim *et al.* 2021). The reaction between AlCl<sub>3</sub> and flavonoid compounds resulted in a yellow solution. About 30- $\mu$ L *Pluchea* infusion was mixed with 0.3 mL NaNO<sub>2</sub> 5% in 10-mL volumetric flask and incubated for 5 min. The mixture was added with 0.3 mL AlCl<sub>3</sub> 10% for 5 min. Then, 2-mL NaOH 1 M and distilled water were added to a 10-mL volume. Then, the red solution was produced after NaOH solution addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda = 510$  nm, with (+)-catechin as

the reference standard compound, and the results were expressed as mg catechin equivalents (CE)/ g samples using the following formula:  $y = 0.00008x - 0.0023$ , with  $R^2 = 0.9980$ .

**Total tannin content (TTC) analysis.** The TTC of the samples was analyzed using the Folin-Ciocalteu method (Chandran and Indira 2016). Approximately 10- $\mu$ L *Pluchea* infusion was added with 1-mL Folin-Ciocalteu's phenol reagent 10% in 10-mL volumetric flask and incubated for 5 min. Then, the mixture was added with 2-mL Na<sub>2</sub>CO<sub>3</sub> 7.5% and filled up to 10-mL volume with distilled water. The blue dark color solution was measured in UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at  $\lambda = 760$  nm, with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/ g samples using the following formula:  $y = 0.00009x + 0.0021$ , with  $R^2 = 0.9993$

### Analysis of the Antioxidant Potential

**DPPH free radical scavenging activity assay.** The DPPH free radical scavenging activity (DPPH) was measured by the spectrophotometric method (Widyawati *et al.* 2017) to determine the ability of the phytochemicals in the *Pluchea* leaf infusion to donate hydrogen atoms to the nitrogen atom in DPPH, resulting in the formation of DPPH-H compound exhibiting a yellow-colored solution. About 25  $\mu$ L *Pluchea* leaf infusion was poured into the reaction tube, into which 3-mL DPPH solution (4 mg/ 100 mL) was added. After incubation for 15 min in a dark room, the absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda = 517$  nm. The reference standard compound was gallic acid, and the results of the analysis were expressed as mg GAE/g samples calculated using the following formula:  $y = 0.146x + 1.7896$ , with  $R^2 = 0.9975$ .

**Ferric-reducing power (FRAP) analysis.** FRAP was determined following the method used by Widyawati *et al.* (2014) method. Approximately 10  $\mu$ L of samples were added to 2.5 mL phosphate buffer pH 6.6 and 2.5 mL of 1% potassium ferricyanide in the reaction tube. Then, the mixture was shaken and incubated for 20 min at 50 °C. Finally, 2.5 mL chloroacetic acid 10% (w/v) was added. Into the 2.5-mL supernatant, 2.5 mL distilled water and 0.5 mL ferric chloride 0.1% w/v were added, and the mixture was incubated for 10 min. The potency of the samples reducing iron (III) to iron (II) ion was indicated by the intensity of blue color formed that was measured using UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda = 700$  nm. The intensity of the blue color indicated a higher reducing capacity. The reducing power, expressed as mg GAE/g samples, was calculated using the following formula:  $y = 0.0002x + 0.0256$ , with  $R^2 = 0.9906$ .

### Analysis of the Antidiabetic Properties

**$\alpha$ -amylase enzyme inhibition (AA) capacity assay.** *In vitro* AA followed the procedure, as described by Widyawati *et al.* (2020). Each 500  $\mu$ L of the samples was mixed with starch 1% (w/v) and sodium acetate buffer pH 5. Into 250  $\mu$ L of the mixture, an  $\alpha$ -amylase solution (0.1 g of this enzyme 12.5 unit/mL) was added and then dissolved in 50 mL of 0.2 M sodium acetate pH 5. The mixture was shaken, into which 2-mL sodium hydroxide 1M was added. Before the analysis, this mixture was incubated at 37 °C for 10 min. Then, the capacity of the  $\alpha$ -amylase enzyme to hydrolyze the starch to release glucose was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at  $\lambda = 540$  nm. The inhibition percentage of  $\alpha$ -amylase was assessed using the formula  $(ACb - ACa) - (As - Ab) / (ACb - ACa) \times 100\%$  – where ACb is the absorbance of 100% enzyme activity (solvent with the enzyme), ACa is the absorbance of 0 % enzyme activity (solvent without the enzyme), As is the absorbance of the test sample with enzyme, and Ab is the absorbance of test sample without enzyme.

**$\alpha$ -glucosidase enzyme inhibition (GA) capacity assay.** The analysis of the  $\alpha$ -glycosidase inhibitor activity (GA) was done using the method of Widyawati *et al.* (2020) with slight modifications. About 150- $\mu$ L samples containing 100- $\mu$ L *Pluchea* infusion and 50  $\mu$ L pNPG (0.0150 g in 100-mL sodium phosphate 0.2 M at pH 7) were reacted with 50- $\mu$ L  $\alpha$ -glycosidase 2 mM (0.0833 unit/mL); then, the mixture was incubated at 37 °C for 15 min. The reaction was stopped with the addition of 1000- $\mu$ L sodium carbonate 0.2 M. The amount of these enzymes that did not react with bioactive compounds of *Pluchea* infusion hydrolyzed pNPG as a substrate to result in p-nitrophenol. The inhibition activity of the *Pluchea* infusion was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at  $\lambda = 405$  nm. The inhibition percentage of  $\alpha$ -glycosidase was calculated using the formula  $(ACb - ACa) - (As - Ab) / (ACb - ACa) \times 100\%$  – where ACb is the absorbance of 100% enzyme activity (solvent with enzyme), ACa is the absorbance of 0% enzyme activity (solvent without enzyme), As is the absorbance of test sample with enzyme, and Ab is the absorbance of test sample without enzyme.

### Analysis of Phenolics

The phenolic compounds of the samples were analyzed using high-performance liquid chromatography (HPLC) based on the method of Kongkiatpaiboon *et al.* (2018) with modifications. Each *Pluchea* infusion was sonicated for 15 min (Branson 1510); then, the sample was filtered using a filter syringe (Whatmann, 0.2  $\mu$ m, NYL). About 20  $\mu$ L of the sample was injected in an HPLC (LC20AD series, Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC LC-20AD pump, CTO-30A

column oven, CBM-20A/20 Alite system controller, and SPD-20A/20 AV UV-Vis detector. Separation of phenolic compounds in samples was carried out using a Shim-pack VP-ODS C18 column (ID 5  $\mu$ m  $\times$  50 mm  $\times$  4.6 mm) with a GVP-ODS Cartridge guard column (two pieces) (ID 10 mm  $\times$  4.6 mm). The mobile phase used consisted of a solution of [A] 0.5% acetic acid in water and [B] absolute methanol. Analysis was carried out using a gradient system in the following order: initial conditions of 10% B in A to 50% B in A were maintained for 40 min; then, 100% B was maintained for 20 min. Next, the column was re-equilibrated with 10% B in A and maintained for 10 min before analysis of the next sample. The sample flow rate was set at 1.0 mL/min with a controlled temperature of 40 °C. Detection was used at a wavelength of 280 nm. The reference standard used were gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,5-dicaffeoylquinic acid, 3,4-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid. All of the reference standard was dissolved in distilled water and prepared similarly to the samples before being injected in HPLC.

**Experiment design and statistical analysis.** The research design used a randomized block design with two factors, *i.e.* the steeping temperature (T) and the storage period. *Pluchea* leaf blades were subjected to 4 steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95 °C (T4), and the storage period of 0 year /unstored (B1), and 5 year /stored (B2) resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic was repeated for six periods. The data analysis of samples was repeated for six periods. The data were analyzed using a paired t-test at  $\alpha \leq 0.05$ , treatment means of specific phenolic compounds that were identified were expressed as the mean  $\pm$  SD. The analysis used SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

## RESULTS AND DISCUSSIONS

### Bioactive Compounds

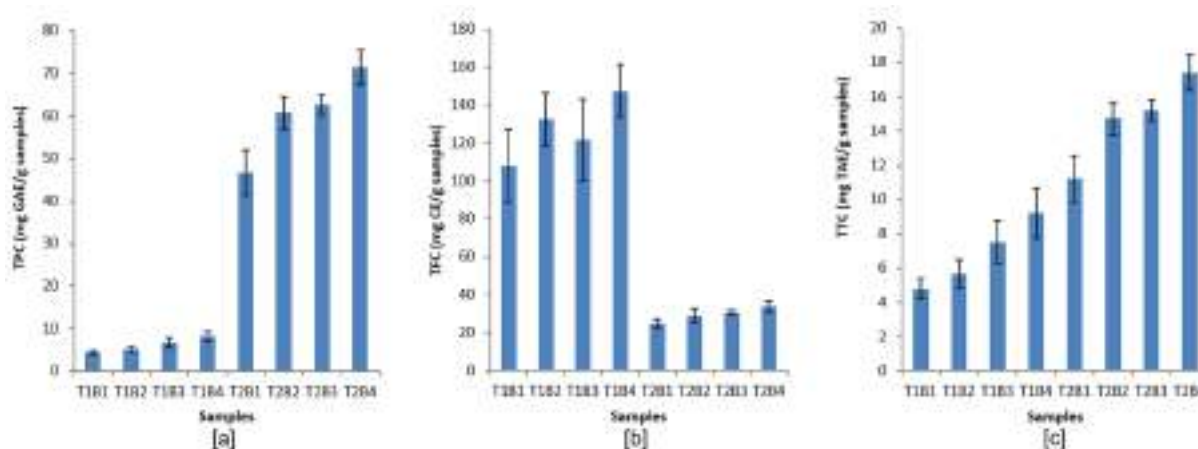
**Phenolic compounds.** Bioactive compounds are active compounds in plants that are essential to protect body health (Nguyen and Chuyen 2020). These compounds usually have many biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer, antimicrobial, antibacterial, anti-cholesterol, and so on (Suriyaphan 2014; Acar *et al.* 2022). Phenolic compounds have potential redox properties that can scavenge free radicals that can cause a number of chronic diseases (Noreen *et al.* 2017; Aryal *et al.* 2019; Acar *et al.* 2022).

The TPC of *Pluchea* infusion at different steeping temperatures and storage periods generally significantly increased with increasing steeping temperature and storage period based on paired t-test at  $\alpha \leq 0.05$  (Figure 1a). Steeped and stored infusion had significantly higher amounts of phenolic compounds than the samples that were steeped and unstored. Further, the highest TPC was observed in samples infused at 95 °C and stored for 5 yr (at  $71.38 \pm 4.14$  mg GAE/g sample), whereas the lowest was measured in the unsteeped samples and infused at 60 °C (at  $4.39 \pm 0.49$  mg GAE/g sample). The phenolic content of stored samples that were steeped only at 60 and 95 °C showed a significant increase in their phenolic content. This implies that the steeping temperature and the storage periods significantly resulted in the high amounts of phenolic compounds in the infusions. Results also indicated that phenolic compounds were generally greater in the infusion at high steeping temperatures and long storage periods. This could have been due to the fact that the steeping temperature and storage period could cause the process of degradation, oxidation, and leaching or release of phenolic compounds. Phenolic compounds are water-soluble and, thus, soaking in hot water for a certain period, as steeping causes the migration process of more phenolic compounds to the water because of longer exposure of phenolic compounds to water (Castiglioni *et al.* 2015; Kilic *et al.* 2017; Acar *et al.* 2022). Su *et al.* (2019) reported that temperature treatment can stimulate the release of phenolic compounds and increase the antioxidant activity of lychee juice stored at different temperatures of 4 and 45 °C and different long storage (fresh and 72 h).

Temperature treatment degrades (or hydrolyzes) the hydrogen bond between phenolic compounds and proteins, resulting in an increase of phenolic compounds

when exposed to higher temperatures (Ali *et al.* 2018; Jayani *et al.* 2022; Ramphinwa *et al.* 2023). Zhang *et al.* (2021) reported that phenolic compounds present in plants are not completely stable but are easily degraded during storage after harvest. Reblova (2012) claimed that antioxidant compounds can be slowly degraded with increasing temperature. Fibrianto *et al.* (2021) also stated that the brewing temperature has an effect on the extracted antioxidant compounds such as alkaloids, catechins, and tannins. Thus, there is an assumption that temperature and storage caused the degradation, oxidation, and hydrolysis of the phenolic compounds period, resulting in the increased amount of the phenolic compounds at higher steeping temperatures and longer storage periods.

Simple phenolic compounds are identified in steeped and stored. *Pluchea* leaf infusion included gallic acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids is shown in Table 1. The treatment effects using the t-test at  $\alpha \leq 0.05$  showed that gallic acid and kaempferol content were insignificantly different at various steeping temperatures and storage periods. The concentration of quercetin and 3,5-di-*O*-caffeoylquinic acid of the unsteeped and stored *Pluchea* infusion was significantly different from the rest of the samples between 70 °C, whereas (+)-catechin concentration of *Pluchea* infusion was only significantly different at 95 °C. The myricetin content was significantly different at 80 and 95 °C. The 3,4-di-*O*-caffeoylquinic acid content showed significant difference at 60, 80, and 95 °C, whereas 4,5-di-*O*-caffeoylquinic acid content was only significantly different at 60 °C.



**Figure 1.** Bioactive compound contents of *Pluchea* infusion at different steeping temperatures and storage periods: [a] total phenolic content, [b] total flavonoid content, and [c] total tannin content. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  $\pm$  standard deviation (n = 6). Samples: T1B1-steeped at 60 °C, unsteeped; T1B2-steeped at 70 °C, unsteeped; T1B3-steeped at 80 °C, unsteeped; T1B4-steeped at 95 °C, unsteeped; T2B1-steeped at 60 °C, stored for 5 yr; T2B2-steeped at 70 °C, stored for 5 yr; T2B3-steeped at 80 °C, stored for 5 yr; T2B4-steeped at 95 °C, stored for 5 yr.

**Table 1.** Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperatures and storage periods.

Phenolic compounds	Steeping temperature (°C)	Mean ± SD (unstored)	Mean ± SD (stored)	Mean difference ±SD	Sig. (two-tailed)
Gallic acid (µg/g samples)	60	0.2132 ± 0.0027	0.2364 ± 0.0015	0.0375 ± 0.0175	0.2030
	70	0.2157 ± 0.0013	0.2324 ± 0.0214	0.0167 ± 0.0227	0.4870
	80	0.2234 ± 0.0122	0.2347 ± 0.0078	0.0386 ± 0.0264	0.2870
	95	0.2316 ± 0.0104	0.2402 ± 0.0169	0.0086 ± 0.1990	0.8500
(+) -Catechin (µg/g samples)	60	0.3425 ± 0.0110	0.5085 ± 0.0111	-0.1576 ± 0.0885	0.241
	70	0.3260 ± 0.0265	0.5448 ± 0.0006	-0.2188 ± 0.0259	0.053
	80	0.3240 ± 0.0222	0.5023 ± 0.0773	-0.1451 ± 0.0248	0.077
	95	0.4039 ± 0.0320	0.5995 ± 0.0372	-0.2049 ± 0.0020	0.004*
Myricetin (µg/g samples)	60	0.1756 ± 0.1234	1.4762 ± 0.0271	-1.2887 ± 0.3222	0.111
	70	0.2587 ± 0.0160	1.4245 ± 0.2526	-1.1657 ± 0.2695	0.103
	80	0.4175 ± 0.0104	1.4570 ± 0.0925	-1.0391 ± 0.0841	0.036*
	95	0.8786 ± 0.0434	2.6138 ± 0.0695	-1.1735 ± 0.1702	0.044*
Quercetin (µg/g samples)	60	0.0220 ± 0.0268	0.6220 ± 0.0706	-0.5999 ± 0.9733	0.544
	70	0.1530 ± 0.0511	1.0708 ± 0.0289	-0.9177 ± 0.0222	0.011*
	80	0.3666 ± 0.0103	0.8629 ± 0.0815	-0.1082 ± 0.4462	0.790
	95	0.6559 ± 0.0570	2.0230 ± 0.0573	-1.4123 ± 0.3203	0.101
Kaempferol (µg/g samples)	60	0.1394 ± 0.0202	0.3675 ± 0.0183	-0.3207 ± 0.1122	0.154
	70	0.0514 ± 0.0037	0.3726 ± 0.0944	0.3213 ± 0.0907	0.125
	80	0.3699 ± 0.0924	0.7966 ± 0.0366	-0.4267 ± 0.2727	0.271
	95	0.5913 ± 0.0239	0.9478 ± 0.0287	-0.3565 ± 0.5256	0.513
3,4-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6103 ± 0.0628	2.4863 ± 0.0270	-1.8760 ± 0.2074	0.050*
	70	0.6271 ± 0.0099	2.3403 ± 0.0325	-1.7131 ± 0.3152	0.082
	80	0.7967 ± 0.03060	2.6278 ± 0.0211	-1.8311 ± 0.0095	0.002*
	95	1.5386 ± 0.0668	4.0211 ± 0.0851	-2.4825 ± 0.1839	0.033*
3,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.6635 ± 0.0628	0.9449 ± 0.0501	-0.2814 ± 0.4458	0.536
	70	0.6162 ± 0.0099	0.9485 ± 0.0794	-0.3323 ± 0.0301	0.041*
	80	0.6601 ± 0.0306	0.9099 ± 0.0387	-0.2498 ± 0.3127	0.461
	95	0.6642 ± 0.0668	1.3156 ± 0.0166	-0.6514 ± 0.2666	0.179
4,5-di-O-Caffeoylquinic acid (µg/g samples)	60	0.4906 ± 0.0060	1.1842 ± 0.0120	-0.6886 ± 0.2723	0.018*
	70	0.4807 ± 0.0034	1.0089 ± 0.0736	-0.5281 ± 0.0702	0.060
	80	0.5299 ± 0.0053	1.2382 ± 0.1435	-0.7082 ± 0.1489	0.094
	95	1.0018 ± 0.0526	1.3797 ± 0.2170	-0.3086 ± 0.3086	0.333

Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean ± standard deviation (n = 6). Samples: T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T3B2-steeped at 70 °C, stored for 5 yr; T3B3-steeped at 80 °C, stored for 5 yr; T3B4-steeped at 95 °C, stored for 5 yr.

Results further showed that gallic acids and kaempferol were relatively stable, as reflected by the insignificant changes when exposed to the different steeping temperatures and storage periods. Myricetin, (+)-catechin, and 3,4-di-O-caffeoylquinic acid showed a drastic increase

at higher steeping temperatures and longer storage periods, implying that these compounds tended to be relatively labile. Quercetin, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid underwent moderate changes. Therefore, myricetin, (+)-catechin, and 3,4-di-



*O*-caffeoylquinic acid were easier to dissolve or degrade to form simple phenolic acids at higher temperatures and storage period (Su *et al.* 2019; Ali *et al.* 2018; Jayani *et al.* 2022; Ramphinwa *et al.* 2023; Zhang *et al.* (2021). Degradable polyphenol compounds have a simple structure and free hydroxyl groups that can react with Folin-Ciocalteu's phenol reagent, resulting in a complex blue solution that can be detected as TPC.

**Flavonoid content (TFC).** Flavonoids are the major phenolic compounds that have potential chemical and biological activities such as radical scavenging and antimicrobial activities (Ayele *et al.* 2022; Chandra *et al.* 2014) that can protect the human body from the oxidative stress caused by many degenerative diseases – especially cancer, cardiovascular problems, and aging (Mathur and Vijayvergia 2017). The TFC of steeped *Pluchea* infusion decreased with a longer storage period. Unstored samples exhibited higher flavonoid content than the stored samples. The statistical analysis using a paired t-test at  $\alpha \leq 0.05$  showed that the TFC of *Pluchea* infusion was significantly different between the steeped unstored and steeped stored samples (Figure 1b). The highest TFC was exhibited by the unstored samples steeped at 95 °C at about  $147.42 \pm 14.03$  mg CE/g sample. The TFC was significantly lower in the stored samples than those of the unstored samples, implying that the increase in the flavonoid content of the infusion was affected primarily by the steeping temperature.

**Tannin content (TTC).** Tannins are bioactive compounds that provide properties, such as astringent, anti-diarrheal, antibacterial, and antioxidant (Malangngi *et al.* 2012). Generally, results indicated that the TTC of *Pluchea* infusion significantly increased with increasing steeping temperature and storage period (Figure 1c). Among the unstored steeped samples, the tannin content was significantly lowest in the samples infused at 60 °C at about  $4.81 \pm 0.58$  to  $17.42 \pm 1.04$  mg TAE/g samples, which was significantly different lower from that of the lowest tannin content of the stored samples. Among the stored and steeped samples, the highest tannin content was observed at samples steeped at 95 °C about  $17.42 \pm 1.04$  mg TAE/g samples and was significantly different from that of the highest tannin content of the unstored steeped samples at 95 °C about  $9.22 \pm 1.48$  mg TAE/g samples. Indicating that the tannin content was primarily affected by a longer storage period than high steeping temperature. The condensation of catechins to tannins is a dominant process occurring in tea leaves that is accelerated during the maceration of raw tea leaves (Kowalska *et al.* 2021) and could have contributed to the observed increase in the tannin content in the treated samples.

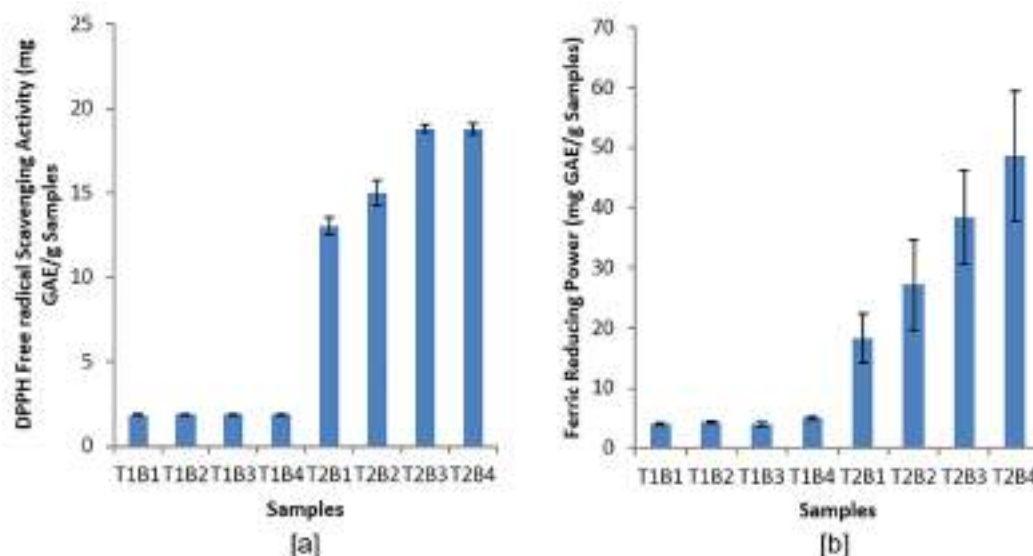
Nonetheless, high temperatures and long storage periods can cause the degradation of tannins to catechins. Rusita *et al.* (2019) emphasized that tannins are polar

thermostable complex compounds that are resistant to heating, indicating that even with the exposure to high temperature, the tannins still remained high in the treated samples period.

**Antioxidant activity.** Antioxidant activity is the capability of compounds to inhibit the oxidation of macromolecules from biological targets that are involved in oxidative chain reactions (Ali *et al.* 2005; Oh *et al.* 2013). The antioxidant activity assay was done in this research using DPPH and FRAP methods. The phenolic compounds are active antioxidants with antioxidant capability that depends on their redox properties. The structure of phenolic compounds determines the effectivity to donate hydrogen atoms, which is negatively correlated with the O-H phenolic bond strength. The antioxidant power of phenolic compounds is due to the weak hydrogen bonds in the OH group of the phenolic compound, so that it is easier to donate hydrogen atoms (Kruk *et al.* 2022). The mechanism of phenolic compounds as antioxidants depends on their ability to donate hydrogen atoms and transfer electrons, as well as reducing agents and singlet oxygen quenchers (Ali *et al.* 2005; Huang *et al.* 2005).

**DPPH free radical scavenging activity (DPPH).** DPPH is a free radical that is often used to evaluate antioxidant activity because this method is simple and is suitable for measuring the donating hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of DPPH to change to a yellow color (Munteanu and Apetrei 2021; Baliyan *et al.* 2022). Figure 2a shows that the free radical scavenging properties of the stored and steeped samples were significantly higher than the unstored steeped samples. It can also be observed that the free radical scavenging property was significantly different among the stored and steeped samples but insignificant among the unstored and steeped sample period. *Pluchea* infusion stored at room temperature for 5 yr resulted in high free radical scavenging activity by more than 10%. Steeping at higher temperatures significantly increased the DPPH free radical scavenging activity in stored *Pluchea* infusion by around 15–25%. This implies that the higher free radical scavenging property was primarily affected by the storage period than the steeping temperature. During the storage process, it is possible to form complex phenolic compounds that provide a high ability to scavenge free radicals (Thanajiruschaya *et al.* 2010).

The scavenging activity of the samples was strongly and positively correlated with total phenolic and tannin contents but inversely with total flavonoid levels (Table 2). The antioxidant activity was strongly and negatively correlated with flavonoid content. The storage period could be reduced flavonoid content. The study also demonstrated that longer storage period and higher infusion temperatures produced many simple phenolic compounds with free



**Figure 2.** Antioxidant activity of *Pluchea* tea at different steeping temperatures and storage periods: [a] DPPH; [b] FRAP. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  $\pm$  standard deviation ( $n = 6$ ). Samples : T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T2B2-steeped at 70 °C, stored for 5 yr; T2B3-steeped at 80 °C, stored for 5 yr; T2B4-steeped at 95 °C, stored for 5 yr.

**Table 2.** Pearson correlation coefficients between bioactive contents (TPC, TFC, and TTC), antioxidant activity (DPPH and FRAP), and antidiabetic activity (AA and GA).

	TPC	TFC	TTC	DPPH	FRAP	$\alpha$ -glucosidase	$\alpha$ -amylase
TPC	1						
TFC	-0.93589	1					
TTC	0.960028	-0.81321	1				
DPPH	0.992776	-0.93992	0.942273	1			
FRAP	0.953366	-0.82636	0.947778	0.956242	1		
$\alpha$ -glucosidase	-0.55512	0.349873	-0.71534	-0.5272	-0.55947	1	
$\alpha$ -amylase	-0.70842	0.429393	-0.8569	-0.69579	-0.80548	0.725161631	1

Significant at the 0.05 level (two-tailed)

hydroxyl groups capable to donate hydrogen atoms to DPPH free radicals. Many phenolic acids such as gallic acids, (+)-catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids have established potential antioxidant activity (Kumar and Goel 2019) (Table 1). Kruk *et al.* (2022) informed that the capability of phenolic compounds to donate hydrogen atom depends on the chemical structure, number, and position of hydroxyl groups attached to a benzene ring, a double bond between C2 and C3 rings, and a carbonyl group (C=O) on the C ring at C4. The effectivity of antioxidant compounds to donate hydrogen atoms is determined by O-H bond dissociation energy.

The free radical scavenging property observed in the

study was not consistent with the results of the study by Moraes-de Souza *et al.* (2008). The research shows that the TPC of herbal infusion is lowly correlated with free radical scavenging activity. However, Dobrinis *et al.* (2021) informed that TPC is positively and significantly correlated with the free radical scavenging property of tea infusion.

**Ferric reducing antioxidant power (FRAP).** FRAP is an analysis of the antioxidant power of the phytochemical compounds that is based on the ability of antioxidant compounds to reduce iron ions of potassium ferricyanide ( $Fe^{3+}$ ) to potassium ferrocyanide ( $Fe^{2+}$ ). Potassium ferrocyanide reacts with ferric chloride to form a ferric-ferrous complex and results green color solution (Widyawati *et al.* 2017; Raharjo and Haryoto 2019).

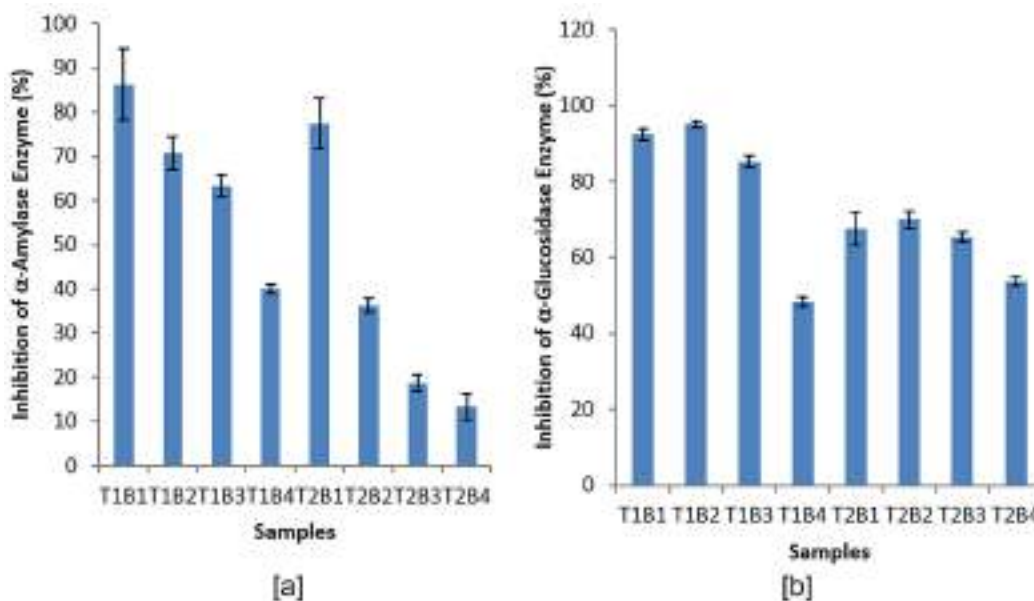
The results showed that the FRAP increased at higher steeping temperatures and longer storage periods. The lowest FRAP was observed in the unstored samples, which were steeped at 60 °C at  $3.95 \pm 0.17$  mg GAE/g samples, and the highest was exhibited in *Pluchea* infusion which was stored for 5 yr at 95 °C at  $48.63 \pm 10.83$  mg GAE/g samples (Figure 2b). FRAP increased significantly as the steeping temperature was increased. FRAP of the samples stored for 5 yr was also significantly higher than the unstored samples at  $\alpha \leq 0.05$ .

This is in contrast with the study on the antioxidant activity of DPPH and FRAP of matcha. The longer storage period reduces the levels of catechin content due to the catechins such as epigallocatechin gallate, epicatechin gallate, epigallocatechin, and epicatechin, which are bioactive compounds that have high antioxidant activity (Kim *et al.* 2020). The ferric-reducing capability of *Pluchea* could have been due to the presence of simple phenolic acid that can transfer electrons from their free hydroxyl groups of samples. The FRAP of *Pluchea* infusion was strongly and positively significantly correlated with the DPPH, TPC, and TTC but inversely to TFC.

### Antidiabetic Activity

**$\alpha$ -amylase enzyme inhibition activity (AA).** Antidiabetic activity is a measure of the potency of phenolic compounds to regulate the uptake of glucose by the cells from the

blood through the mediation of two digestive enzymes, *i.e.*  $\alpha$ -amylase and  $\alpha$ -glucosidase, which are involved in the control of dietary carbohydrate digestion and release in the postprandial blood glucose in human body (Fu *et al.* 2017). The phenolic compounds have the capability to bind with the protein component of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Martinez-Solis *et al.* 2022), resulting in the reduced activity of the enzymes. The results showed that lower steeping *Pluchea* leaf infusion was able to inhibit the action of the  $\alpha$ -amylase enzymes (Figure 3a). The *Pluchea* infusion exhibited a good AA of more than 50% and even almost 100% in unstored *Pluchea* infusion steeped at 60, 70, and 80 °C, with the highest at 60 °C and in stored *Pluchea* leaf infusion, which was steeped at 60 °C. The stored *Pluchea* leaf infusion steeped at 70, 80, and 95 °C for 5 min had lower enzyme inhibition activity of less than 50%, with the lowest at 95 °C around 13%. Widyawati *et al.* (2017) found that the ability to inhibit the  $\alpha$ -amylase enzyme in unstored *Pluchea* infusion steeped at 95 °C for 5 min was also low at 28.79%. Increasing the steeping temperature and storage period reduced the ability of the phytochemicals in the *Pluchea* infusion to inhibit the  $\alpha$ -amylase enzyme activity period. Table 2 further shows that the AA of *Pluchea* infusion was strongly and negatively significantly correlated with TPC, TTC, DPPH, and FRAP, but it was weakly and positively significantly correlated with TFC.



**Figure 3.** Antidiabetic activity of pluchea tea at different steeping temperatures and storage periods: [a]  $\alpha$ -amylase; [b]  $\alpha$ -glucosidase. Data analysis using ANOVA at  $\alpha \leq 0.05$  continued analysis using a paired t-test at  $\alpha \leq 0.05$ . Data were expressed as mean  $\pm$  standard deviation ( $n = 6$ ). Samples: T1B1-steeped at 60 °C, unstored; T1B2-steeped at 70 °C, unstored; T1B3-steeped at 80 °C, unstored; T1B4-steeped at 95 °C, unstored; T2B1-steeped at 60 °C, stored for 5 yr; T2B2-steeped at 70 °C, stored for 5 yr; T2B3-steeped at 80 °C, stored for 5 yr; T2B4-steeped at 95 °C, stored for 5 yr.

This inhibitory activity was thought to be contributed by other bioactive compounds besides phenolics, which are sensitive to steeping temperature and storage period. Li *et al.* (2018) stated that there are flavonoid compounds that contribute to the ability to inhibit the  $\alpha$ -amylase enzyme. Akah *et al.* (2011) reported that phytochemical compounds such as terpenoids, saponins, flavonoids, glycosides/carbohydrates, and alkaloids are good antidiabetic metabolites or  $\alpha$ -amylase enzyme activity inhibitors. Sangeetha and Vedesree (2012) explained that the ability of *Threspesia populnea* extract to inhibit the  $\alpha$ -amylase enzyme was determined by their phenolic compound content and protein. Moreover, the presence of the  $\alpha$ -amylase enzyme inhibitor in this extract may be proteinaceous or nonproteinaceous in nature. It was assumed that this enzyme inhibitory activity in *Pluchea* infusion also was determined by their protein and polyphenolic content. Aleixandre *et al.* (2022) also stated that phenolic acids have inhibition activity to  $\alpha$ -amylase enzyme depending on their structures. There are C=C double bonds conjugated with a carbonyl group of phenolic structures that stabilize the binding forces to the active site of the  $\alpha$ -amylase. The hydroxyl groups can bind by non-covalent interaction (hydrogen bonding, cation- $\pi$  interactions, salt bridge interactions, ionic interactions, or electrostatic forces) with amino acid residue at the active site in the  $\alpha$ -amylase enzyme. Elevated steeping temperatures and longer storage periods can easily cause the removal of the hydroxyl groups of phenolic compounds, which can reduce their ability to enzyme inhibition. The phenolic acids with a greater number of hydroxyl groups exhibits stronger capability to obstruct the  $\alpha$ -amylase enzyme.

**$\alpha$ -glucosidase enzyme inhibition activity (GA).**  $\alpha$ -glucosidase is an important enzyme in carbohydrate digestion, that catalysis the hydrolysis of 1,4- $\alpha$ -bonds of the unabsorbed oligo- and disaccharides, and converts them into monosaccharides (glucose), thereby resulting in hyperglycemia (Nurcholis *et al.* 2014; Proenca *et al.* 2017). The ability of bioactive compounds to inhibit the  $\alpha$ -glucosidase enzyme is used to determine their antidiabetic activity. This is supported by Werdani and Widyawati (2018) stated that *Pluchea* infusion has the potential as an antidiabetic agent. Widyawati *et al.* (2020) found that the steeping of unstored *Pluchea* infusion at 95 °C for 5 min has an inhibitory effect on the  $\alpha$ -glucosidase enzyme of 67.857%.

Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme decreased with increasing steeping temperature and storage period. Steeping at 95 °C of the unstored *Pluchea* leaf infusion obtained the lowest inhibitory ability, *i.e.*  $48.32 \pm 1.27\%$ , and the highest inhibitory activity was at 70 °C at  $95.11 \pm 0.70\%$ . The results of a paired t-test showed that GA of *Pluchea* infusion was significantly different between

steeping temperature and long storage. Figure 3 further shows that the ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher than the ability to inhibit the  $\alpha$ -amylase enzyme. Data analysis in Table 2 showed that the TFC of the *Pluchea* leaf infusion was influenced weakly and positively by GA and AA, but the GA and AA were not affected by TPC, TTC, DPPH, and FRAP. Li *et al.* (2018) stated that flavonoid compounds can inhibit the action of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. Dias *et al.* (2021) stated that flavonoid compounds such as rutin, myricetin, kaempferol, and quercetin have antioxidant and antihyperglycemic activities. The ability to inhibit the action of enzymes from flavonoid compounds is determined by the position and number of hydroxyl groups, the number of double bonds in rings A and B, and the heterocyclic ring in ring C. Tadera *et al.* (2006) and Zhang *et al.* (2014) also explained that flavonoid compounds of samples significantly inhibit the  $\alpha$ -glucosidase enzyme activity.

The ability to inhibit the  $\alpha$ -glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping temperature and long storage. Figure 3 also showed that the capability of *Pluchea* infusion to obstruct the  $\alpha$ -glucosidase enzyme was greater than the  $\alpha$ -amylase enzyme because the mechanism of the two enzymes was different, according to the opinion of McCue *et al.* (2005). The mechanism of the  $\alpha$ -glucosidase enzyme inhibitor includes making the sugar mimic structure, binding using ionic bonds with nucleophilic, making the transition state-like structure, binding hydrogen with catalytic acid residue, interacting ionic and hydrophobic with site other than the active site, and binding covalent with enzymes through an epoxy or aziridine group (Moorthy *et al.* 2012). Then, the mechanism of the  $\alpha$ -amylase enzyme inhibitor includes blocking carbohydrates, thereby limiting the digestibility and absorption of carbohydrates, as well as blocking the active centers of several subsites of the enzyme (Gong *et al.* 2020).

Widyawati *et al.* (2017) stated that phenolic and non-phenolic compounds can inhibit the  $\alpha$ -glucosidase enzyme activity. The ability of bound phenolic compounds to inhibit  $\alpha$ -glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, which may occur in *Pluchea* infusion during storage, affects the structure and profile of phenolic and non-phenolic compounds. Arsiningtyas *et al.* (2014) explained that the methyl-esterified quinic acid with the caffeic groups such as 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid methyl ester, 3,4,5-tri-*O*-caffeoylquinic acid, and 1,3,4,5-tetra-*O*-caffeoylquinic acid of *Pluchea* leaves inhibits the  $\alpha$ -glucosidase enzyme activity. The resulting analysis of

caffeoylquinic acids (3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid) in stored *Pluchea* leaf infusion higher concentration than in unsteeped *Pluchea* infusion, and the concentrations of the simple phenolic compounds were increased at higher steeping temperature, but the GA of them was reduced. It means that the methyl-esterified quinic acid with the caffeic groups had more potential to inhibit  $\alpha$ -glucosidase enzyme than free caffeoylquinic acid.

This study showed that the increasing steeping temperature and storage period caused degradation of polyphenolic compounds to produce simple phenolic compounds such as gallic acid, (+)-catechin, myricetin, quercetin, kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid that increased the TPC and TTC. The increase in the simple phenolic concentration of the *Pluchea* leaf infusion caused higher antioxidant activity and lower antidiabetic activity.

## CONCLUSION

The TPC of *Pluchea* infusion at different steeping temperatures and storage periods generally significantly increased with increasing steeping temperature and storage periods. Steeped and stored infusion had significantly higher amounts of phenolic compounds than the samples that were steeped and unsteeped. TPC was highest in the stored and steeped at 95 °C and lowest in the unsteeped and steeped at 60 °C. Unsteeped steeped samples exhibited significantly higher flavonoid content than the stored steeped samples. The highest TFC was exhibited by the unsteeped samples steeped at 95 °C. The TTC of *Pluchea* leaf infusion significantly increased with increasing steeping temperature and storage period. Among the unsteeped steeped samples, the tannin content was significantly the lowest in the samples steeped at 60 °C and the highest in the samples steeped at 95 °C.

The DPPH of the stored and steeped *Pluchea* leaf infusion was significantly higher than the unsteeped steeped samples. The free radical scavenging property was highest in the stored samples steeped at 80 and 95 °C. The free radical scavenging activity of the samples was strongly and positively correlated with total phenolic and tannin contents but inversely with total flavonoid levels. The FRAP significantly increased with increasing steeping temperature and longer storage periods. The lowest FRAP was found in the unsteeped samples that were steeped at 60 °C, and the highest was exhibited in *Pluchea* samples that were stored for 5 yr and steeped at 95 °C. The FRAP of *Pluchea* leaf infusion was significantly strong and positively correlated with the free radical scavenging property, TPC, and TTC but inversely with TFC. The

AA was generally found to be higher at lower steeping temperatures of the unsteeped *Pluchea* leaf infusion than at higher steeping temperatures of the stored sample. The AA capacity of the *Pluchea* leaf infusion showed a significantly strong and negative correlation with TPC, TTC, DPPH, and FRAP, but it was weakly and positively correlated significantly with TFC.

The ability of the *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme decreased at high steeping temperatures and long storage periods. The highest inhibitory activity was obtained in the unsteeped *Pluchea* leaf infusion that was steeped at 70 °C, whereas the lowest was obtained in the unsteeped sample that was steeped at 95 °C. The ability of *Pluchea* leaf infusion to inhibit the  $\alpha$ -glucosidase enzyme tended to be higher than the ability to inhibit the  $\alpha$ -amylase enzyme. The GA was significantly strong and negative TPC, TTC, DPPH, and FRAP, and it was weakly and positively correlated significantly with TFC.

The simple phenolic compounds identified in *Pluchea* leaf infusion may affect the presence of the bioactive compounds, antioxidant potential, and antidiabetic properties at different steeping temperatures and storage periods – including gallic acids, (+)-catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids.

## ACKNOWLEDGMENTS

The authors would like to thank the Ministry of Education and Culture of the Republic of Indonesia for a fundamental research grant to higher education institutions in 2022, with contract number 260L/WM01.5/N/2022.

## STATEMENT ON CONFLICT OF INTEREST

The authors declare no conflict of interest.

## REFERENCES

- ACARA, AYDIN M, ARSLAN D. 2022. Development of infusion tea formulations with food wastes: evaluation of temperature and time effects on quality parameters. *Applied Food Res* 2(100087): 1–8.
- ALEIXANDRE A *et al.* 2022. Understanding phenolic acids inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase and influence of reaction conditions. *Food Chem* 372(131231): 1–9.

- ALI *et al.* 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile. *Food Chem* 89: 27–36.
- ALI A *et al.* 2018. Impact of storage conditions on the stability of predominant phenolic constituents and antioxidant activity of dried *Piper betle* extracts. *Molecules* 23(24): 1–13.
- AKAH PA, UZODINMA SU, OKOLO CE. 2011. Antidiabetic activity of aqueous and methanol extract and fractions of *Gongronema latifolium* (*Asclepidaceae*) leaves in alloxan diabetic rats. *J Applied Pharm Sci* 01(09): 99–102.
- ARSININGTYAS IS *et al.* 2014. Identification of  $\alpha$ -glucosidase inhibitors from the leaves of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product use. *Natural Product Res* 28(17): 1350–1353.
- ARYAL S *et al.* 2019. Total phenolic content, flavonoid content, and antioxidant of wild vegetables from Western Nepal. *Plants* 8(96): 1–12.
- AYELE DT, AKELE ML, MELESE AT. 2022. Analysis of total phenolic contents, flavonoids, antioxidant, and antibacterial activities of *Croton macrostachyus* root extracts. *BMC Chem* 16(30): 1–9.
- BALIYAN S *et al.* 2022. Determination of antioxidants by DPPH radical scavenging activity and quantitative phytochemical analysis of *Ficus religiosa*. *Molecules* 27(1326): 1–19.
- CASTIGLIONI S *et al.* 2015. Influence of steeping conditions (time, temperature, and particle size) on antioxidant properties and sensory attributes of some white and green teas. *Intern J Food Sci Nut Online*. p. 1–7.
- CHAN EWC *et al.* 2022. *Pluchea indica*: an updated review of its botany, uses, bioactive compounds, and pharmacological properties. *Pharmaceutical Sciences Asia* 49(1): 77–85.
- CHANDRA S *et al.* 2014. Assessment of total phenolic and flavonoid content, antioxidant properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit crops: a comparative study. *Hindawi Publishing Corporation Evidence-based Complementary Alter Med*. p. 1–9.
- CHANDRAN CI K, INDIRA G. 2016. Quantitative estimation of total phenolic, flavonoids, tannin, and chlorophyll content of leaves of *Strobilanthes kunthiana* (neelakurinji). *J Med Plants Studies* 4(4): 282–286.
- DIAS MC, PINTO DCGA, SILVA AMS. 2021. Plant flavonoids: chemical characteristics and biological activity. *Molecules* 26(5377): 1–16.
- DOBRINAS S, SOCEANU A, POPESCU V, POPOVICI IC, JITARIU D. 2021. Relationship between total phenolic content, antioxidant capacity, and Fe and Cu content from tea plant samples at different brewing times. *Processes* 9(1311): 1–11.
- FERRARIS Z. 2023. *Pluchea indica* Less. iNaturalist.org. <https://www.gbif.org/occurrence/4022273660>
- FIBRIANTO K, YUWONO SS, WAHIBAH LY. 2021. Brewing optimization for functional properties and visual appearance of Dampit Robusta coffee leaves tea. *Advances in Food Sci Sustain Agric Agro-industrial Engineering* 4(1): 25–32.
- FU L *et al.* 2017. Total phenolic contents and antioxidant capacities of herbal and tea infusions. *Intern J Molecular Sci* 12: 2112–2124.
- GAO MR *et al.* 2019. A theoretical and experimental study: the influence of different standards on the determination of total phenol content in the Folin-Ciocalteu assay. *J Food Measurement and Characteristic* 13: 1349–1356.
- GONG L, FENG D, WANG T, REN Y, LIU Y, WANG J. 2020. Inhibitors of  $\alpha$ -amylase and  $\alpha$ -glucosidase: potential linkage for whole cereal foods on prevention of hyperglycemia. *Food Sci Nut* 2020(8): 6320–6337.
- HUANG D, OU B, PRIOR RL. 2005. The chemistry behind antioxidant capacity assays. *J Agric Food Chem* 53: 1841–1856.
- ILYASOGLU H, ARPA TE. 2017. Effect of brewing conditions on antioxidant properties of rosehip tea beverage: study by response surface methodology. *J Food Sci Tech* 54(11): 3737–3743.
- JAYANI NIE *et al.* 2022. Evaluation of stability and quality characteristics of moringa (*Moringa oleifera*) herbal tea during storage. *Food Res* 6(4): 399–406.
- KIM JM *et al.* 2020. Effect of storage temperature on the antioxidant activity and catechins stability of matcha (*Camellia sinensis*). *Food Science and Biotechnology* 29(9): 1261–1271.
- KILIC *et al.* 2017. Antioxidant properties of some herbal teas (green tea, senna, corn silk, rosemary) brewed at different temperatures. *Intern J Secondary Metabolite* 4(3): 142–248.
- KONGKIATPAIBOONA S, CHEWCHINDA S, VONGSAK B. 2018. Optimization of extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves from different provenances in Thailand. *Revista Brasileira de Farmacognosia* 28: 145–150.
- KOWALSKA J *et al.* 2021. Influence of tea brewing parameters on the antioxidant potential of infusions and

- extracts depending on the degree of processing of the leaves of *Camellia sinensis*. *Molecules* 26(4773): 1–17.
- KRUK J, ABOUL-ENAIN BH, DUCHNIK E, MARCHLEWICZ M. 2022. Antioxidative properties of phenolic compounds and their effect on oxidative stress induced by severe physical exercise. *J Physiol Sci* 72(19): 1–24.
- KUMARN, GOEL N. 2019. Phenolic acids: natural versatile molecules with promising therapeutic applications. *Biotechnology Reports* 24: e00370.
- LI K *et al.* 2018. Inhibitory effects against  $\alpha$ -glucosidase and  $\alpha$ -amylase of the flavonoids rich extract from *Scutellaria baicalensis* shoots and interpretation of structure activity relationship of its eight flavonoids by a refined assign score method. *Chem Central J* 12(82): 1–11.
- LIN YS. *et al.* 2020. Effects of storage time and temperature on antioxidants in juice from *Momordica charantia* L. and *Momordica charantia* L. var. *abbreviata* Ser. *Molecules* 25(3614): 1–12.
- MALANGNGI LP, SANGI MS, PAENDONG JJE. 2012. Determination of tannin content and antioxidant activity test of avocado seed extract (*Persea americana* Mill). *Jurnal MIPA Unsrat Online* 1(1): 5–10 [in Bahasa Indonesia].
- MARTINEZ-SOLIS J *et al.* 2022. Antidiabetic and toxicological effect of the tea infusion of summer collection from *Annona cherimola* Miller leaves. *Plants* 11(3224): 1–17.
- MATHUR R, VIJAYVERGIAR. 2017. Determination of total flavonoid and phenol content in *Mimusops elengi* Linn. *Intern J Pharm Sci Res* 8(12): 5282–5285.
- MCCUE P, KWON YI, SHETTY K. 2005. Anti-diabetic and antihypertensive potential of sprouted and solid-state bioprocessed soybean. *Asia Pacific J Clinical Nut* 14(2): 145–152.
- MOORTHY NS, RAMOS MJ, A FERNANDES P. 2012. Studies on  $\alpha$ -glucosidase inhibitors development: magic molecules for the treatment of carbohydrate mediated diseases. *Mini Rev Med Chem* 12: 713–720.
- MORAES-DE SOUZA RA, OLDONI TLC, REGITANO-D'ARCE MAB, ALENCAR SM. 2008. Antioxidant activity and phenolic composition of herbal infusions consumed in Brazil. *Cienc Tecnol Aliment* 6(1): 41–47.
- MUNTEANU IG, APETREI C. 2021. Analytical methods used in determining antioxidant activity: a Review. *Intern J Molecular Sci* 22(3380): 1–30.
- NGUYEN QV, CHUYEN HV. 2020. Processing of herbal tea from roselle (*Hibiscus sabdariffa* L.): effect of drying temperature and brewing conditions on total soluble solid, phenolic content, antioxidant capacity, and sensory quality. *Beverages* 6(2): 1–11.
- NOREEN H *et al.* 2017. Measurement of total phenolic content and antioxidant activity of aerial parts of medicinal plant *Coronopus didymus*. *Asian Pacific J Tropical Med* 10(8): 792–801.
- NURCHOLIS GW *et al.* 2014. Phytochemical analysis,  $\alpha$ -glucosidase inhibition activity *in vitro*, and enzyme kinetics of ethyl acetate and hexane extracts of *Graptophyllum pictum* (L.). *Current Biochem* 1(2): 58–65.
- OH J *et al.* 2013. Antioxidant and antimicrobial activities of various leafy herbal teas. *Food Control* 31: 403–409.
- PROENCA C *et al.* 2017.  $\alpha$ -Glucosidase inhibition by flavonoids: an *in vitro* and *in silico* structure-activity relationship study. *J Enzyme Inhibition Med Chem* 32(1): 1216–1228.
- PUREWAL SS *et al.* 2022. Unraveling the effect of storage duration on antioxidant properties, physico-chemical and sensorial parameters of ready to serve Kinnow-Amla beverages. *Applied Food Res* 2(1): 1–8.
- RAHARJO D, HARYOTO. 2019. Antioxidant activity of mangrove *Sonnenatia caseolaris* L. using the FRAP method. *ISETH*. p. 623–629.
- RAMPHINWA ML *et al.* 2023. Eco-physiological response of secondary metabolites of teas: review of quality attributes of herbal tea. *Frontiers in Sustain Food Systems* 7(990334): 1–14.
- REBLOVA Z. 2012. Effect of temperature on the antioxidant activity of phenolic acids. *Czech J Food Sci* 30(2): 171–177.
- RUAN J *et al.* 2019. Comprehensive chemical profiling in the ethanol extract of *Pluchea indica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel column chromatography fractions. *Molecules* 24(2784): 1–20.
- RUSITA YD *et al.* 2019. Comparison of tannin concentration on fresh and dried tea leaf (*Camellia sinensis* L.) extract using UV-visible spectrophotometric method. 1st International Conference of Health, Science, & Technology (ICOHETECH) 2019 ;16 Nov 2019; LPPM Universitas, Duta Bangsa Surakarta, Surakarta.
- SANGEETHA R, VEDASREE N. 2012. *In vitro*  $\alpha$ -amylase inhibitory activity of the leaves of *Thespesia populnea*. *Intern Scholarly Res Network*. p. 1–4.

- SHRAIM AM *et al.* 2021. Determination of total flavonoid content by aluminum chloride assay: a critical evaluation. *LWT Food Sci Tech* 150(111932): 1–11.
- SILVA-RAMIREZ KA *et al.* 2020. Effect of drying and steeping temperature on the phenolic content, antioxidant activity, aromatic compounds, and sensory properties of *Culila polyantha* Benth. *Infusions Processes* 8(1378): 2–20.
- SRISOOK K *et al.* 2012. Antioxidant and anti-inflammatory activities of hot water extract from *Pluchea indica* Less. herbal tea. *J Med Plants Res* 6(23): 4077–4081.
- SURIYAPHAN O. 2014. Nutrition, health benefits, and applications of *Pluchea indica* (L) Less leaves. *Pharm Sci Asia* 41: 1–10.
- SUD *et al.* 2019. Impact of thermal processing and storage temperature on phenolic profile and antioxidant activity of different varieties of lychee juice. *LWT–Food Tech* 116(108578): 1–9.
- TADERA K, MINAMI Y, TAKAMATSU K, MATSUOKA T. 2006. Inhibition of alpha-glucosidase and alpha-amylase by flavonoids. *J Nutr Sci Vitaminol* 52: 149–153.
- THANAJIRUSCHAYA P *et al.* 2010. Effect of storage period and temperature on antioxidant components and properties of milled rice. *Khon Kaen Univ Res J* 15(9): 843–851.
- VONGSAK B *et al.* 2018. Comparison of active constituents, antioxidant capacity, and  $\alpha$ glucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. *Food Biosci* 25: 68–73.
- WANG SC *et al.* 2022. Effect of brewing conditions on polyphenols in the dark tea (*Camellia sinensis* L.) infusions: content, composition, and antioxidant activities. *Food Sci Tech* 42(e36322): 1–9.
- WERDANI YDW, WIDYAWATI PS. 2018. Antidiabetic effect on tea of *Pluchea indica* Less as functional beverage in diabetic patients. *ASSEHR* 98: 164–167.
- WIDYAWATI PS *et al.* 2014. Potency of beluntas (*Pluchea indica* Less) leaves extract as antioxidant and anti-warmed-over flavor (WOF) of duck meat. International Congress on Challenges of Biotechnological Research in Food and Health; 15 Nov 2014; Agricultural Technology Faculty, Slamet Riyadi University, Surakarta, Indonesia.
- WIDYAWATI PS *et al.* 2016. The physicochemical and antioxidant properties of *Pluchea indica* Less drink in tea bag packaging. *IJFNS* 5: 13–120.
- WIDYAWATI PS *et al.* 2017. *In vitro* antioxidant capacities and antidiabetic properties of *Pluchea* leaves. *IJPPS* 9(8): 203–208.
- WIDYAWATI PS *et al.* 2020. Effect of lemon (*Citrus limon* L.) addition to *Pluchea indica* Less beverage. *CJFST* 12(4): 125–139.
- WIDYAWATI PS *et al.* 2022. The effect of  $\kappa$ -carrageenan proportion and hot water extract of the *Pluchea indica* Less leaf tea on the quality and sensory properties of stink lily (*Amorphophallus muelleri*) wet noodles. *Molecules* 27(5062): 1–16.
- XU P, CHEN L, WANG Y. 2019. Effect of storage period on antioxidant activity and inhibition on  $\alpha$ -amylase and  $\alpha$ -glucosidase of white tea. *Food Sci Nut* 7(2): 636–644.
- ZARWINDA, SARTIKA D. 2019. Effect of temperature and extraction period on caffeine in coffee. *Lantanida J* 6(2): 103–202. [in Bahasa Indonesia].
- ZHANG H, WANG G, DONG J. 2014. Inhibitory properties of aqueous ethanol extracts of propolis on alpha-glucosidase. *J Evid Based Complement Alternat Med.* p. 1–8.
- ZHANG Y *et al.* 2021. Effect of storage conditions and period on the polyphenol content of wheat flours. *Processes* 9(248): 1–11.





Paini Sri Widyawati <paini@ukwms.ac.id>

---

## First Draft of PJS Article Ms 23-158

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Wed, Jun 19, 2024 at 10:08 PM

Dear Mr. ALLYSTER A. ENDOZO  
Managing Editor

Greetings,  
After I read and discuss with the coauthor that I agree with this manuscript and Editor can publish the manuscript.  
Thanks for attention

Regards

Paini SW  
[Quoted text hidden]



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

## First Draft of PJS Article Ms 23-158

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Thu, Jun 20, 2024 at 6:54 AM

Dear Dr. Widyawati,

We acknowledge the receipt of your approval for publication of the final draft of your article.

At this point, we kindly request that you send the duly signed pre-publication forms. Scanned copies [with electronic signatures in blue color](#), as per ISO 9001:2015, are acceptable.

Thank you for your support!

Sincerely,  
**Mr. ALLYSTER A. ENDOZO**  
Managing Editor  
[Quoted text hidden]

---

### 3 attachments

 **[PRO] Form - Copyright Transfer Agreement.doc**  
52K

 **[PRO] Form - Co-author Agreement.doc**  
50K

 **[PRO] Form - Author's Approval.doc**  
52K



**PHILIPPINE JOURNAL OF SCIENCE**  
Science and Technology Information Institute  
Department of Science and Technology  
Gen. Santos Avenue, Bicutan, Taguig 1631  
Philippines

**AUTHOR APPROVAL FORM**  
(To be Accomplished in Duplicate)

This is to certify that I have read the page proof of the attached article entitled “**title of manuscript**” and that I have approved the corrections on the same article. I understand that all errors that I find henceforth on my article will be my sole responsibility and not of the **Philippine Journal of Science**.

**Type name here**

\_\_\_\_\_  
CORRESPONDING AUTHOR'S NAME

\_\_\_\_\_  
SIGNATURE

\_\_\_\_\_  
DATE



**PHILIPPINE JOURNAL OF SCIENCE**  
Science and Technology Information Institute  
Department of Science and Technology  
Gen. Santos Avenue, Bicutan, Taguig 1631  
Philippines

**AUTHOR APPROVAL FORM**  
(To be Accomplished in Duplicate)

This is to certify that I have read the page proof of the attached article entitled “**title of manuscript**” and that I have approved the corrections on the same article. I understand that all errors that I find henceforth on my article will be my sole responsibility and not of the **Philippine Journal of Science**.

**Type name here**

\_\_\_\_\_  
CORRESPONDING AUTHOR'S NAME

\_\_\_\_\_  
SIGNATURE

\_\_\_\_\_  
DATE



**PHILIPPINE JOURNAL OF SCIENCE**  
Science and Technology Information Institute  
Department of Science and Technology  
Gen. Santos Avenue, Bicutan, Taguig 1631  
Philippines

---

Date

**CO-AUTHOR AGREEMENT FORM**  
(To be Accomplished in Duplicate)

This is to certify that I have conferred with my co-authors and that we agree to submit our article entitled "**title of manuscript**" for publication in the **Philippine Journal of Science**. We also certify that we as joint authors are responsible for the content of the article, that we can defend it should there be any queries and that we may be asked to make revisions on the content if necessary. We also agree to have the corresponding author sign the copyright transfer agreement in our behalf once our manuscript is accepted for publication.

---

Corresponding AUTHOR  
(Signature over printed name)

---

Senior AUTHOR  
(Signature over printed name)

---

AUTHOR  
(Signature over printed name)

---

AUTHOR  
(Signature over printed name)

---

AUTHOR  
(Signature over printed name)

---

AUTHOR  
(Signature over printed name)



**PHILIPPINE JOURNAL OF SCIENCE**  
Science and Technology Information Institute  
Department of Science and Technology  
Gen. Santos Avenue, Bicutan, Taguig 1631  
Philippines

### COPYRIGHT TRANSFER AGREEMENT

Date \_\_\_\_\_

A Memorandum of Agreement is made between \_\_\_\_\_ hereinafter called the  
AUTHORS (represented by the Corresponding Author) of the manuscript entitled “ \_\_\_\_\_  
\_\_\_\_\_ ”

and the **Philippine Journal of Science**, Department of Science and Technology (DOST), which shall hereafter be  
referred to as the PUBLISHER, whereby it is mutually agreed that:

#### COPYRIGHT

The Author hereby grant and assign to the Publisher the exclusive right to take out the copyright for this work and to  
hold said copyright.

#### WARRANTY

The Authors guarantee to the Publisher that the work in no way violates any copyright belonging to any other party,  
that he/she is the sole Author of the work and has full power to make this agreement; that for multiple authors, all  
authors were meaningfully involved in the work and mutually agree on the content of the submitted version; that the  
work contains nothing of a libelous character; and the Authors further undertake that he/she and his/her legal  
representatives shall and will hold the Publisher harmless from all suits and all manners of claims and proceedings  
which may be taken on ground that said work is a violation of propriety right or copyright or contains anything libelous  
or anything that gives to any one a right of action of any nature.

#### EDITING AND REVISION OF MANUSCRIPT

The text and illustration of said material shall be subjected to editing and revision by the Publisher prior to first  
publication, or prior to any subsequent printing provided, however, that such editing or revision shall not materially  
change the meaning or materially alter the text of said work without the Author's consent. Editing to correct infelicities  
of expression, misstatement of facts, misquotations, errors in grammar, sentence structure and spelling, and editing to  
make the work conform to the Publisher's style of punctuation, capitalization, and like details, shall not be considered  
as material changes.

#### PRODUCTION AND PROMOTION

All details as to the manner of production and advertisement, and the number of distribution of free copies to editors  
and others shall be left to the sole discretion of the Publisher, who shall bear all expense of production, publication and  
advertisement, unless otherwise arranged with the Authors.

#### RIGHTS OF THE HEIR

In consideration of the mutuality of this contract, each said parties intending to be legally bound hereby, agrees to all its  
provisions for himself/herself, his/her heirs, assigns, or legal representatives.

IN WITNESS WHEREOF, the Authors have hereunto set his/her hand and seal, as the DEPARTMENT OF  
SCIENCE AND TECHNOLOGY has caused its named to be signed and its seal to be affixed.

Corresponding Author:

\_\_\_\_\_  
**NAME OF CORRESPONDING  
AUTHOR**

\_\_\_\_\_  
**CAESAR A. SALOMA**, Editor-in-Chief  
*Philippine Journal of Science*

Witnesses



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**First Draft of PJS Article Ms 23-158**

---

**Paini Sri Widyawati** <paini@ukwms.ac.id>  
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Thu, Jun 20, 2024 at 1:22 PM

Dear Mr. ALLYSTER A. ENDOZO  
Managing Editor

Greetings,

I sent back the documents that I had to sign.  
Thanks for attention

Regards

Paini SW

[Quoted text hidden]

---

**3 attachments**

**[PRO] Form - Author's Approval.pdf**  
54K



**[PRO] Form - Co-author Agreement.pdf**  
58K



**[PRO] Form - Copyright Transfer Agreement.pdf**  
79K



**PHILIPPINE JOURNAL OF SCIENCE**  
Science and Technology Information Institute  
Department of Science and Technology  
Gen. Santos Avenue, Bicutan, Taguig 1631  
Philippines

**AUTHOR APPROVAL FORM**  
(To be Accomplished in Duplicate)

This is to certify that I have read the page proof of the attached article entitled “Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea indica* Less Tea” and that I have approved the corrections on the same article. I understand that all errors that I find henceforth on my article will be my sole responsibility and not of the **Philippine Journal of Science**.

20<sup>th</sup> June 2024

PAINI SRI WIDYAWATI



**PHILIPPINE JOURNAL OF SCIENCE**  
Science and Technology Information Institute  
Department of Science and Technology  
Gen. Santos Avenue, Bicutan, Taguig 1631  
Philippines

**AUTHOR APPROVAL FORM**  
(To be Accomplished in Duplicate)

This is to certify that I have read the page proof of the attached article entitled “Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea indica* Less Tea” and that I have approved the corrections on the same article. I understand that all errors that I find henceforth on my article will be my sole responsibility and not of the **Philippine Journal of Science**.

20<sup>th</sup> June 2024

PAINI SRI WIDYAWATI



**PHILIPPINE JOURNAL OF SCIENCE**  
Science and Technology Information Institute  
Department of Science and Technology  
Gen. Santos Avenue, Bicutan, Taguig 1631  
Philippines

20<sup>th</sup> June 2024

**CO-AUTHOR AGREEMENT FORM**  
(To be Accomplished in Duplicate)

This is to certify that I have conferred with my co-authors and that we agree to submit our article entitled “Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea indica* Less Tea” for publication in the **Philippine Journal of Science**. We also certify that we as joint authors are responsible for the content of the article, that we can defend it should there be any queries and that we may be asked to make revisions on the content if necessary. We also agree to have the corresponding author sign the copyright transfer agreement in our behalf once our manuscript is accepted for publication.

Painsri Widyawati

---

Corresponding AUTHOR  
(Signature over printed name)

Painsri Widyawati

---

Senior AUTHOR  
(Signature over printed name)

Yufita Ratnasari Wilianto

---

AUTHOR  
(Signature over printed name)





**PHILIPPINE JOURNAL OF SCIENCE**  
Science and Technology Information Institute  
Department of Science and Technology  
Gen. Santos Avenue, Bicutan, Taguig 1631  
Philippines

### **COPYRIGHT TRANSFER AGREEMENT**

Date 20<sup>th</sup> June 2024

A Memorandum of Agreement is made between Painsi Sri Widyawati hereinafter called the AUTHORS (represented by the Corresponding Author) of the manuscript entitled “**Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea indica* Less Tea**” and the **Philippine Journal of Science**, Department of Science and Technology (DOST), which shall hereafter be referred to as the PUBLISHER, whereby it is mutually agreed that:

#### **COPYRIGHT**

The Author hereby grant and assign to the Publisher the exclusive right to take out the copyright for this work and to hold said copyright.

#### **WARRANTY**

The Authors guarantee to the Publisher that the work in no way violates any copyright belonging to any other party, that he/she is the sole Author of the work and has full power to make this agreement; that for multiple authors, all authors were meaningfully involved in the work and mutually agree on the content of the submitted version; that the work contains nothing of a libelous character; and the Authors further undertake that he/she and his/her legal representatives shall and will hold the Publisher harmless from all suits and all manners of claims and proceedings which may be taken on ground that said work is a violation of propriety right or copyright or contains anything libelous or anything that gives to any one a right of action of any nature.

#### **EDITING AND REVISION OF MANUSCRIPT**

The text and illustration of said material shall be subjected to editing and revision by the Publisher prior to first publication, or prior to any subsequent printing provided, however, that such editing or revision shall not materially change the meaning or materially alter the text of said work without the Author’s consent. Editing to correct infelicities of expression, misstatement of facts, misquotations, errors in grammar, sentence structure and spelling, and editing to make the work conform to the Publisher’s style of punctuation, capitalization, and like details, shall not be considered as material changes.

#### **PRODUCTION AND PROMOTION**

All details as to the manner of production and advertisement, and the number of distribution of free copies to editors and others shall be left to the sole discretion of the Publisher, who shall bear all expense of production, publication and advertisement, unless otherwise arranged with the Authors.

#### **RIGHTS OF THE HEIR**

In consideration of the mutuality of this contract, each said parties intending to be legally bound hereby, agrees to all its provisions for himself/herself, his/her heirs, assigns, or legal representatives.

IN WITNESS WHEREOF, the Authors have hereunto set his/her hand and seal, as the DEPARTMENT OF SCIENCE AND TECHNOLOGY has caused its named to be signed and its seal to be affixed.

Corresponding Author:

---

**PAINI SRI WIDYAWATI**  
**CORRESPONDING AUTHOR**

---

**CAESAR A. SALOMA**, Editor-in-Chief  
*Philippine Journal of Science*

Witnesses



Paini Sri Widyawati &lt;paini@ukwms.ac.id&gt;

---

**First Draft of PJS Article Ms 23-158**

---

**Philippine Journal of Science** <pjs@stii.dost.gov.ph>  
To: Paini Sri Widyawati <paini@ukwms.ac.id>

Thu, Jun 20, 2024 at 1:41 PM

Dear Dr. Widyawati,

We acknowledge the receipt of the duly signed pre-publication forms.

Your paper titled "Effect of Steeping Temperature and Storage Time on the Bioactive Compounds Antioxidant and Antidiabetic Activities of *Pluchea indica* Less Tea" has been provisionally designated as the 28th article of PJS Vol. 153 No. 3 (June 2024 Issue), which is set for full upload to our website within five (5) working days.

Once accessible, kindly review your online article for possible errors in textual or visual content. We at the PJS Editorial Office wish to thank you and your co-author for your indispensable contribution to the Philippine scientific community!

Lastly, we highly encourage you to promote the PJS website (<https://philjournalsci.dost.gov.ph/>) and Facebook page (<https://www.facebook.com/pjs.dost/>) by sharing the access link of your article on social media. Congratulations on your success!

Sincerely,  
Mr. ALLYSTER A. ENDOZO  
Managing Editor

[Quoted text hidden]