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   -Authorship Statement

Gmail

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

(no subject)

Paini Sri Widyawati <paini@ukwms.ac.id> Wed, Apr 26, 2023 at 11:08 AM
To: Philippine Journal of Science <philjournsci@gmail.com>

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

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

COVER LETER

Indonesia, April 26th 2023

Dear the Editorial Board of the PJS

Greetings,

I, the undersigned below:

Name :

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2. Yufita Ratnasari Wilianto, M.Farm.Klinis, Apt. Email : yufitaratnasari@ukwms.ac.id

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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. Paini Sri Widyawati, S.Si., M.Si.

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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 certifiesthat 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): PainiSri Widyawati, Yufita Ratnasari Wilianto

PJS Authorship Statement

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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		April26th 2023
Yufita Ratsanasari Wilianto		April26th 2023

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

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

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ABSTRACT

This study was done to determine 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^{o}C) and storage time (0 and 5 years). The steeping temperature and storage time influenced the bioactive compounds, antioxidant and antidiabetic activities of samples. Total phenolic content and total tannin content went up along with increased antioxidant activity. Treatment resulted simple phenolic compounds, such as gallic acids, (+)-catechins, kaempferols, myricetins, quercetins, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, 4,5-di-*O*-caffeoylquinic acids. Total flavonoid content was decreased for storage time and significant different at higher steeping temperatures. The total flavonoid content had graph pattern similar with α-amylase and α-glucosidase inhibition activities. This means, the antidiabetic activity was largely determined by the total flavonoid content and structure of phenolic compounds. In order, to get high antioxidant activity, it was chosen pluchea tea stored at high steeping temperature, but high antidiabetic activity was fresh pluchea tea steeped at a low temperature.

INTRODUCTION

Pluchea tea is a product of 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 an herbal plant that has been widely used for traditional medicine and food (Chan et al., 2022). Pluchea tea is composed many nutrients and bioactive

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compounds useful to body health. The nutrient compositions in the pluchea tea include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates, calcium, β-carotene, and vitamin C, whereas bioactive compounds is 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, β-carotene, and total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et al., 2022, Chan et al., 2022).

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

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

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

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

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

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

MATERIALS AND METHODS

MATERIALS

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

REAGENTS

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

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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). Aquadest and aquabidest were purchased by PT Aqua Surabaya.

METHODOLOGY

TOTAL PHENOLIC CONTENT ANALYSIS

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

TOTAL FLAVONOID CONTENT ASSAY

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

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

TOTAL TANNIN CONTENT ANALYSIS

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

DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

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

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FERRIC REDUCING POWER ANALYSIS

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

α-AMYLASE ENZYME INHIBITION CAPACITY ASSAY

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

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α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

The analysis of the α-glycosidase inhibitor activity was done by Widyawati et al. (2020) method with slight modification. The samples were reacted with the α-glycosidase enzyme, and then the residue of this enzyme hydrolyzed p-nitrophenyl-α-D-glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The inhibitor activity of steeping pluchea tea to enzyme was measured by spectrophotometer UV-Vis (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at λ 405 nm. The inhibition percentage of α-glycosidase was assessed by the following formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 100% Where, ACb was absorbance of 100% enzyme activity (only solvent with enzyme), ACa was absorbance of 0% enzyme activity (only solvent without enzyme), As was absorbance of tested sample with enzyme, Ab was absorbance of tested sample without enzyme.

HPLC ANALYSIS OF PHENOLICS

The phenolic compounds of samples were analyzed by HPLC based on Kongkiatpaiboona et al. (2018) method with modification. HPLC separation was achieved on LC20AD series (Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC LC-20AD pump, SIL-20 AC_{HT} autosampler, CTO-10AS VP column oven, CBM-20A system controller, and SPD-40 detector. The separation was done in a Shim-pack VP-ODS C18 column (5µm × 50 mm x 4.6 mm I.D.) with a GVP-ODS Cartridges (2pcs) guard column (10 mm x 4.6 mm I.D.). The mobile phases were (A) 0.5% acetic acid in water and (B) methanol using gradient elution: 10% B in A to 50% B in A for 40 min; 100% B for 20 min. This column was re-equilibrated with 10% B in A for 10 min prior to each

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

EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

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

RESULTS AND DISCUSSIONS

Pluchea leaf tea is produced by young pluchea leaf from 1-6 level on each branch the shoot (Widyawati et al., 2022), that is steeped at 95^{o}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 pluchea tea involve alkaloids, flavonoids, phenolics, sterols, cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2 g/100 mL steeping pluchea tea has total phenolic content 9.3 mg gallic acid equivalents (GAE)/g

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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 related to the composition of phytochemical compounds in pluchea leaves, such as phenolic acids such as chlorogenic acids, caffeic acids, 3-*O*-caffeoylquinic acids, 4-*O*-caffeoylquinic acids, 5-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin; β-carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Chan et al., 2022; Widyawati et al., 2022). Presence of phytochemical compounds in herbal product were 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 of 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). Therefore, this study emphasized the effect of steeping temperature and storage time of pluchea tea on levels of the bioactive compounds, antioxidant and antidiabetic properties and phenolic compound profile.

BIOACTIVE COMPOUNDS

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

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

The steeping temperature (60, 70, 80 and 95^{o}C) and storage time (fresh and stored) determined total phenolic content, with values ranging from 4.39±0.49 to 71.38±4.14 mg GAE/g samples. The total phenolic content of pluchea tea infused at different temperature and stored at different time that statistical analyzed by ANOVA at $\alpha \leq 5\%$ shown at Figure 1. The total phenolic content of samples was significantly influenced by the steeping temperature and storage time. The highest total phenolic content was detected in the L95 sample infused at 95^{o}C and stored for 5 years (71.38±4.14 mg GAE/g samples) with was followed by L80 sample infused at 80^{o}C and stored for 5 years (62.60±2.49 mg GAE/g samples) and L70 sample infused at 70^{o}C and stored for 5 years (60.68±3.79 mg GAE/g samples) and L60 sample infused at 60^{o}C and stored for 5 years (46.67±5.38 mg GAE/g samples). The total phenolic contents of steeping fresh pluchea tea (B60) had a lower total phenolic content (4.39±0.48 mg GAE/g samples) than the steeping stored pluchea tea for 5 years (48.67±5.38 until 71.38±4.14 mg GAE/g samples). 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 infusion influenced total phenolic content, it could relate to migration process of phenolic compounds to the water because of increasing contact between this compounds and water. The same phenomena also occurred in Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al. (2022).

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This occurrence showed that steeping temperature and storage time caused the process of degradation and oxidation of phenolic 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^{o}C and different storage times (fresh and 72 hours). Hydrogen bonding is affected by temperature treatment because the hydrogen bond between phenolic compounds and proteins can be degraded that the measured levels of phenolic compounds are higher. The phenomena were supported by Ali et al. (2018); Jayani et al. (2022) and 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. Besides that, 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 the phenolic compounds in pluchea tea are degraded due to oxidation and hydrolysis because of temperature and storage time and can be easily extracted during brewing, thus increasing the phenolic content as the steeping temperature and storage time increase.

Based on using of a reference standard could be informed that phenolic compounds in steeping pluchea tea, including 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 was showed in Table 1. Gallic acids and (+)-catechins were relative stable phenolic acid because of very small changes at different steeping temperature and storage time with concentration about 0.21± 0.00 – 0.24±0.02 µg/g

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296 samples and 0.32±0.02 – 0.60±0.04 µg/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*-cafffeoylquinic 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^{o}C (147.42±14.03 mg CE/g samples) and the lowest was owned by

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pluchea tea which had been stored for 5 years at various brewing temperatures (between 24.75±2.47-33.71±3.06 mg CE/g samples). Statistical analysis by ANOVA analysis at α≤5% proven that brewing temperature and storage time of fresh pluchea tea had a significant effect on the total flavonoid content, but the stored pluchea tea (L) had no significant effect. Storage time had a significant effect on the total flavonoid content of brewing pluchea tea. Ali et al. (2018) reported that the degradation of bioactive compounds can take place through several stages, such as pre-treatment, processing, and storage, as is the case with medicinal plants which are dried, extracted and stored in the long term. Brewing temperature and storage time have an influence on the oxidation and polymerization processes that are stimulated by light. According to Noree et al. (2017), that the total flavonoid content test with $AlCl_3$ and $NaNO_2$ reagents measures flavone compounds, these compounds have activity due to the presence of a free hydroxyl functional group at position 4' in the compound. Degradation of flavone compounds due to temperature and storage causes the breaking of methylation bonds. Kim et al. (2020) also confirmed, that the total phenolic content and total flavonoid content of matcha are decreased with increasing brewing temperature and storage time. Xu et al. (2019) informed, that storage time can give a big impact on chemical composition changes with trending not the same.

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

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temperature and storage time, as seen in Figure 1. Steeping pluchea tea contained tannins ranging from 4.81±0.58-17.42±1.04 (mg TAE/g samples). The tannin content increased with increasing storage time and brewing temperature. The results of the ANOVA statistical analysis at α≤5%, showed a significant increase in tannin content levels with increasing brewing temperature and storage time. The fresh pluchea tea brewed at 60^{o}C had the lowest tannin content level, was 4.81 ± 0.58 mg TAE/g samples. The stored pluchea tea brewed at 95^{o}C had the highest tannin content level, was 17.42 ± 1.04 mg TAE/g samples. The results showed, that the higher the brewing temperature and the longer the storage time caused the tannin compound polymerization process to occur. 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. Rusita et al. (2019) emphasized that tannins are a polar compound, that is resistant to heating, as a result the tannin content in pluchea tea increases with increasing brewing temperature and storage time, this is caused tannins are thermostable complex compounds.

ANTIOXIDANT ACTIVITY

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

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

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

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

FRAP is method that identifies the antioxidant capacity of the phytochemical component through measured absorbance, as a result of the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a color complex, that can be measured at λ 700 nm (Fu et al., 2011; Al-Temimi and Choudhary, 2013). The principle of testing the ability to reduce iron ions is that antioxidants can reduce potassium ferrocyanide (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).

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

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

ANTIDIABETIC ACTIVITY

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

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

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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 α-amylase enzyme.

457 α-glucosidase is an important enzyme in carbohydrates digestion, that catalysis
458 the hydrolysis of 1,4-α-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 α-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^{o}C for 5 minutes has
464 an inhibitory effect on the α-glucosidase enzyme of 67.857%.

465 The results showed, that the ability to inhibit the α-glucosidase enzyme decreased
466 with increasing brewing temperature and storage time. Brewing at 95^{o}C for fresh pluchea
467 tea (0 days of storage) obtained the lowest inhibitory ability, i.e., 48.32 ± 1.27%, and the
468 highest inhibitory activity was found at 70^{o}C brewing temperature for fresh pluchea tea,
469 which was 95.11 ± 0.70% (Figure 3). The test results showed that the ability to inhibit the
470 enzyme α-glucosidase tended to be higher than the ability to inhibit the enzyme α-
471 amylase. Li et al. (2018) informed that flavonoid compounds have the ability to inhibit the
472 action of the α-amylase and α-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 α-amylase and α-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

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bonds in rings A and B and the heterocyclic ring in ring C. The ability to inhibit the α-glucosidase enzyme from pluchea tea was significantly affected by the brewing temperature and storage time. The capability of pluchea tea infusion to obstruct the α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of two enzymes was different, according to the opinion of McCue et al. (2005). Widyawati et al. (2017) informed that phenolic and non-phenolic compounds determine the inhibitory activity of the α-glucosidase enzyme. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, that may be occurred in pluchea tea during storage, affects the structure and profile of phenolic and non-phenolic compounds. Asriningtyas et al. (2014) claimed that pluchea leaves contain 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. Quinic acid is methyl esterified with the number of caffeic groups in the molecule that determines the activity of inhibiting the α-glucosidase enzyme. Analysis of caffeoylquinic acids in pluchea tea infusion was obtained that the higher steeping temperature and longer storage time caused increased concentration of them, but the α-glucosidase inhibition of them was reduced. Aleixandre et al. (2022) reported that the simple phenolic acids forming a dipole-dipole interaction of active site from α-glucosidase enzyme are effectively inhibiting the enzyme.

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

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

CONCLUSION

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

DATA AVAILABILITY

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

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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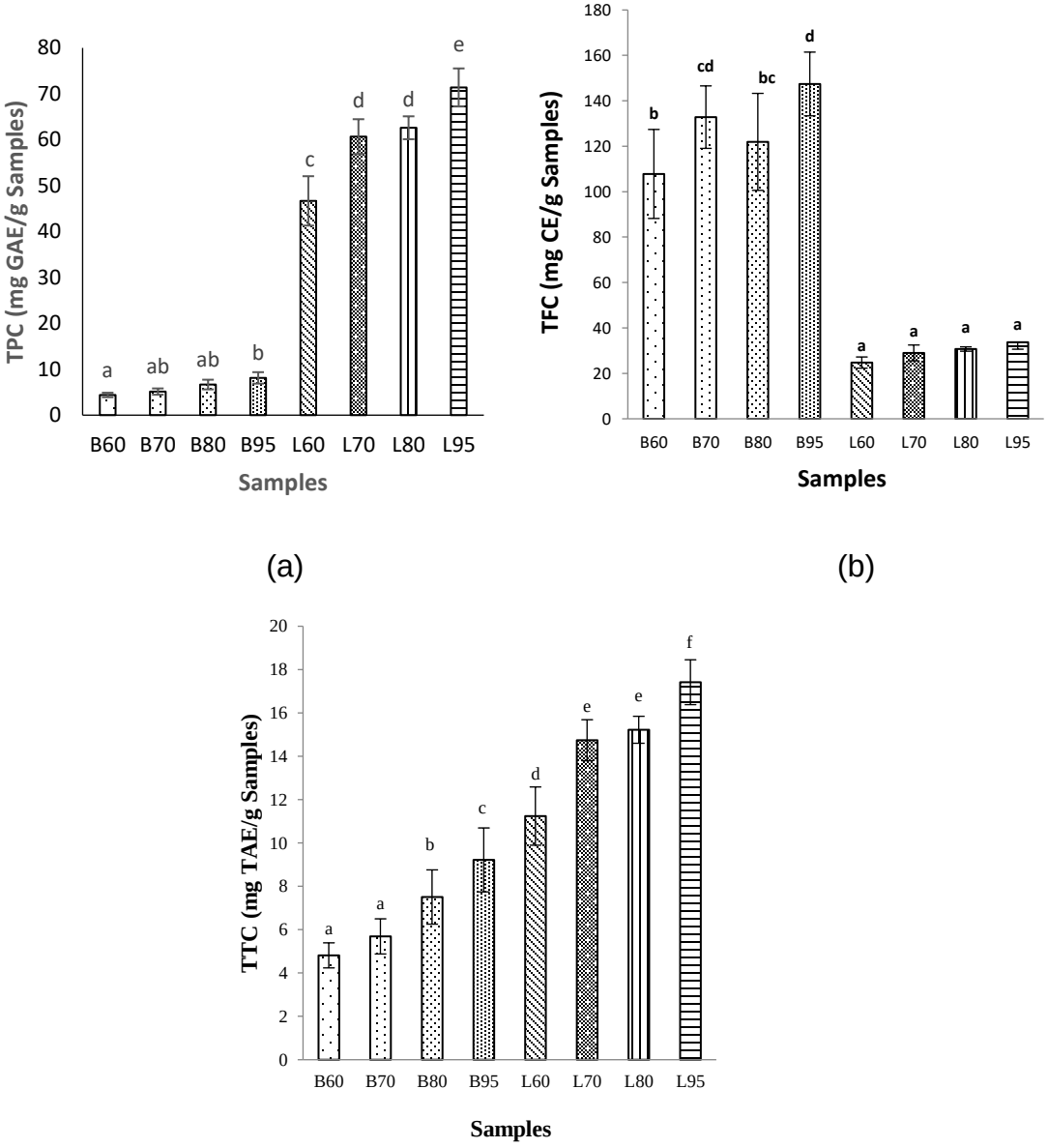

Figure 1. Bioactive compound content of pluchea tea at different steeping temperature and storage time (a) Total phenolic content (b) Total flavonoid content (c) Total tannin content (Values were means ±standard deviations (n=6). Different supercripts in graph showed a significant difference based on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

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

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*-Caffeoylquinic acid (µg/g samples)	3,5-di-*O*-Caffeoylquinic acid (µg/g samples)	4,5-di-*O*-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

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

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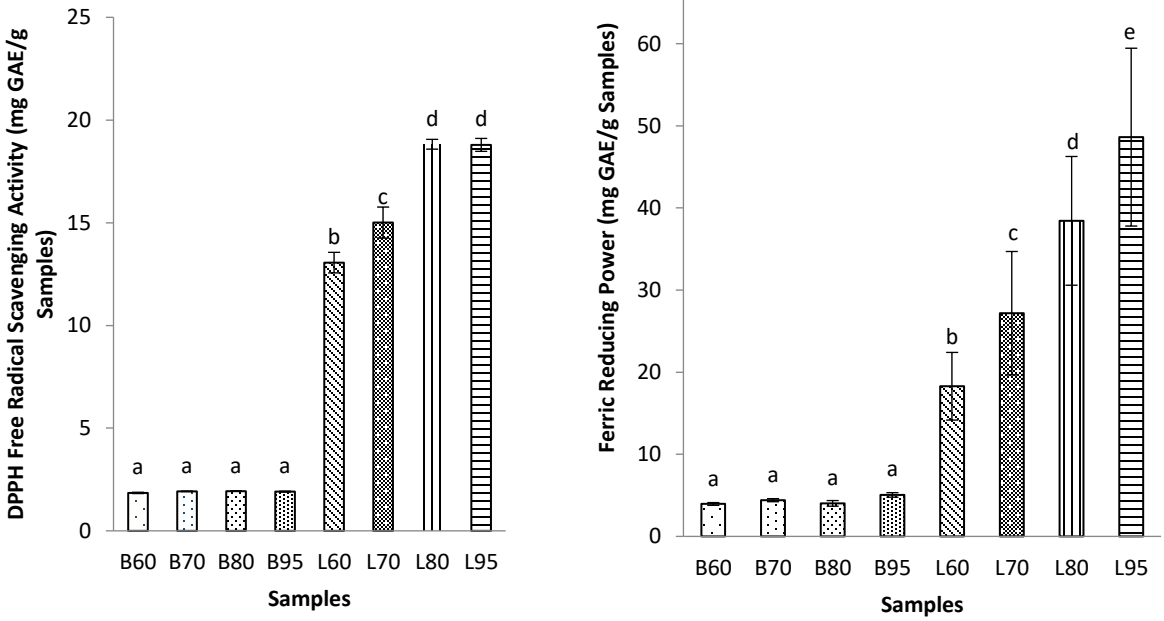

(a) (b)

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

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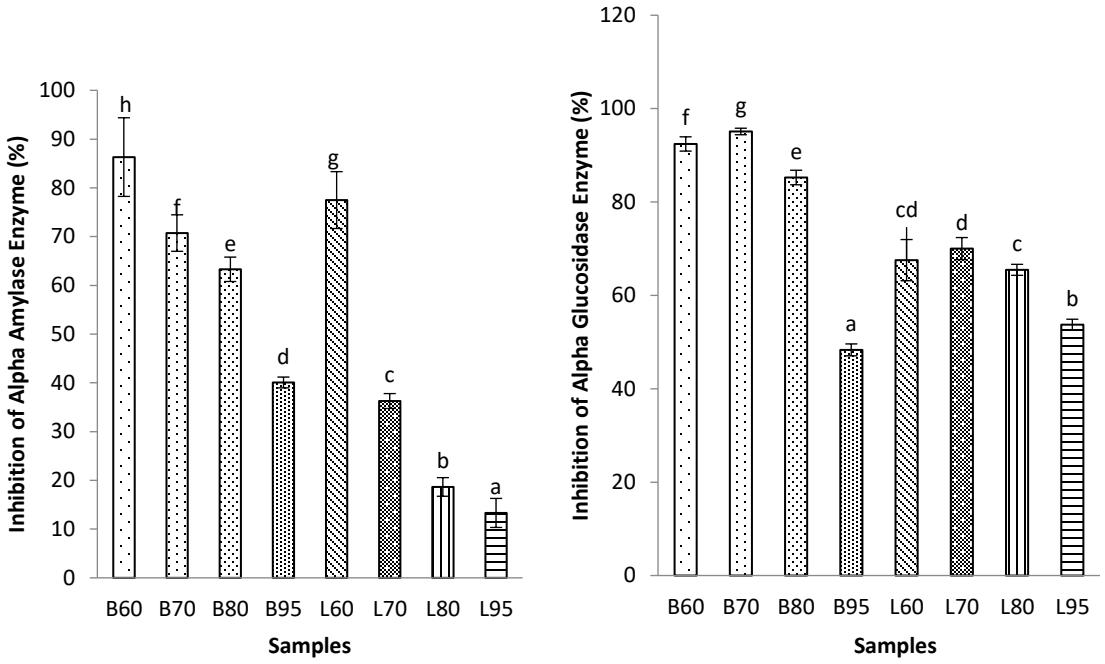

(a) (b)

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

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2. First Revision: Format Revision Based on Philippine Journal of Science (28-4-2023)
    -Correspondence
    -Document
    -Cover Letter
    -List Reviewer
    -Authorship Statement

Gmail

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

(no subject)

Philippine Journal of Science <philjournsci@gmail.com> Fri, Apr 28, 2023 at 12:23 PM
To: Paini Sri Widyawati <paini@ukwms.ac.id>

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

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

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ABSTRACT

This study was done to determine 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 ^{o}C) and storage time (0 and 5 years). The steeping temperature and storage time influenced the bioactive compounds, antioxidant and antidiabetic activities of samples. Total phenolic content and total tannin content went up along with increased antioxidant activity. Treatment resulted simple phenolic compounds, such as gallic acids, (+)-catechins, kaempferols, myricetins, quercetins, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, 4,5-di-*O*-caffeoylquinic acids. Total flavonoid content was decreased for storage time and significant different at higher steeping temperatures. The total flavonoid content had graph pattern similar with α-amylase and α-glucosidase inhibition activities. This means, the antidiabetic activity was largely determined by the total flavonoid content and structure of phenolic compounds. In order, to get high antioxidant activity, it was chosen pluchea tea stored at high steeping temperature, but high antidiabetic activity was fresh pluchea tea steeped at a low temperature.

INTRODUCTION

Pluchea tea is a product of 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 an herbal plant that has been widely used for traditional medicine and food (Chan *et al.* 2022). Pluchea tea is composed many nutrients and bioactive

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compounds useful to body health. The nutrient compositions in the pluchea tea include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates, calcium, β-carotene, and vitamin C, whereas bioactive compounds is 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, β-carotene, and total carotenoid (Suriyaphan 2014; Vongsak *et al*. 2018; Ruan *et al*. 2019; Widyawati *et al*. 2022, Chan *et al.* 2022).

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

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

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

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

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

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bioactive compounds, antioxidant and antidiabetic properties of pluchea tea. The study was emphasized to determine total phenolic content, total flavonoid content, total tannin content, scavenging activity of DPPH free radical, ferric reducing power, α-amylase and α-glycosidase inhibition activities, and phenolic compound profile.

MATERIALS AND METHODS

MATERIALS

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

REAGENTS

The compounds used to analyze including 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid, α-amylase, α-glucosidase, pNPG (p-nitrophenyl-α-glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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

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

METHODOLOGY

TOTAL PHENOLIC CONTENT ANALYSIS

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

TOTAL FLAVONOID CONTENT ASSAY

Total flavonoid content of the samples was determined by the spectrophotometric method based on the reaction between $AlCl_3$ and $NaNO_2$ with an aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim *et al*. 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted a yellow solution. Then, the red solution was produced after NaOH solution addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ 510 nm. A (+) catechin was

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

TOTAL TANNIN CONTENT ANALYSIS

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

DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

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

FERRIC REDUCING POWER ANALYSIS

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

α-AMYLASE ENZYME INHIBITION CAPACITY ASSAY

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

α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

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The analysis of the α-glycosidase inhibitor activity was done by Widyawati *et al.* (2020) method with slight modification. The samples were reacted with the α-glycosidase enzyme, and then the residue of this enzyme hydrolyzed p-nitrophenyl-α-D-glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The inhibitor activity of steeping pluchea tea to enzyme was measured by spectrophotometer UV-Vis (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at λ 405 nm. The inhibition percentage of α-glycosidase was assessed by the following formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 100 % Where, ACb was absorbance of 100 % enzyme activity (only solvent with enzyme), ACa was absorbance of 0 % enzyme activity (only solvent without enzyme), As was absorbance of tested sample with enzyme, Ab was absorbance of tested sample without enzyme.

HPLC ANALYSIS OF PHENOLICS

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

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SPD-40 detector was set at λ 280 nm and injection volume was 20 µL for every sample and reference standard.

EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

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

RESULTS AND DISCUSSIONS

Pluchea leaf tea is produced by young pluchea leaf from 1-6 level on each branch the shoot (Widyawati *et al*. 2022), that is steeped at 95 ^{o}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 pluchea tea involve alkaloids, flavonoids, phenolics, sterols, cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2 g/100 mL steeping pluchea 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

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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 related to the composition of phytochemical compounds in pluchea leaves, such as phenolic acids such as chlorogenic acids, caffeic acids, 3-*O*-caffeoylquinic acids, 4-*O*-caffeoylquinic acids, 5-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin; β-carotene; and total carotenoids (Suriyaphan 2014; Vongsak *et al*. 2018; Ruan *et al.* 2019; Chan *et al.* 2022; Widyawati *et al*. 2022). Presence of phytochemical compounds in herbal product were 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 of 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). Therefore, this study emphasized the effect of steeping temperature and storage time of pluchea tea on levels of the bioactive compounds, antioxidant and antidiabetic properties and phenolic compound profile.

BIOACTIVE COMPOUNDS

The bioactive compounds are active compounds in plants that are essential to protect a 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.*

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

The steeping temperature (60, 70, 80 and 95 ^{o}C) and storage time (fresh and stored) determined total phenolic content, with values ranging from 4.39 ± 0.49 to 71.38 ± 4.14 mg GAE/g samples. The total phenolic content of pluchea tea infused at different temperature and stored at different time that statistical analyzed by ANOVA at $\alpha \leq 5$ % shown at Figure 1. The total phenolic content of samples was significantly influenced by the steeping temperature and storage time. The highest total phenolic content was detected in the L 95 sample infused at 95 ^{o}C and stored for 5 years (71.38 ± 4.14 mg GAE/g samples) with was followed by L 80 sample infused at 80 ^{o}C and stored for 5 years (62.60 ± 2.49 mg GAE/g samples) and L70 sample infused at 70 ^{o}C and stored for 5 years (60.68 ± 3.79 mg GAE/g samples) and L60 sample infused at 60 ^{o}C and stored for 5 years (46.67 ± 5.38 mg GAE/g samples). The total phenolic contents of steeping fresh pluchea tea (B60) had a lower total phenolic content (4.39 ± 0.48 mg GAE/g samples) than the steeping stored pluchea tea for 5 years (48.67 ± 5.38 until 71.38 ± 4.14 mg GAE/g samples). 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 infusion influenced total phenolic content, it could relate to migration process of phenolic compounds to the water because of increasing contact between this compounds and water. The same phenomena also occurred in Castiglioni *et al*. (2015); Kilic *et al.* (2017), and Acar *et al.* (2022).

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This occurrence showed that steeping temperature and storage time caused the process of degradation and oxidation of phenolic 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 ^{o}C and different storage times (fresh and 72 hours). Hydrogen bonding is affected by temperature treatment because the hydrogen bond between phenolic compounds and proteins can be degraded that the measured levels of phenolic compounds are higher. The phenomena were supported by Ali *et al.* (2018); Jayani *et al.* (2022) and 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. Besides that, 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 the phenolic compounds in pluchea tea are degraded due to oxidation and hydrolysis because of temperature and storage time and can be easily extracted during brewing, thus increasing the phenolic content as the steeping temperature and storage time increase.

Based on using of a reference standard could be informed that phenolic compounds in steeping pluchea tea, including 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 was showed in Table 1. Gallic acids and (+)-catechins were relative stable phenolic acid because of very small changes at different steeping temperature and storage time with concentration about 0.21 ± 0.00 – 0.24 ± 0.02 µg/g

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samples and 0.32 ± 0.02 – 0.60 ± 0.04 µg/g samples, respectively. However, myricetin, quercetin and 3,4-di-*O*-caffeoylquinic acid showed drastic increasing at higher steeping temperature and longer storage time. It's meant that these compounds tended relatively labile. Kaempferol, 3,5-di-*O*-cafffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid underwent moderate changes compared to the other two groups of phenolic acids. Therefore myricetin, quercetin and 3,4-di-*O*-caffeoylquinic acid were easier to dissolve at higher steeping temperature and storage time can cause macromolecules of three phenolic acids in herbal tea convenient degradable to form simple phenolic compounds for storage, as explained by 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.

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

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316 pluchea tea which had been stored for 5 years at various brewing temperatures (between
317 24.75 ± 2.47-33.71 ± 3.06 mg CE/g samples). Statistical analysis by ANOVA analysis at
318 α ≤ 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 $AlCl_3$ and $NaNO_2$ 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

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temperature and storage time, as seen in Figure 1. Steeping pluchea tea contained tannins ranging from 4.81 ± 0.58 - 17.42 ± 1.04 (mg TAE/g samples). The tannin content increased with increasing storage time and brewing temperature. The results of the ANOVA statistical analysis at $\alpha \leq 5$ %, showed a significant increase in tannin content levels with increasing brewing temperature and storage time. The fresh pluchea tea brewed at 60 ^{o}C had the lowest tannin content level, was 4.81 ± 0.58 mg TAE/g samples. The stored pluchea tea brewed at 95 ^{o}C had the highest tannin content level, was 17.42 ± 1.04 mg TAE/g samples. The results showed, that the higher the brewing temperature and the longer the storage time caused the tannin compound polymerization process to occur. 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. Rusita *et al.* (2019) emphasized that tannins are a polar compound, that is resistant to heating, as a result the tannin content in pluchea tea increases with increasing brewing temperature and storage time, this is caused tannins are thermostable complex compounds.

ANTIOXIDANT ACTIVITY

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

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

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

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

FRAP is method that identifies the antioxidant capacity of the phytochemical component through measured absorbance, as a result of the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a color complex, that can be measured at λ 700 nm (Fu *et al*. 2011; Al-Temimi and Choudhary 2013). The principle of testing the ability to reduce iron ions is that antioxidants can reduce potassium ferrocyanide (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).

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

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

ANTIDIABETIC ACTIVITY

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

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

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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 α-amylase enzyme.

454 α-glucosidase is an important enzyme in carbohydrates digestion, that catalysis
455 the hydrolysis of 1,4-α-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 α-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 ^{o}C for 5 minutes has
461 an inhibitory effect on the α-glucosidase enzyme of 67.857%.

462 The results showed, that the ability to inhibit the α-glucosidase enzyme decreased
463 with increasing brewing temperature and storage time. Brewing at 95^{o}C for fresh pluchea
464 tea (0 days of storage) obtained the lowest inhibitory ability, i.e., 48.32 ± 1.27%, and the
465 highest inhibitory activity was found at 70 ^{o}C brewing temperature for fresh pluchea tea,
466 which was 95.11 ± 0.70% (Figure 3). The test results showed that the ability to inhibit the
467 enzyme α-glucosidase tended to be higher than the ability to inhibit the enzyme α-
468 amylase. Li *et al*. (2018) informed that flavonoid compounds have the ability to inhibit the
469 action of the α-amylase and α-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 α-amylase and α-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

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bonds in rings A and B and the heterocyclic ring in ring C. The ability to inhibit the α-glucosidase enzyme from pluchea tea was significantly affected by the brewing temperature and storage time. The capability of pluchea tea infusion to obstruct the α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of two enzymes was different, according to the opinion of McCue *et al.* (2005). Widyawati *et al.* (2017) informed that phenolic and non-phenolic compounds determine the inhibitory activity of the α-glucosidase enzyme. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, that may be occurred in pluchea tea during storage, affects the structure and profile of phenolic and non-phenolic compounds. Asriningtyas *et al.* (2014) claimed that pluchea leaves contain 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. Quinic acid is methyl esterified with the number of caffeic groups in the molecule that determines the activity of inhibiting the α-glucosidase enzyme. Analysis of caffeoylquinic acids in pluchea tea infusion was obtained that the higher steeping temperature and longer storage time caused increased concentration of them, but the α-glucosidase inhibition of them was reduced. Aleixandre *et al*. (2022) reported that the simple phenolic acids forming a dipole-dipole interaction of active site from α-glucosidase enzyme are effectively inhibiting the enzyme.

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

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

CONCLUSION

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

DATA AVAILABILITY

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

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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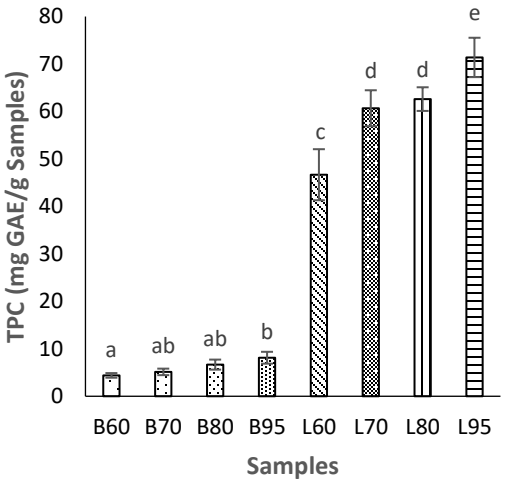

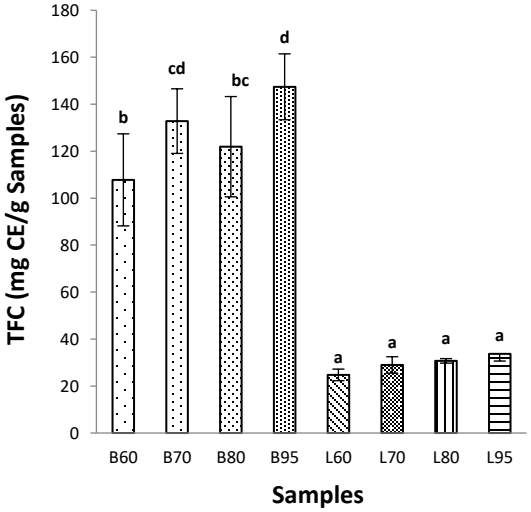

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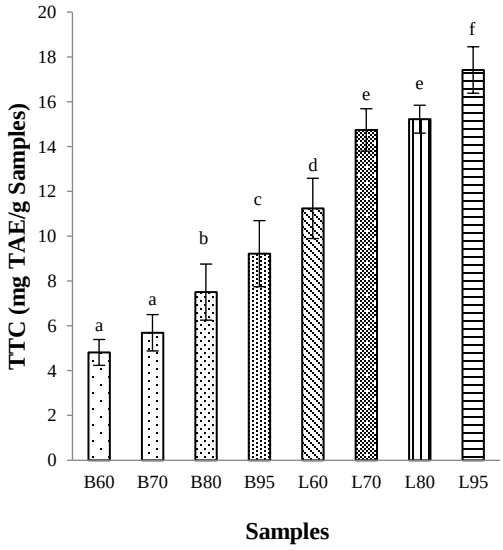

(c)

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 ± standard deviations (n=6). Different supercripts in graph showed a significant difference based on the DMRT test ($\alpha \leq 5$ %)

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

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*-Caffeoylquinic acid (µg/g samples)	3,5-di-*O*-Caffeoylquinic acid (µg/g samples)	4,5-di-*O*-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

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

711

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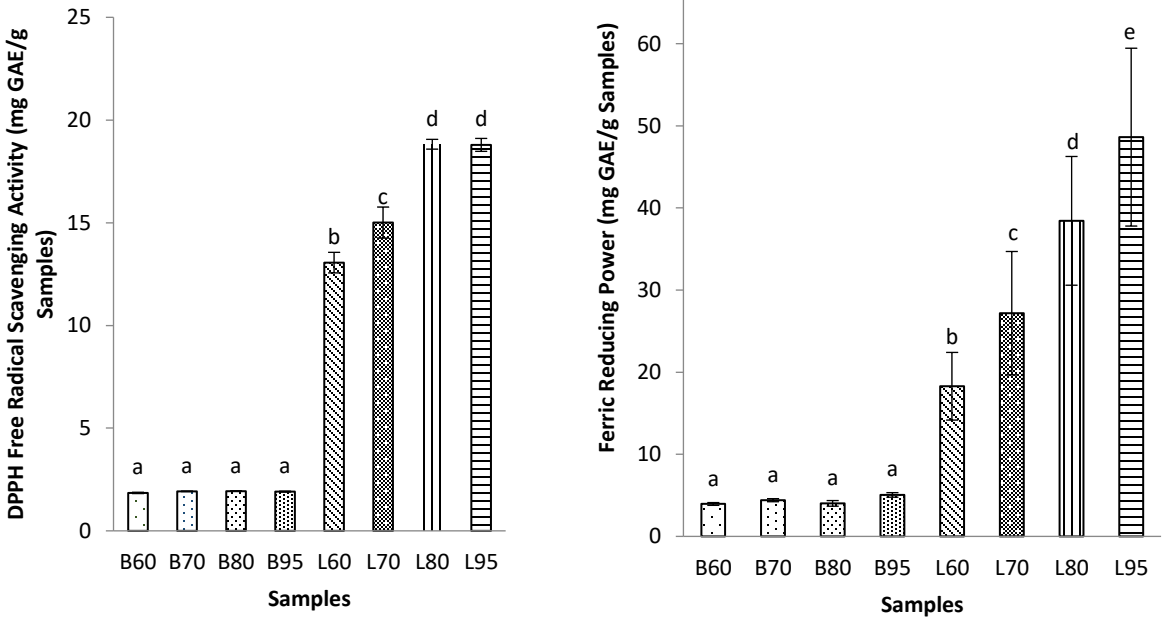

(a) (b)

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

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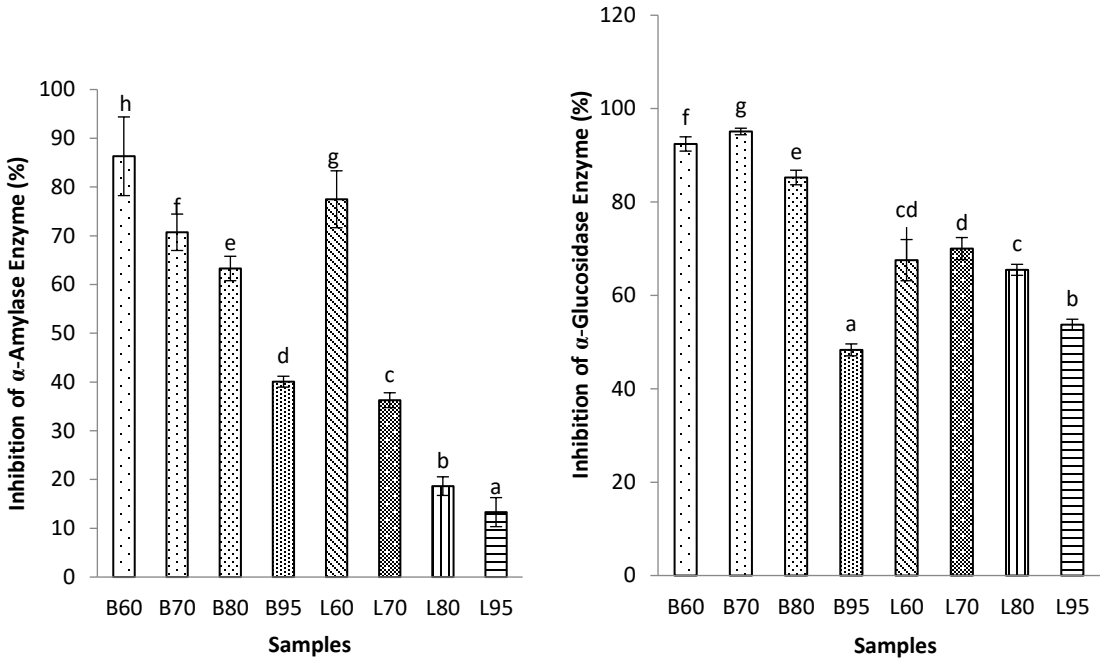

(a) (b)

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

Corresponding Author: paini@ukwms.ac.id

Gmail

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

Gmail

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

(no subject)

Paini Sri Widyawati <paini@ukwms.ac.id> Fri, May 5, 2023 at 8:52 AM
To: Philippine Journal of Science <philjournsci@gmail.com>

Dear Ms Caryl Maria Minette

Thanks for your attention

Regards

Paini Sri W
[Quoted text hidden]

Gmail

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

Acknowledgment - PJS Paper Ms -158

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

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" submitt 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> Wed, Apr 26,
To: Philippine Journal of Science <philjournsci@gmail.com>

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 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> Fri, Apr 28,
To: Paini Sri Widyawati <paini@ukwms.ac.id>

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 inclu 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://philjournalsci.dost.gov.ph/author-s-guide). A reference number will be provided once you have 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

Gmail

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

status my manuscript

Paini Sri Widyawati <paini@ukwms.ac.id> Sat, Jul 8, 2023 at 9:45 PM
To: Philippine Journal of Science <philjournsci@gmail.com>

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

Gmail

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

status my manuscript

Philippine Journal of Science <philjournsci@gmail.com> Sun, Jul 9, 2023 at 2:11 PM
To: Paini Sri Widyawati <paini@ukwms.ac.id>

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]
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PHILIPPINE JOURNAL OF SCIENCE

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Science and Technology Information Institute
Department of Science and Technology
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Taguig City, Metro Manila, Philippines
Telephone no. : 837 - 2191
Email: pjs@stii.dost.gov.ph; philjournsci@gmail.com
Website: https://philjournalsci.dost.gov.ph
Scopus: https://www.scopus.com/sourceid/19700175735

Gmail

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

status my manuscript

Paini Sri Widyawati <paini@ukwms.ac.id> Sun, Jul 9, 2023 at 3:01 PM
To: Philippine Journal of Science <philjournsci@gmail.com>

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

Gmail

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

Fwd: Comments on PJS Paper Ms 23-158

Philippine Journal of Science <pjs@stii.dost.gov.ph> Fri, Oct 13, 2023 at 4:26 PM
To: Paini Sri Widyawati <paini@ukwms.ac.id>

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

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

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Email: pjs@stii.dost.gov.ph; philjournsci@gmail.com
Website: https://philjournalsci.dost.gov.ph
Scopus: https://www.scopus.com/sourceid/19700175735

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Taguig City, Metro Manila, Philippines
Telephone no. : 837 - 2191
Email: pjs@stii.dost.gov.ph; 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

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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 or 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		
Page	**Line**	**Comments and Recommendations**
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.

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

Paini Sri Widyawati[1*)], Yufita Ratnasari Wilianto[2)]

[1)]Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

[2)]Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic University, Kalisari Street Number 1, Surabaya 60272, Indonesia

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

Corresponding Author: paini@ukwms.ac.id

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.

ABSTRACT

This study was done to determine the effects of steeping? temperature and storage time on the bioactive compounds, antioxidant and antidiabetic activities of ~~*Plucheа*~~*Pluchea indica* Less ~~tea infusion~~ infusion~~.~~ The research used a randomized block design with two factors, i.e., steeping temperature (60, 70, 80, and 95 ^{o}C) and storage time (0 and 5 years). The steeping temperature and storage time influenced the bioactive compounds, antioxidant and antidiabetic activities of samples. Total phenolic ~~content~~ and total tannin contents went up along with increased antioxidant activity. Treatment resulted simple phenolic compounds, such as gallic acids, (+)-catechins, kaempferols, myricetins, quercetins, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, 4,5-di-*O*-caffeoylquinic acids. Total flavonoid content was decreased for storage time and significant different at higher steeping temperatures. The total flavonoid content had graph pattern similar with α-amylase and α-glucosidase inhibition activities. This means, the antidiabetic activity was largely determined by the total flavonoid content and structure of phenolic compounds. In order, to get high antioxidant activity, it was chosen ~~plucheа~~*Pluchea* ~~tea~~ infusion stored at high steeping temperature, but high antidiabetic activity was fresh ~~plucheа~~*Pluchea* tea steeped at a low temperature.

INTRODUCTION

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

Corresponding Author: paini@ukwms.ac.id

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 ie., infusion/or drink; fresh leaf, dried leaf, powdered leaf

nutrients and bioactive compounds useful to body health. The nutrient compositions in the ~~plucheaPluchea tea~~ include protein, fat, ash, insoluble fiber, soluble fiber, carbohydrates, calcium, β-carotene, and vitamin C, whereas bioactive compounds is 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, β-carotene, and total carotenoid (Suriyaphan 2014; Vongsak *et al*. 2018; Ruan *et al*. 2019; Widyawati *et al*. 2022, Chan *et al.* 2022).

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

Steeping ~~plucheaPluchea~~ tea with hot water at 95 ^{o}C for 5 min certainly determines the stability and amount of extracted bioactive compounds, that influences the biological

Corresponding Author: paini@ukwms.ac.id

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

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

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

Corresponding Author: paini@ukwms.ac.id

kinnow-amla beverages (Purewal *et al*. 2022), whole wheat flour (Zhang *et al*. 2021). Therefore, this research studied effect of steeping temperature and storage time on the bioactive compounds, antioxidant and antidiabetic properties of ~~plucheaPluchea~~ tea. The study was emphasized to determine total phenolic content, total flavonoid content, total tannin content, scavenging activity of DPPH free radical, ferric reducing power, α-amylase and α-glycosidase inhibition activities, and phenolic compound profile.

MATERIALS AND METHODS

~~MATERIALS 4~~

The ~~plucheaPluchea~~ leaves were collected from ~~gardens in~~the Mangrove areas in ~~,~~ Wonorejo and, ~~–~~Surabaya, East Java, Indonesia. The ~~plucheaPluchea~~ plants were included in Asteraceae family with specification according to the GBIF taxon ID number database:3132728. Then, the material was treated based on Widyawati *et al*. (2022) method and Widyawati *et al*. (2023). The ~~plucheaPluchea~~ ~~tea~~ dried leaves were packed in ~~tea~~ infusion bag (2 g/~~tea~~ bag) was steeped with hot water temperatures of 60, 70, 80, and 95 ^{o}C for 5 min and storage times of 0 (control) and 5 years (stored) with infusion method. Then, the extracts of the control and treated samples were obtained following the procedure ~~samples preparation was done based on~~ by Widyawati *et al.* (2016) and Widyawati *et al.* (2022) methods.

REAGENTS

The compounds used to analyze including 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid, α-amylase, α-glucosidase, pNPG (p-nitrophenyl-α-

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Commented [A14]: The introduction appears to be more of /Review of Related Literature RRL. This section must contain primarily the scientific basis of the study/conceptual framework, statement of the problem, its significance/importance (ie as a functional food, industrial use) and the research objectives/research questions. RRL may be included to describe/identify the research gap. Generally, sentences must be restructured for clarity.

Commented [A15]: This section as presented also lacks clarity, and organization. It should be written as if you were describing a schematic diagram of the protocol of the methodology. For this part, procedure/method/treatment must be clearly decribed then, cited.

For this section suggestion: Create subtopics ie.,
MATERIALS
Raw Materials – revision of lines 98-101

Example: Preparation of the *Pluchea* dried leaf powder
eg. *Pluchea* fresh leaf blades harvested 1 to 6 inches from the base of the flowering tip were collected from the mangrove areas in Wonorejo and Subaraya, East Java, Indonesia. Authentication was made …… The leaf blades were washed, leaf blade stalks trimmed then, dried at ambient temperature until the leaf blades were easily crushed with the hand. Dried leaves were pulverized using (mention the equipment) to 28 mesh size. Two grams of the powder was weighed in an analytical balance, then packed in an infuser filter paper bag with dimension of 2(length) x 2.5(width) inches.

Reagents – lines 111-120

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 *Pluchea* powder was stored at 0 and 5 year -storage period.

Commented [A16R15]:

Commented [A17]: Cite the source of the authentication,

Commented [A18]: Describe the trt…….. (Widyawati *et al*., 2022; Widyawati *et al*., 2023).

Commented [A19]: 0 yr is not the control; control grp remains constant throughout the entire duration of the study; no treatment applied (no storage, no steeping). As such, 0 yr is a treatment

Commented [A20]: Lacks clarity.

Describe the drying process/processing procedure of the powdered sample. It must be noted that during the drying process losses occur because the bioactive compounds are generally, volatile or easily oxidized, and soluble in water

Commented [A21]: Describe the extraction procedure.

glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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 Aquadest ~~and aquabidest~~ which ~~were~~ was purchased from~~by~~ PT Aqua Surabaya.

~~METHODOLOGY~~Describe the preparation of the Pluchea leaf either dried leaf or powder.

Describe the preparation of the infusion samples.

Describe sampling and extraction procedure.

ANALYSIS OF THE BIOACTIVE COMPOUNDS

Total Phenolic Content Analysis

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

Commented [A22]: If you followed the procedure of Widyawati, then this is……..*Pluchea* 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 *Pluchea* 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 completetely 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.

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

(formula)

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

TOTAL FLAVONOID CONTENT ASSAY

Total flavonoid content of the samples was ~~determined~~ measured based on the reaction ~~by the spectrophotometric method based on the reaction~~ between $AlCl_3$ and $NaNO_2$ with an aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim *et al*. 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted a yellow solution. Then, the red solution was produced after NaOH solution addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ 510 nm with catechin as ~~. A (+) catechin was used as a~~the reference standard compound, and the results were expressed as mg catechin equivalents (CE)/g samples using the formula~~.~~

(show the formula)

TOTAL TANNIN CONTENT ANALYSIS

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

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

ANALYSIS OF THE ANTI-OXIDANT POTENTIAL

DPPH FREE RADICAL SCAVENGING ACTIVITY ASSAY

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

FERRIC REDUCING POWER ANALYSIS

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

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Commented [A31]: Lines 163 -168Description 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

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

α-AMYLASE ENZYME INHIBITION CAPACITY ASSAY

In vitro inhibition of α-amylase enzyme followed the procedure as described by~~was determined by~~ Widyawati *et al.* (2020)~~. method~~. ~~Samples of steeping pluchea tea at various steeping temperatures and storage times were analyzed by spectrophotometer UV-Vis (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) based on reaction between bioactive compounds and α-amylase enzyme.~~ Then, the residue enzyme was reacted with starch and the capacity of the α-amylase enzyme hydrolyzed the starch to release glucose that could be analyzed based on absorbance at λ 540 nm. The inhibition percentage of α-amylase was assessed ~~by~~ using the ~~following~~ formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 100 %. Where, ACb ~~was~~ is the absorbance of 100% enzyme activity (~~only~~ solvent? with the enzyme), ACa ~~was~~ is the absorbance of 0 % enzyme activity (~~only~~ solvent? without the enzyme), As is the ~~was~~ absorbance of test~~ed~~ sample with enzyme, Ab ~~was~~ is absorbance of test~~ed~~ sample without enzyme.

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

Commented [A35]: Check the formula

α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

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

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

Commented [A37]: Describe the modification

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glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The inhibitor activity of steeping ~~plucheaPluchea~~ tea to enzyme was measured by spectrophotometer UV-Vis (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at λ 405 nm. The inhibition percentage of α-glycosidase was assessed by the following formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 100 .~~%~~ Where, ACb was absorbance of 100 % enzyme activity (only solvent with enzyme), ACa was absorbance of 0 % enzyme activity (only solvent without enzyme), As was absorbance of tested sample with enzyme, Ab was absorbance of tested sample without enzyme.

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

HPLC ANALYSIS OF PHENOLICS

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

The phenolic compounds of samples were analyzed by HPLC based on Kongkiatpaiboona *et al.* (2018) method with modification. HPLC separation was achieved on LC-20AD series (Shimadzu, Japan) equipped with a Shimadzu HPLC Prominence UFLC LC-20AD pump, SIL-20 AC_{HT} autosampler, CTO-10AS VP column oven, CBM-20A system controller, and SPD-40 detector. The separation was done in a Shim-pack VP-ODS C18 column (5 µm × 50 mm x 4.6 mm I.D.) with a GVP-ODS Cartridges (2 pcs) guard column (10 mm x 4.6 mm I.D.). The mobile phases were (A) 0.5 % acetic acid in water and (B) methanol using gradient elution: 10 % B in A to 50 % B in A for 40 min; 100 % B for 20 min. This column was re-equilibrated with 10 % B in A for 10 min prior to each analysis and the flow rate was set at 1.0 mL/min with the controlled temperature at 40 ^{o}C. SPD-40 detector was set at λ 280 nm and injection volume was 20 µL for every sample and reference standard.

Commented [A40]: Describe the modification

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

How was the specific phenolic compounds identified?

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EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

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

RESULTS AND DISCUSSIONS

~~Pluchea~~*Pluchea* leaf infusion ~~tea~~ is produced by young ~~pluchea~~*Pluchea* leaf from 1-6 level on each branch the shoot (Widyawati *et al*. 2022), that is steeped at 95 ^{o}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 ~~pluchea~~*Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols, cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2 g/100 mL steeping ~~pluchea~~*Pluchea* tea has total phenolic content 9.3 mg gallic acid equivalents (GAE)/g samples, total flavonoid content 22.0 mg catechin

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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 sedtion.

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

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 related to the composition of phytochemical compounds in ~~plucheaPluchea~~ leaves, such as phenolic acids such as chlorogenic acids, caffeic acids, 3-*O*-caffeoylquinic acids, 4-*O*-caffeoylquinic acids, 5-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin; β-carotene; and total carotenoids (Suriyaphan 2014; Vongsak *et al*. 2018; Ruan *et al.* 2019; Chan *et al.* 2022; Widyawati *et al*. 2022). Presence of phytochemical compounds in herbal product were 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 of 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). Therefore, this study emphasized the effect of steeping temperature and storage time of ~~plucheaPluchea~~ tea on levels of the bioactive compounds, antioxidant and 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.

BIOACTIVE COMPOUNDS

Phenolic Compounds

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

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

~~The steeping temperature (60, 70, 80 and 95 °C) and storage time (fresh and stored) determined total phenolic content, with values ranging from 4.39 ± 0.49 to 71.38 ± 4.14 mg GAE/g samples.~~ The total phenolic content of ~~plucheaPluchea~~ ~~tea~~the ~~infused~~infusion at different steeping temperature and storage period ~~ed at different time that statistical analyzed by ANOVA at α ≤ 5 % shown at Figure 1~~ generally, significantly increased with increasing steeping temperatures and storage periods. ~~.~~Steeped and stored ~~-~~infusion had significantly higher amounts of phenolic compounds that the samples were steeped and unstored. Further, ~~The total phenolic content of samples was significantly influenced by the steeping temperature and storage time.~~ The highest total phenolic content was ~~detected~~ observed in the~~the L 95~~ sample infused at 95 °C and stored for 5 years ~~(~~at 71.38 ± 4.14 mg GAE/g samples~~)~~ while the lowest was measured in in the unstored samples and infused at 60C, Phenolic content of the samples that were infused at different temperatures then stored were significantly higher than the steeped unstored samples while samples that were steeped only at 60 and 95C also showed a significant increase in their phenolic. This implies that the steeping temperature and the storage periods significantly resulted in the high amounts of the phenolic compounds of the infusions. Results also indicated that phenolic compounds were 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

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

This occurrence showed that steeping temperature and storage time caused the process of degradation and oxidation of phenolic 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 ^{o}C and different storage times (fresh and 72 hours). Hydrogen bonding is affected by temperature treatment because the hydrogen bond between phenolic compounds and proteins can be degraded that the measured levels of phenolic compounds are higher. The phenomena were supported by Ali *et al.* (2018); Jayani *et al.* (2022) and 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

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Sentences have to be reconstructured for clarity

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

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(2012) claimed that antioxidant compounds can be slowly degraded with increasing temperature. Besides that, 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 the phenolic compounds in ~~plucheaPluchea~~ tea are degraded due to oxidation and hydrolysis because of temperature and storage time and can be easily extracted during brewing, thus increasing the phenolic content as the steeping temperature and storage time increase.

Based on using of a reference standard could be informed that phenolic compounds in steeping ~~plucheaPluchea~~ tea, including 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 was showed in Table 1. Gallic acids and (+)-catechins were relative stable phenolic acid because of very small changes at different steeping temperature and storage time with concentration about 0.21 ± 0.00 – 0.24 ± 0.02 µg/g samples and 0.32 ± 0.02 – 0.60 ± 0.04 µg/g samples, respectively. However, myricetin, quercetin and 3,4-di-*O*-caffeoylquinic acid showed drastic increasing at higher steeping temperature and longer storage time. It's meant that these compounds tended relatively labile. Kaempferol, 3,5-di-*O*-cafffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid underwent moderate changes compared to the other two groups of phenolic acids. Therefore myricetin, quercetin and 3,4-di-*O*-caffeoylquinic acid were easier to dissolve at higher steeping temperature and storage time can cause macromolecules of three phenolic acids in herbal tea convenient degradable to form simple phenolic compounds for storage, as explained by Su *et al.* (2019), Ali *et al.* (2018); Jayani *et al.* (2022); Ramphinwa *et al.* (2023), and Zhang *et al.* (2021). Degradable

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Commented [A55]: Indicate Stat treatment means significance

Commented [A56]: Same as gallic and catechin

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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.

Flavonoid Content

Flavonoids are the major phenolic compounds ~~having~~ that have potential ~~as~~ chemical and biological activities, ~~especially~~ such as radical scavenging and antimicrobial activities (Ayele *et al.* 2022; Chandra *et al*. 2014) . ~~These compounds are the bioactive compounds~~ that can protect the human body from the oxidative stress caused many degenerative diseases, especially cancer, cardiovascular problems and ageing (Mathur and Vijayvergia 2017). ~~Total flavonoid content analysis for pluchea tea at various steeping temperatures and storage times were showed in Figure 1~~. The total flavonoid~~s~~ content of steeped~~ing~~ ~~pluchea~~*Pluchea* ~~tea~~ infusion decreased with longer ~~increasing~~ storage period~~time~~. Unstored samples exhibited higher flavonoid content than the stored samples.~~, but increased with increasing brewing temperature.~~ The highest total flavonoid content was ~~owned~~ exhibited by ~~fresh pluchea tea which was brewe~~stored samples steeped~~d~~ at 95 ^{o}C ~~(~~at 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 unstored and steeped samples at 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. ~~and the lowest was~~ This implies that ~~owned by pluchea tea which had been stored for 5 years at various brewing temperatures (~~24.75 ± 2.47 to 33.71 ± 3.06 mg CE/g samples~~between 24.75 ± 2.47 33.71 ± 3.06 mg CE/g samples). Statistical~~

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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.

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~~analysis by ANOVA analysis at α ≤ 5 % proven that brewing temperature and storage time of fresh pluchea tea had a significant effect on the total flavonoid content, but the stored pluchea tea (L) had no significant effect. Storage time had a significant effect on the total flavonoid content of brewing pluchea tea. Ali *et al.* (2018) reported that the degradation of bioactive compounds can take place through several stages, such as pre-treatment, processing, and storage, as is the case with medicinal plants which are dried, extracted and stored in the long term. Brewing temperature and storage time have an influence on the oxidation and polymerization processes that are stimulated by light. According to Noree *et al.* (2017), that the total flavonoid content test with $AlCl_3$ and $NaNO_2$ reagents measures flavone compounds, these compounds have activity due to the presence of a free hydroxyl functional group at position 4' in the compound. Degradation of flavone compounds due to temperature and storage causes the breaking of methylation bonds. Kim *et al.* (2020) also confirmed, that the total phenolic content and total flavonoid content of matcha are decreased with increasing brewing temperature and storage time. Xu *et al.* (2019) informed, that storage time can give a big impact on chemical composition changes with trending not the same.~~

Tannin content

~~The tannins have a various type of compounds are water-soluble polyphenols that are current in many plant foods and have a number of effects on health (Balaky *et al.* 2021).~~ Tannins are bioactive compounds that provide properties, such as astringent, anti-diarrheal, antibacterial and antioxidant (Malangngi *et al*. 2012). ~~Data analysis~~Generally, results indicated ~~showed,~~ that the total tannin content of ~~brewing pluchea~~*Pluchea* ~~tea~~ infusion signficantly increased with increasing steeping~~brewing~~ temperature and storage

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.

Commented [A67]: Needs revision, sentences reconstruction

Commented [A68]: Do these support your observations

Commented [A69]: Reconstruct to support the findings

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periodtime (, as seen in Figure 1). . Steeping pluchea tea contained tannins ranging from 4.81 ± 0.58 - 17.42 ± 1.04 (mg TAE/g samples). The tannin content increased with increasing storage time and brewing temperature. The results of the ANOVA statistical analysis at α ≤ 5 %, showed a significant increase in tannin content levels with increasing brewing temperature and storage time. Among, the unstored steeped samples, theThe fresh pluchea tea brewed tannin content was significantly lowest in samples infused at 60 ^{o}C had the lowest tannin content level, wasat 4.81 ± 0.58 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 The stored pluchea tea brewed at 95 ^{o}C had the highest tannin content level, was 17.42 ± 1.04 mg TAE/g samples and is significantly different from that of the highest tannin content of the unstored steeped samples at _____. Indicating that the tannin content was affected by both high steeping temperature and long storage period and that the presence of high tannin content was primarily brought about by long storage period. The results showed, that the higher the brewing temperature and the longer the storage time caused the tannin compound polymerization process to occur. 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. Rusita *et al.* (2019) emphasized that tannins are a polar compound, that is resistant to heating, as a result the tannin content in pluchea*Pluchea* tea increases with increasing brewing temperature and storage time, this is caused tannins are thermostable complex compounds.

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ANTIOXIDANT ACTIVITY

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

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DPPH Free Radical Scavenging Activity

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

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~~s~~teeping at higher temperatures ~~could~~ significantly increase~~d~~ the DPPH free radical scavenging activity in stored ~~plucheaPluchea tea~~ infusion around 15 to ~~–~~25 %. ~~Brewing~~ Steeping at 80 - 95 ºC in stored ~~plucheaPluchea~~ infusion~~tea~~ insignificantly affected the free radical scavenging property of the bioactive compounds ~~this antioxidant activity.~~ (Figure 2a).

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

Ferric Reducing Antioxidant Power (FRAP)

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

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

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color complex, that can be measured at λ 700 nm (Fu *et al*. 2011; Al-Temimi and Choudhary 2013). The principle of testing the ability to reduce iron ions is that antioxidants can reduce potassium ferrocyanide (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).

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

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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

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ANTIDIABETIC ACTIVITY

Enzyme Inhibition Activity

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

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

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

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

The results showed, that the ability to inhibit the α-glucosidase enzyme decreased with increasing brewing temperature and storage time. Brewing at 95ºC for fresh ~~plucheaPluchea~~ tea (0 days of storage) obtained the lowest inhibitory ability, i.e., 48.32 ± 1.27%, and the highest inhibitory activity was found at 70 ºC brewing temperature for fresh ~~plucheaPluchea~~ tea, which was 95.11 ± 0.70% (Figure 3). The test results showed that the ability to inhibit the enzyme α-glucosidase tended to be higher than the ability to inhibit the enzyme α-amylase. Li *et al*. (2018) informed that flavonoid compounds have the ability to inhibit the action of the α-amylase and α-glucosidase enzymes. This is due to the total flavonoids in brewing ~~plucheaPluchea~~ tea which tended to have the same pattern as the ability to inhibit the activity of the α-amylase and α-glucosidase enzymes. Flavonoid compounds, such as rutin, myricetin, kaempferol, and quercetin which 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 and the number of double bonds in rings A and B and the heterocyclic ring in ring C. The ability to inhibit the α-glucosidase enzyme from ~~plucheaPluchea~~ tea was significantly

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affected by the brewing temperature and storage time. The capability of ~~plucheaPluchea~~ tea infusion to obstruct the α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of two enzymes was different, according to the opinion of McCue *et al.* (2005). Widyawati *et al.* (2017) informed that phenolic and non-phenolic compounds determine the inhibitory activity of the α-glucosidase enzyme. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, that may be occurred in ~~plucheaPluchea~~ tea during storage, affects the structure and profile of phenolic and non-phenolic compounds. Asriningtyas *et al.* (2014) claimed that ~~plucheaPluchea~~ leaves contain 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. Quinic acid is methyl esterified with the number of caffeic groups in the molecule that determines the activity of inhibiting the α-glucosidase enzyme. Analysis of caffeoylquinic acids in ~~plucheaPluchea~~ tea infusion was obtained that the higher steeping temperature and longer storage time caused increased concentration of them, but the α-glucosidase inhibition of them was reduced. Aleixandre *et al*. (2022) reported that the simple phenolic acids forming a dipole-dipole interaction of active site from α-glucosidase enzyme are effectively inhibiting the enzyme.

This study was obtained information that the increasing of steeping temperature and storage time caused a degradation reaction 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, supported the results of total phenolic content and total tannin content

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

CONCLUSION

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

DATA AVAILABILITY

Table and figures used to support ~~of thisthe~~ study were included in the article.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Explain the decreasing trend in the enzyme iinhition 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

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ACKNOWLEDGEMENT~~S~~

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

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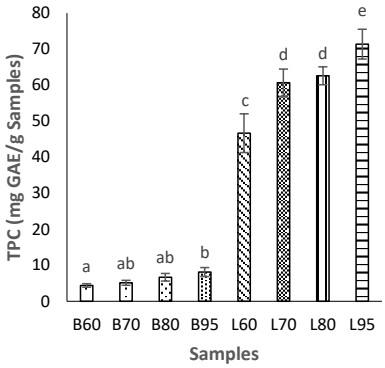

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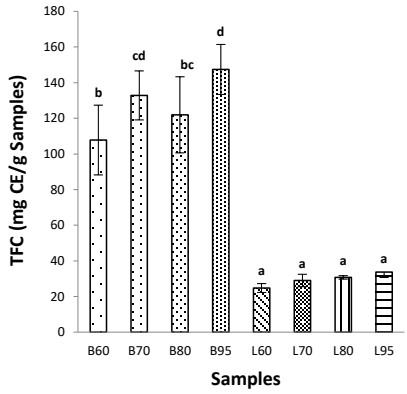

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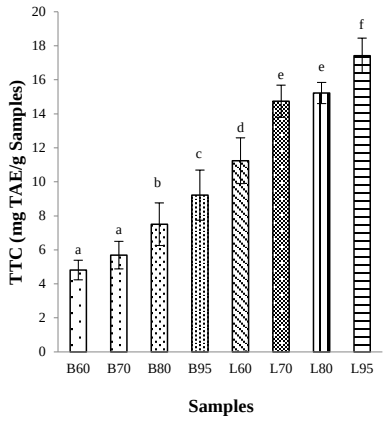

(c)

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

~~(Values were~~ ~~Mm~~eans are expressed as ± 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

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~~Different supercripts in graph showed a significant difference based on the DMRT test (α ≤ 5 %)~~

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Table 1. Simple Pphenolic Ccompound compound Profile profile of Plucheа*Pluchea* Tea Infusion at dDifferent sSteeping tTemperature and sStorage tTime

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*-Caffeoylquinic acid (µg/g samples)	3,5-di-*O*-Caffeoylquinic acid (µg/g samples)	4,5-di-*O*-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

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

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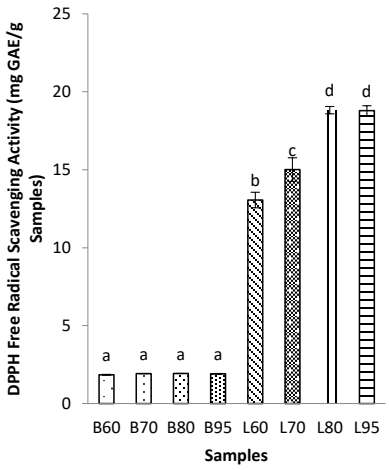

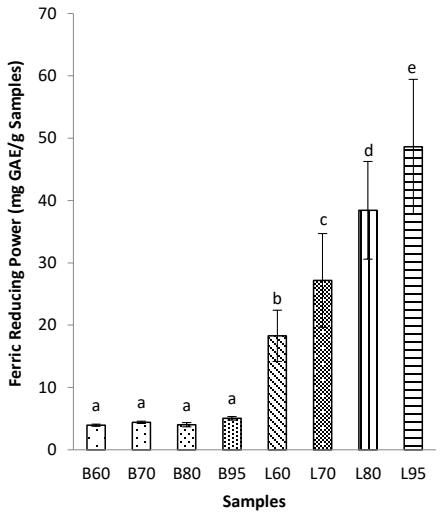

(a) (b)

Figure 2. Antioxidant activity of ~~plucheaPluchea~~ ~~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 ± 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
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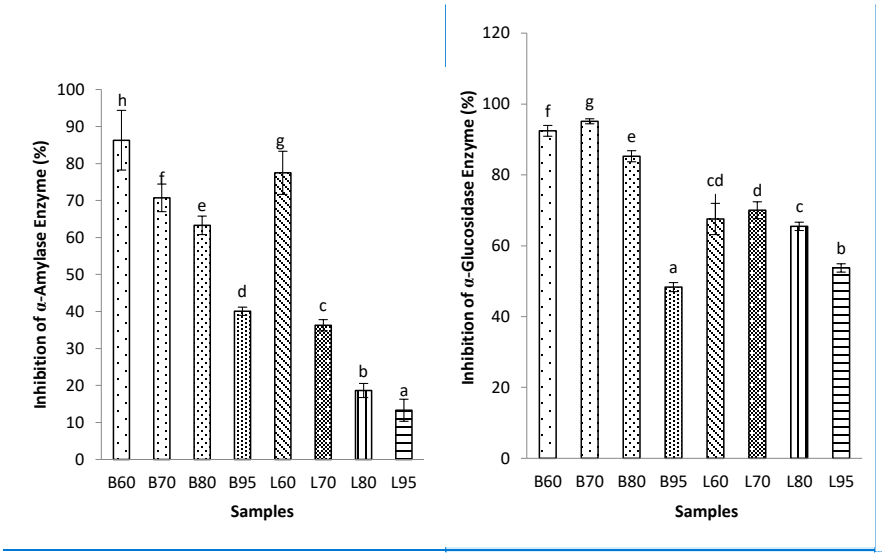

(a) (b)

Figure 3. ~~Antidiabetic activity~~Percent (%) inhibition of ~~pluchea~~ Pluchea ~~tea~~ infusion at different steeping temperature and storage time (a) α-amylase (b) α-glucosidase

Treatment means are expressed as ± 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 ± standard deviations (n=6). Different supercripts in graph showed a significant difference based on the DMRT test (α ≤ 5 %)~~

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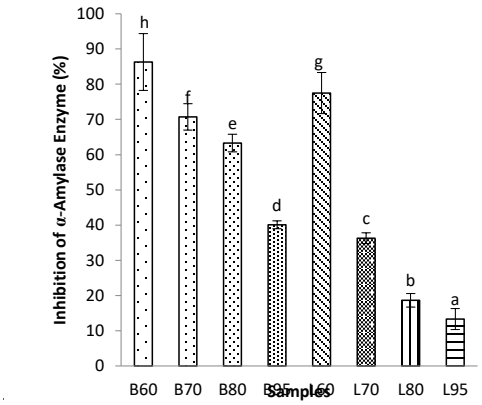

(a) (b)

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

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

Fwd: Comments on PJS Paper Ms 23-158

Paini Sri Widyawati <paini@ukwms.ac.id> Fri, Oct 13, 2023 at 10:45 PM
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Dear Ms. CARYL MARIA MINETTE I. ULAY
Editorial Assistant

Greeting,
Thanks for information
Regards

Paini Sri Widyawati
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Gmail

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

Fwd: Comments on PJS Paper Ms 23-158

Paini Sri Widyawati <paini@ukwms.ac.id> Tue, Oct 24, 2023 at 10:49 AM
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

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

Fwd: Comments on PJS Paper Ms 23-158

Philippine Journal of Science <pjs@stii.dost.gov.ph> Tue, Oct 24, 2023 at 12:49 PM
To: Paini Sri Widyawati <paini@ukwms.ac.id>

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

Fwd: Comments on PJS Paper Ms 23-158

Paini Sri Widyawati <paini@ukwms.ac.id> Tue, Oct 24, 2023 at 2:04 PM
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

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
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Gmail

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

Fwd: Comments on PJS Paper Ms 23-158

Philippine Journal of Science <pjs@stii.dost.gov.ph> Tue, Oct 24, 2023 at 3:07 PM
To: Paini Sri Widyawati <paini@ukwms.ac.id>

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

Fwd: Comments on PJS Paper Ms 23-158

Paini Sri Widyawati <paini@ukwms.ac.id> Tue, Oct 24, 2023 at 3:26 PM
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Dear Ms Ceryl Maria

Greetings,

Thanks for your attention

Regards

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

Fwd: Comments on PJS Paper Ms 23-158

Paini Sri Widyawati <paini@ukwms.ac.id> Wed, Nov 22, 2023 at 6:37 PM
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

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
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Effect of Brewing Temperature and Storage Time on Antioxidant and Antidiabetic Properties of Pluchea Tea Final.docx
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Effect of Steeping Temperature and Storage Time on the Bioactive Compounds, Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea Indica* Less

Paini Sri Widyawati[1*)], Yufita Ratnasari Wilianto[2)]

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[2)]Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic University, Kalisari Street Number 1, Surabaya 60272, Indonesia

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

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ABSTRACT

This study was done to determine the effects of steeping temperature and storage time on the bioactive contents, 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 time (B). The variety of the steeping temperatures included 60 (T1), 70 (T2), 80 (T3), and 95 (T4) (^oC) with the storage time of 0 (B1) and 5 (B2) (year). The research resulted 8 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 influenced the bioactive contents (total phenol (TPC), total tannin (TTC), total flavonoid (TFC)), antioxidant (DPPH scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)) and antidiabetic (α-amylase (AA) and α-glucosidase (GA) inhibitors) activities of samples. The bioactive contents influenced antioxidant and antidiabetic activities. TFC was decreased for storage time and significant increased at higher steeping temperature. The AA and GA of *Pluchea* infusion increased until 70 ^oC of the steeping temperature, but deceased until 95 ^oC. The AA and GA were strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively correlated with TFC. Between the antioxidant activity of DPPH and FRAP and the antidiabetic activity of AA and GA of Pluchea infusion were strongly and positively correlated with correlation coefficient (r) values of 0.956 and 0.725, respectively. The treatments gave different effect of simple phenolic compounds, such as 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 of *Pluchea* infusion at different steeping temperature and long storage. To obtain high antioxidant activity, *Pluchea*

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

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 an herbal plant that has been widely used for traditional medicine and food (Chan et al., 2022). *Pluchea* leaves are composed 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, β-carotene, and vitamin C, whereas bioactive compounds is 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, β-carotene, and total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et al., 2022, Chan et al., 2022).

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

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content, the ability to scavenge DPPH free radicals, and the capability of reduce ferric ions 9.3 mg gallic acid equivalent (GAE)/g samples, 22.0 mg gallic acid equivalent (GAE)/g samples, 27.2 mg gallic acid equivalent (GAE)/g samples, and 10.2 mg gallic acid equivalent (GAE)/g samples, respectively. Werdani and Widyawati (2018) reported that drinking of *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 ^{o}C for 5 minutes certainly determines the stability and amount of extracted bioactive compounds, that influences the biological activity, especially antioxidant and antidiabetic activities. Silva-Ramirez et al. (2020) reported that the infusion process can influence their content and composition of the bioactive compounds and antioxidant activity of tea. Acar et al. (2022) informed that infusion quality of herbal tea extract depends on several factors, i.e., time and temperature. Polyphenol profile and antioxidant properties of herbal tea infusion decline with an increase in steeping/brewing and storage temperatures and longer exposure times.

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

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

On the other hand, storage time of *Pluchea* herbal tea also affects the levels of the bioactive compounds and biological activity because this herbal tea usually is stored for a several months until years (Jayani et al., 2022). Tea or herbal tea is generally stored in ambient temperature and packed in tea bag or Alu foil standing proud or a combination of both. Many researchers informed that storage time decreases the bioactive compounds, antioxidant and antidiabetic activities, i.e., juice from *Momordica charantia* L. (Lin et al., 2020), dried *Piper betlle* extracts (Ali et al., 2018), white tea (Xu et al., 2019), kinnow-amla beverages (Purewal et al., 2022), whole wheat flour (Zhang et al., 2021). Therefore, this research studied the effect of steeping temperature and storage time on the bioactive compounds, antioxidant and antidiabetic activities of infusion from powdered *Pluchea* leaves. 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 antioxidant power (FRAP), α-amylase (AA) and α-glycosidase (GA) inhibition activities, and 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 *Asteraceae* family with specification according to the GBIF taxon ID number database:3132728 (Ferraris, 2023). *Pluchea* leaves at 1-6 level of each branch of the shoot were collected, sorted, washed

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

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

REAGENTS

The compounds used to analyze including 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid, α-amylase, α-glucosidase, pNPG (p-nitrophenyl-α-glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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 distillated water which was purchased from PT Aqua Industry Surabaya.

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METHODOLOGY

ANALYSIS OF THE BIOACTIVE COMPOUNDS

TOTAL PHENOLIC CONTENT ANALYSIS

Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the technique by Gao et al. (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. And then 2 mL Na_2CO_3 7.5 % was entered and distillated water was added until 10 mL volume. The color intensity of solution was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at λ 760 nm with gallic acid as the reference standard. The total phenolic content was calculated using the formula: $y=0.00009x+0.008$ with $R^2=0.9941$. The results were expressed as mg gallic acid equivalent (GAE)/g samples.

TOTAL FLAVONOID CONTENT ASSAY

Total flavonoid content (TFC) of the samples was measured based on the reaction between $AlCl_3$ and $NaNO_2$ with an aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim et al., 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted a yellow solution. About 30 µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5 % in 10 mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 10 % for 5 min. And then, 2 mL NaOH 1 M and distillated water were added until 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 λ 510 nm with (+)-catechin as the reference standard compound,

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

TOTAL TANNIN CONTENT ANALYSIS

Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method (Chandran and Indira, 2016). Approximately 10 µL *Pluchea* infusion was added 1 mL Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flash and incubated for 5 min. Then, the mixture was added 2 mL Na_2CO_3 7.5 % and distillated water was added until 10 mL volume. The blue dark color solution that measured UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at λ 760 nm with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/g samples used the 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 antioxidant activity of the *Pluchea* leaf infusion to donor hydrogen atom to nitrogen atom in DPPH resulting DPPH-H compound with a yellow-colored solution. About 25 µL *Pluchea* leaf infusion was entered in reaction tube and added 3 mL DPPH solution (4 mg/100 mL). And then the solution was incubated for 15 min in a dark room and absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ. 517 nm. The reference standard compound was gallic acid and the results of analysis were

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

FERRIC REDUCING POWER ANALYSIS

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

α-AMYLASE ENZYME INHIBITION CAPACITY ASSAY

In vitro inhibition of α-amylase enzyme (AA) followed the procedure as described by Widyawati et al. (2020). Each 500 µL of samples, starch 1 % (w/v) and sodium acetate buffer pH 5 were mixed. Then, each 250 µL of mixture and α-amylase solution (0.1 g of this enzyme 12.5 unit/mL was dissolved in 50 mL of 0.2 M sodium acetate pH 5) was shaken and added 2 mL sodium hydroxide 1M. Before analysis, this mixture was incubated at 37 ^{o}C for 10 min. Then, the capacity of the α-amylase enzyme hydrolyzed

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the starch to release glucose that could be analyzed based on absorbance at λ 540 nm. The inhibition percentage of α-amylase was assessed using the formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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 test sample with enzyme, Ab is absorbance of test sample without enzyme.

α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

The analysis of the α-glycosidase inhibitor activity (GA) was done by Widyawati et al. (2020) method with slight modification. About 150 µL samples contained 100 µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH 7) were reacted with 50 µL α-glycosidase 2 mM (0.0833 unit/mL), and then the mixture was incubated at 37 ^{o}C for 15 min. Finally, the reaction was stopped with addition of 1000 µL sodium carbonate 0.2 M. The residue of this enzyme hydrolyzed p-nitrophenyl-α-D-glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The inhibitor activity of steeping *Pluchea* tea to enzyme was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at λ 405 nm. The inhibition percentage of α-glycosidase was calculated using formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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, Ab is the absorbance of test sample without enzyme.

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HPLC ANALYSIS OF PHENOLICS

The phenolic compounds of the samples were analyzed by HPLC based on Kongkiatpaiboona et al. (2018) method with modifications. Each *Pluchea* infusion was sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter syringe (Whatmann, 0.2 µm, NYL). About 20 µL of 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 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (2 pieces) (ID 10 mm x 4.6 mm). Analytical conditions: 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 minutes; then 100 % B was maintained for 20 minutes. Next the column was re-equilibrated with 10 % B in A maintained for 10 minutes before analysis of the next sample. The sample flow rate was set at 1.0 ml/min with a controlled temperature at 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 distillated water and prepared similar to the samples before injected in HPLC.

EXPERIMENT DESIGN AND STATISTICAL ANALYSIS

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

RESULTS AND DISCUSSIONS

Pluchea leaf infusion is produced by young *Pluchea* leaf from 1-6 level on each branch the shoot (Widyawati et al., 2022), that is steeped at 95 ^{o}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 *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols, cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2 g/100 mL steeping *Pluchea* 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 related to the composition of phytochemical compounds in *Pluchea* leaves, such as phenolic acids such as chlorogenic acids, caffeic

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acids, 3-*O*-caffeoylquinic acids, 4-*O*-caffeoylquinic acids, 5-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin; β-carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Chan et al., 2022; Widyawati et al., 2022). Presence of phytochemical compounds in herbal product were 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 of 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). Therefore, this study was conducted to determine the effect of steeping temperature and storage time of *Pluchea* tea on levels of the bioactive compounds, antioxidant and antidiabetic properties and phenolic compound profile.

BIOACTIVE COMPOUNDS

Phenolics Compounds

The bioactive compounds are active compounds in plants that are essential to protect a 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

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

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 based on paired T test at $\alpha \leq 0.05$. Steeped and stored infusion had significantly higher amounts of phenolic compounds that the samples were steeped and un-stored. Further, the highest total phenolic content was observed in samples infused at 95 ^{o}C and stored for 5 years (at 71.38±4.14 mg GAE/g samples) while the lowest was measured in the un-stored samples and infused at 60 ^{o}C. Phenolic content of samples that were infused at different temperatures then stored were steeped only at 60 and 95 ^{o}C also showed a significant increase in their phenolic. This implies that the steeping temperature and the storage periods significantly resulted in the high amounts of the phenolic compounds of the infusions. Results also indicated that phenolic compounds were generally greater in the infusion at high steeping temperatures and long storage (Figure 1a). This could have been due to that 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 sleeping temperature also caused the greater the extracted total phenolic content. The temperature of infusion influenced total phenolic content, it could relate to migration process of phenolic compounds to the water because of increasing contact between this compounds and water. The same phenomena also occurred in Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al. (2022).

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

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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 ^{o}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 ^{o}C. The (+)-catechin

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concentration of *Pluchea* infusion was significantly different at 95 ^{o}C, but the myricetin was different concentration at 80 and 95 ^{o}C. The 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid compounds from *Pluchea* infusion were significantly different at 60 ^{o}C, however the concentration of 3,4-dicaffeoylquinic acid was also significantly different at 80 and 95 ^{o}C. Based on the analysis of concentration of simple phenolic compounds showed that gallic acids and kaempferol were relative stable phenolic acid because of no changes at different steeping temperature and storage 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, (+)-catechin and 3,4-di-*O*-caffeoylquinic acid showed drastic increasing at higher steeping temperature and longer storage time. It's meant that these compounds tended relatively labile. Quercetin, 3,5-di-*O*-cafffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid underwent moderate changes compared to the other two groups of phenolic acids. Therefore, myricetin, (+)-catechin and 3,4-di-*O*-caffeoylquinic acid were easier to dissolve at higher steeping temperature and storage time can cause macromolecules of three phenolic acids in herbal tea convenient degradable to form simple phenolic compounds for storage, as explained by 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.

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.,

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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 α= 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 ^{o}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 ^{o}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 ^{o}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 ^{o}C about 9.22 ± 1.48 mg TAE/g

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samples. Indicating that the tannin content was affected by both high steeping temperature and long storage period and that the presence of high tannin content was primarily brought about by long storage period. Kowalska et al. (2021) informed that the condensation of catechins to tannins of polyphenolic compounds is a dominant process occurred in tea leaves that is accelerated during maceration of raw material. However, the high temperature can degrade polyphenolic compounds to form simple phenolic 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 concentration 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, Rusita et al. (2019) emphasized that tannins are a polar compound, that is resistant to heating, as a result the tannin content in *Pluchea* tea increases with increasing steeping temperature and storage time, this is caused tannins are thermostable complex compounds.

ANTIOXIDANT ACTIVITY

Antioxidant activity is capability of compounds to inhibit the oxidation of macromolecules from biological target that involve 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 antioxidant that have antioxidant

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

DPPH Free Radical Scavenging Activity

DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate antioxidant activity because this method is simple that is suitable to measure the donating hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of DPPH to change to yellow color (Munteanu and Apetrei, 2021; Baliyan et al., 2022). The result of DPPH assay indicates that the DPPH values accrued at higher steeping temperature and longer storage time. Statistical analysis by ANOVA using a paired T test at $\alpha \leq 0.05$ 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. *Pluchea* infusion stored at room temperature for 5 years resulted in the DPPH free radical scavenging activity by more than 100 %. Steeping at higher temperatures significantly increased the DPPH free radical scavenging activity in stored *Pluchea* infusion around 15 to 25 %. Steeping at 80-95 ^{o}C in stored *Pluchea* infusion insignificantly affected the free radical scavenging property of the bioactive compounds (Figure 2a).

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Scavenging activity of DPPH free radicals was strongly and positively correlated with total phenolic and tannin levels, but inversely to total flavonoid levels. Based on Pearson correlation at Table 2, the correlated coefficient values (r) between DPPH and TPC, TTC and TFC were 0.993, 0.942, and -0.940, respectively. During the storage process it is possible to form complex phenolic compounds which provide a high ability to scavenge DPPH free radicals (Thanajiruschaya et al., 2010). This research also demonstrated that longer storage time and higher infusion temperature produced many simple phenolic compounds with free hydroxyl groups capable to donor hydrogen atom to DPPH free radical. Many phenolic acids, such as gallic acids, (+)-catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 4,5-di-*O*-caffeoylquinic acids have established potential antioxidant activity (Kumar and Goel, 2019). Kruk et al (2022) informed that the capability of phenolic compounds to donor hydrogen atom depends on 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 donor hydrogen atom is determined by O-H bond dissociation energy.

The DPPH 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 total phenolic content of herbal infusion is low correlated with DPPH free radical scavenging activity. However, Dobrinas et al. (2021) informed that total phenolic content of tea infusion is positively and significantly correlated with Inhibitor activity of DPPH.

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Ferric Reducing Antioxidant Power (FRAP)

FRAP is an analysis of antioxidant power of the phytochemical compounds based on the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a color complex, that can be measured at λ 700 nm (Fu et al., 2011; Al-Temimi and Choudhary, 2013). The principle of the assay measures of the ability of antioxidant compounds to reduce iron ions of potassium ferrocyanide (Fe^{3+}) to be 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 ferric reducing antioxidant power (FRAP) increased with higher steeping temperature and long storage time. The lowest FRAP was observed in the un-stored samples which was steeped at 60 ^{o}C at 3.95 ± 0.17 mg gallic acid equivalents (GAE)/g samples, and the highest was owned by *Pluchea* infusion which was stored for 5 years at 95 ^{o}C at 48.63 ±10.83 mg gallic acid equivalents (GAE)/g samples (Figure 2b). FRAP increased significantly as steeping temperature was increased. FRAP of the samples stored for 5 years was also significantly higher than the stored samples at $\alpha \leq 0.05$. 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) between FRAP and DPPH, TPC, TTC and TFC were 0.956, 0.953, 0.948 and -0.826, respectively.

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

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epigallocatechin (EGC), and epicatechin (EC) are bioactive compounds that have high antioxidant activity (Kim et al. 2020), and also the case of the effect of temperature and storage time in betel (*Piper bettle* 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). Thanajiruschaya et al. (2010) revealed that the antioxidant activity of rice stored at high temperatures is greater than that stored at low temperatures. The ferric reducing capability of *Pluchea* infusion corresponded to simple phenolic acid values, presence of them in samples could accrue antioxidant activity because of ability of the electron transfer from free hydroxyl groups of phenolic acids.

ANTIDIABETIC ACTIVITY

α-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 2-degestive enzymes i.e., α-amylase and α-glucosidase, which are involved 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 α-amylase and α-glucosidase enzymes (Martinez-Solis et al., 2022) resulting in the reduced activity of the enzymes. The results showed, that the steeping *Pluchea* infusion was able to inhibit the action of the α-amylase enzymes (Figure 3a). The *Pluchea* infusion had very good activity, more than 50 % and even almost 100 % for fresh

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

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

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

α-Glucosidase enzyme inhibition activity (GA)

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

The results showed, that the ability to inhibit the α-glucosidase enzyme decreased with increasing steeping temperature and storage time. Steeping at 95 ^{o}C for fresh *Pluchea* infusion (un-stored) obtained the lowest inhibitory ability, i.e., 48.32 ± 1.27 %,

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548 and the highest inhibitory activity was found at 70 ^{o}C steeping temperature for fresh
549 *Pluchea* infusion, which was 95.11 ± 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 α-glucosidase enzyme tended to be higher than the ability to inhibit
553 the α-amylase enzyme. Li et al. (2018) informed that flavonoid compounds have the ability
554 to inhibit the action of the α-amylase and α-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 α-amylase and α-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 α-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 α-glucosidase enzyme was greater than the α-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 α-glucosidase enzyme. The ability of bound phenolic compounds to inhibit

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α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, that may be occurred in *Pluchea* infusion during storage, affects the structure and profile of phenolic and non-phenolic compounds. Asriningtyas et al. (2014) claimed that *Pluchea* leaves contain 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. Quinic acid is methyl esterified with the number of caffeic groups in the molecule that determines the activity of inhibiting the α-glucosidase enzyme. Analysis of caffeoylquinic acids in *Pluchea* infusion was obtained that the higher steeping temperature and long storage caused increased concentration of them, but the α-glucosidase inhibition activity of them was reduced. Aleixandre et al. (2022) reported that the simple phenolic acids forming a dipole-dipole interaction of active site from α-glucosidase enzyme are effectively inhibiting the enzyme.

This study was obtained information that the increasing of steeping temperature and storage time caused a degradation reaction 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, supported the results of total phenolic content and total tannin content assays. Increased concentration of simple phenolic compounds determined the ability of these compounds as antioxidant agents, but reduced their capability as antidiabetic agents.

CONCLUSION

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The steeping temperature and storage time of *Pluchea* infusion significantly influenced bioactive contents, antioxidant and antidiabetic activities. TPC, TTC, and TFC were significantly different at various steeping temperature and storage period based on statistical analysis using a paired T test at $\alpha \leq 0.05$. There was the difference of the phenolic compound profile in fresh and stored of *Pluchea* infusion and various steeping temperature. The simple phenolic compounds were detected in *Pluchea* infusion including gallic acid, (+)-catechin, quercetin, myricetin, kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoilquinic acid and 4,5-di-*O*-caffeoilquinic acid. The results of statistical analysis using a paired T test at $\alpha \leq 0.05$ showed that gallic acid and kaempferol of *Pluchea* infusion were insignificantly different at various steeping temperature and long storage. Nevertheless, the concentration of quercetin and 3,5-dicaffeoylquinic acid of *Pluchea* infusion was significantly different of two treatments except at 70 ^{o}C. The (+)-catechin concentration of *Pluchea* infusion was significantly different at 95 ^{o}C, but the myricetin was different concentration at 80 and 95 ^{o}C. The 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid compounds from *Pluchea* infusion were significantly different at 60 ^{o}C, however the concentration of 3,4-dicaffeoylquinic acid was also significantly different at 80 and 95 ^{o}C. TPC, TTC and TFC of *Pluchea* infusion were significantly different at various steeping temperature and storage period. TPC and TTC significantly increased with increasing steeping temperature and long storage, but TFC significantly increased at various steeping temperature and significantly decreased at long storage. The bioactive compounds of *Pluchea* infusion influenced antioxidant activities (DPPH and FRAP) and antidiabetic activity (AA and GA). The DPPH was strongly and positively correlated with TPC and TTC, but it was strongly and negatively

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

DATA AVAILABILITY

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

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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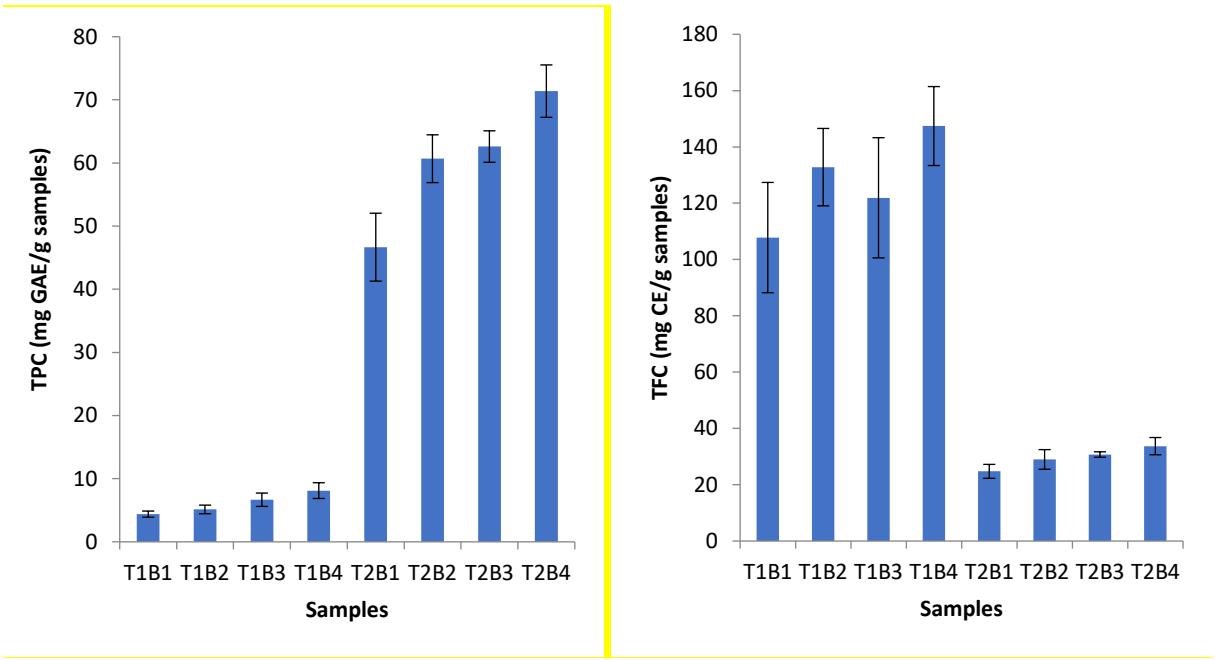
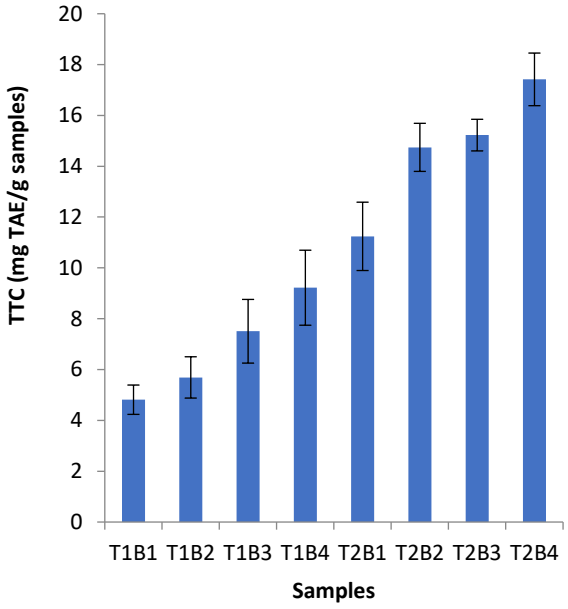


(a) (b)



(c)

Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping temperature and storage time (a) Total phenolic content (b) Total flavonoid content (c) Total tannin content. Data were expressed as mean ±standard

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deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}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 $\alpha \leq 0.05$.

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828 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

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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

Note : Data were expressed as mean ±standard deviation (n=2). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}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 $\alpha \leq 0.05$. * $\alpha \leq 0.05$.

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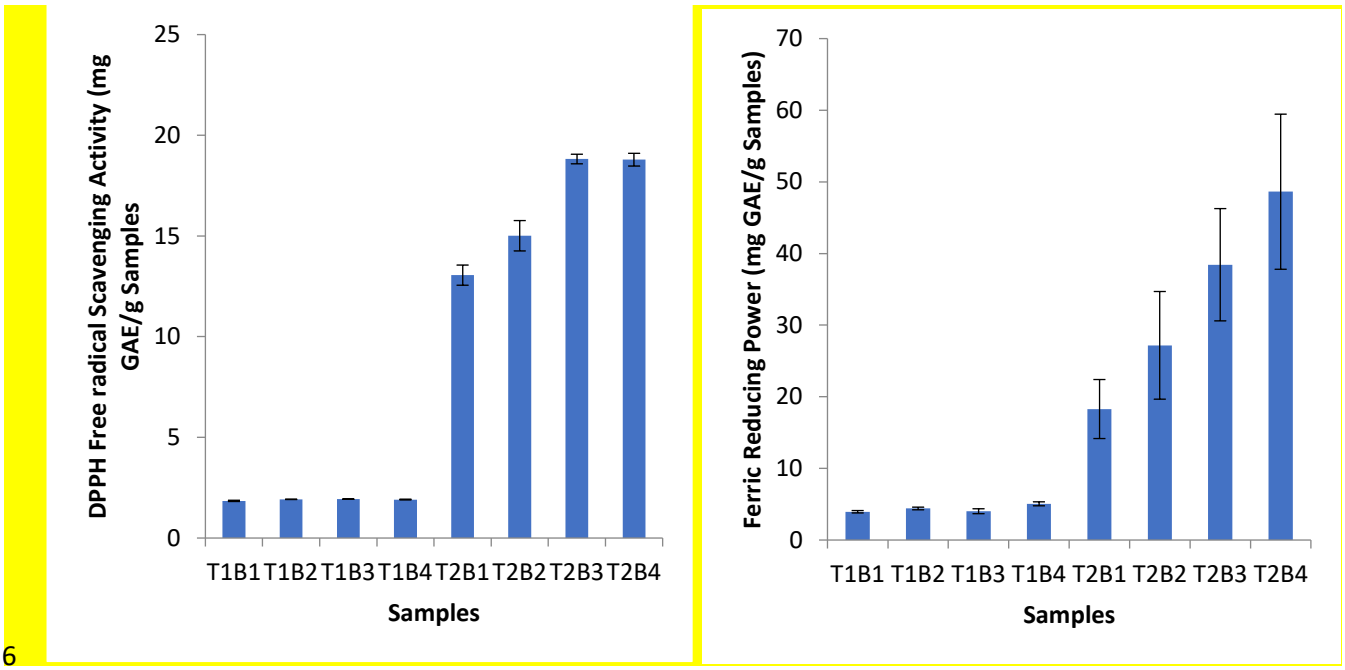

(a) (b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage time (a) DPPH (b) FRAP. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}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 $\alpha \leq 0.05$

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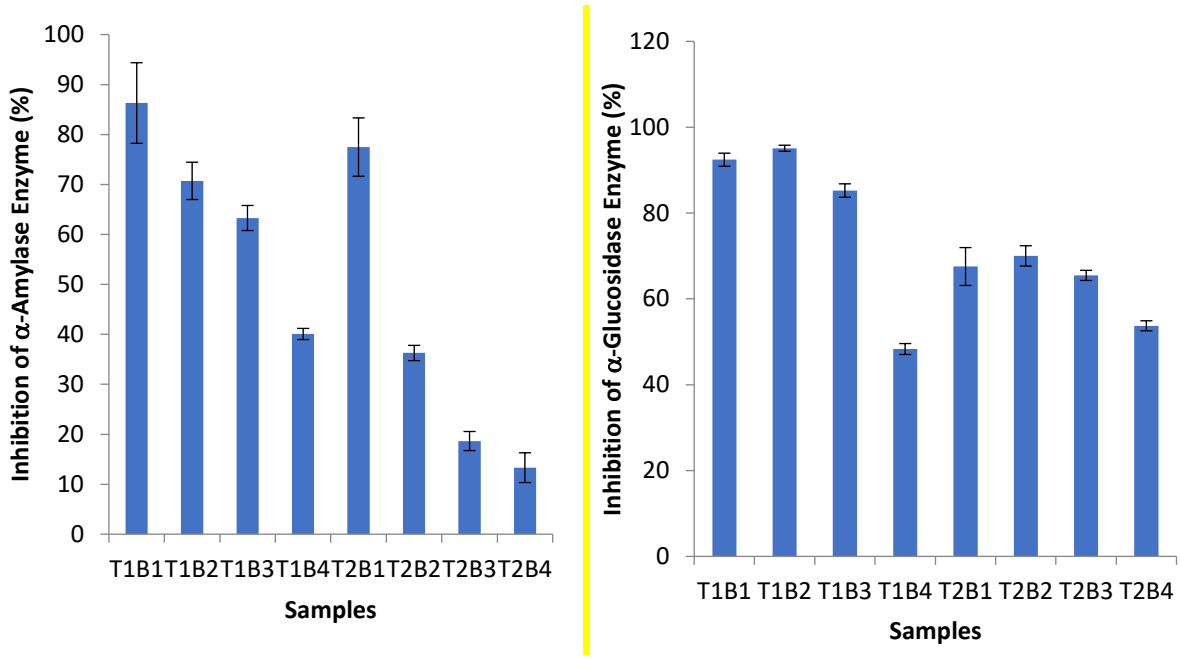

(a) (b)

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage time (a) α-amylase (b) α-glucosidase. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}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 $\alpha \leq 0.05$

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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

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

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Fwd: Comments on PJS Paper Ms 23-158

Philippine Journal of Science <pjs@stii.dost.gov.ph> Thu, Nov 23, 2023 at 7:56 AM
To: Paini Sri Widyawati <paini@ukwms.ac.id>

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

Gmail

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

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

Caesar Saloma <caesar.saloma@gmail.com> Mon, Jan 15, 2024 at 4:16 PM
To: paini@ukwms.ac.id
Cc: DOST STII PJS <pjs@stii.dost.gov.ph>, Philippine Journal of Science <philjournsci@gmail.com>

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

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; 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.

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

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[1)]Food Technology Study Program, Agricultural Technology Faculty, Widya Mandala Surabaya Catholic University, Dinoyo Street Number 42-44, Surabaya 60265, Indonesia

[2)]Pharmacy Study Program, Pharmacy Faculty, Widya Mandala Surabaya Catholic University, Kalisari Street Number 1, Surabaya 60272, Indonesia

Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea indica* Less, storage ~~time~~period

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ABSTRACT

This study was done to determine the effects of steeping temperature and storage ~~time~~period on the bioactive contents, 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 ~~time~~period (B). The ~~variety of the~~ *Pluchea* leaf blades were exposed to 4 steeping temperatures ~~included~~ of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) ~~(~~ºC~~)~~ with the storage ~~time~~period~~–~~period of 0 (B1) and 5 (B2) ~~(~~year~~)~~. ~~The research resulted~~resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). Statistical analysis using a paired t~~-T~~ test at $\alpha \leq 0.05$ showed that treatments significantly affected ~~influenced~~ the bioactive contents (total phenol (TPC), total tannin (TTC), total flavonoid (TFC)), antioxidant [(DPPH scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)]~~)~~ potential ~~–~~and antidiabetic [(α-amylase (AA) and α-glucosidase (GA) ~~inhibitors~~inhibition)] ~~activities~~ properties of the *Pluchea* leaf infusion~~samples~~. TFC decreased during storage period but significantly increased at higher steeping temperature. ~~The AA and GA of *Pluchea* infusion increased until 70 ºC of the steeping temperature, but deceased until 95 ºC. The bioactive contents influenced antioxidant and antidiabetic activities. TFC was decreased for storage time and significant increased at higher steeping temperature. The AA and GA of *Pluchea* infusion increased until 70 ºC of the steeping temperature, but deceased until 95 ºC.~~ The AA ~~–~~and GA were strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively correlated with TFC. ~~Between~~ The antioxidant activity ~~of DPPH and FRAP~~ and the antidiabetic activity ~~of AA and GA~~ of Pluchea infusion were 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.

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0.725, respectively. The treatments gave different effect of simple phenolic compounds derived from *Pluchea* leaf infusion at different steeping temperatures and storage included, such as 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. of *Pluchea* infusion at different steeping temperature and long storage. To obtain high antioxidant activity, *Pluchea* infusion selected was stored and steeped at high temperature, however high antidiabetic activity obtained was fresh *Pluchea* infusion and steeped at low temperature.

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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 an herbal plant that has been widely used for traditional medicine and food (Chan et al., 2022). *Pluchea* leaves are composed 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, β-carotene, and vitamin C, whereas bioactive compounds is 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, β-carotene, and total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et al., 2022, Chan et al., 2022).

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Steeping process of *Pluchea* leaves can be performed with fresh or dry leaves ~~infusion by~~in hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et al., 2020; Jayani et al., 2022). In Asia~~n area~~, especially in Indonesia~~n~~, people usually consume the *Pluchea* infusion ~~with brewing of~~by steeping 2 g of powdered *Pluchea* leaves in tea bag ~~by~~in 100 mL of hot ~~water~~ or boiling water. ~~Each tea bag contained 2 g of *Pluchea* leaf powder is steeped with 100 mL hot water or boiling water.~~ Widyawati et al. (2016) claimed that steeping of 2 g of *Pluchea* leaf powder at 95 ^{o}C for 5 minutes ~~results~~ rexhibits total phenolic ~~content,~~and total flavonoid content~~s~~, the ability to scavenge DPPH free radicals, and the capability ~~of~~ to reduce ferric ions at 9.3 mg gallic acid equivalent (GAE)/g sample~~s~~, 22.0 mg gallic acid equivalent (GAE)/g sample~~s~~, 27.2 mg gallic acid equivalent (GAE)/g sample~~s~~, and 10.2 mg gallic acid equivalent (GAE)/g sample~~s~~, respectively. Werdani and Widyawati (2018) reported that drinking of *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 ^{o}C for 5 minutes certainly determines the stability and amount of extracted bioactive compounds~~,~~ that influences the biological activity~~,~~ especially antioxidant and antidiabetic activities. Silva-Ramirez et al. (2020) reported that the infusion process can influence the~~ir~~ content and composition of the bioactive compounds and antioxidant activity of tea. Acar et al. (2022) informed that infusion quality of herbal tea extract depends on several factors, i.e., ~~time~~ storage and temperature. Polyphenol profile and antioxidant properties of herbal tea infusion decline with an increase in steeping/brewing and storage temperatures, and longer exposure ~~time~~period~~s~~.

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Several studies have mentioned the effect of steeping temperature ~~to~~ on the bioactive compound contents and antioxidant activity, such as some white and green teas are effective with hot water at 90 ºC for 7 min (Castiglioni et al., 2015), on roseship tea is effective~~ly~~ at infusion ~~time~~period around 6-8 min at temperatures of 84-86 ºC (Ilyasoglu and Arpa, 2017), on the caffeine content extracted ~~the coffee~~at the brewing temperature of coffee~~influences the caffeine content extracted~~ (Zarwinda and Sartika, 2018), and ~~the steeping~~ the high total phenol content and antioxidant activity of dark tea at 92 ºC for 27 min ~~results the highest total phenol content and antioxidant activity~~ (Wang et al., 2022). The study of the effect of steeping temperature to ***Pluchea*** infusion was carried out to afford information about the most efficient preparation of powdered ***Pluchea*** leaves ~~most efficiently~~ to get higher ~~the~~ bioactive compounds, antioxidant and antidiabetic activities.

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

Therefore, this research studied the effect of steeping temperature and storage ~~time~~period on the bioactive compounds [(total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging

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activity (DPPH), ferric reducing antioxidant power (FRAP)] and antidiabetic activities [(α-amylase (AA) and α-glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea* leaves.~~- 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 antioxidant power (FRAP), α-amylase (AA) and α-glycosidase (GA) inhibition activities,~~ 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 *Asteraceae* family with specification according to the GBIF taxon ID number database:3132728 (Ferraris, 2023). *Pluchea* leaves at 1-6 level of each branch ~~of~~from the shoot were collected, sorted, washed and dried to ~~get a~~moisture content of around 11.16 ± 0.09 % dry bas~~is~~ (Widyawati et al., 2022). The ~~powdering of~~ dried *Pluchea* leaves was ~~done~~ pulverized to ~~get~~ a 45-mesh size powder. ~~And then, the heating of T~~the *Pluchea* leaf powder was ~~done using a drying~~dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 °C for 10 min to reduce microbial organisms. ~~and~~ Then, 2 g of the powder were packed ~~using~~ into a paper filter infusion bag.~~ that made from paper filter around 2 g/bag~~. ~~And then all of samples called~~Packed samples were ~~*Pluchea* herbal tea was~~ stored for 0 (un-stored) and 5 (stored)years in standing pouch before analysis.

In the research, the one tea bag of *Pluchea* herbal tea that stored 0 (B1) and 5 (B2) year, was steeped with 100 mL hot water at various temperatures, including 60 (T1),

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70 (T2), 80 (T3), and 95 (T4) ºC for 5 min with infusion method ~~that obtained~~obtaining 8 treatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2. After the temperature of *Pluchea* infusion similar to ambient temperature was analyzed further.

REAGENTS

The ~~compounds~~reagents used ~~to analyze~~in the analyses ~~including~~ include 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid, α-amylase, α-glucosidase, pNPG (p-nitrophenyl-α-glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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 distillated water which was purchased from PT Aqua Industry Surabaya.

METHODOLOGY

ANALYSIS OF THE BIOACTIVE COMPOUNDS

TOTAL PHENOLIC CONTENT ANALYSIS

Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the technique by Gao et al. (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. And

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then 2 mL Na_2CO_3 7.5 % was ~~entered~~ added and filled up to 10 mL volume with distilled water.~~and distillated water was added until 10 mL volume~~. The color intensity of solution was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at λ 760 nm with gallic acid as the reference standard. The total phenolic content was calculated using the formula: y=0.00009x+0.008 with R^2=0.9941. The results were expressed as mg gallic acid equivalent (GAE)/g samples.

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TOTAL FLAVONOID CONTENT ASSAY

Total flavonoid content (TFC) of the samples was measured based on the reaction between $AlCl_3$ and $NaNO_2$ with ~~an~~ the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim et al., 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted in a yellow solution. About 30 µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5 % in 10 mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 10 % for 5 min. And then, 2 mL NaOH 1 M and distillated water were added until 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 λ 510 nm with (+)-catechin as the reference standard compound, and the results were expressed as mg catechin equivalents (CE)/g samples using the formula: y=0.00008x-0.0023 with R^2= 0.9980.

TOTAL TANNIN CONTENT ANALYSIS

Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method (Chandran and Indira, 2016). Approximately 10 µL *Pluchea* infusion was added with 1 mL

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Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flash and incubated for 5 min. Then, the mixture was added with 2 mL Na_2CO_3 7.5 % and filled up to 10 mL volume with distillated water. ~~was added until 10 mL volume~~. The blue dark color solution ~~that~~ was measured in UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at λ 760 nm with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/g samples used the formula: y=0.00009x+0.0021 with R^2=0.9993

ANALYSIS OF THE ANTIOXIDANT POTENTIAL

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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 ~~antioxidant activity of~~in the *Pluchea* leaf infusion to ~~donor~~ donate hydrogen atom to the nitrogen atom in DPPH resulting in the formation of –DPPH-H compound ~~with~~ exhibiting a yellow-colored solution. About 25 µL *Pluchea* leaf infusion was ~~entered~~ poured into reaction tube ~~and~~ into which was added added 3 mL DPPH solution (4 mg/100 mL). ~~And then the solution was~~After incubationed for 15 min in a dark room , the ~~and~~ absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ. 517 nm. The reference standard compound was gallic acid and the results of analysis were expressed as mg gallic acid equivalents (GAE)/g samples that calculated using formula: y=0.146x+1.7896 with R^2=0.9975.

FERRIC REDUCING POWER ANALYSIS

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

α-AMYLASE ENZYME INHIBITION CAPACITY ASSAY

In vitro inhibition of α-amylase enzyme (AA) followed the procedure as described by Widyawati et al. (2020). Each 500 µL of samples, was mixed with starch 1 % (w/v) and sodium acetate buffer pH 5.~~ were mixed~~. ~~Then,~~ Into a~~each~~ 250 µL of the mixture ~~and~~ was added an α-amylase solution (0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in 50 mL of 0.2 M sodium acetate pH 5~~)~~. Mixture was shaken ~~and~~ into which was added 2 mL sodium hydroxide 1M. Before the analysis, this mixture was incubated at 37 ºC for 10 min. Then, the capacity of the α-amylase enzyme to hydrolyze~~d~~ the starch to release glucose was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) ~~that could be analyzed based on absorbance~~ at λ 540 nm. The

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inhibition percentage of α-amylase was assessed using the formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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 test sample with enzyme, Ab is absorbance of test sample without enzyme.

α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

The analysis of the α-glycosidase inhibitor activity (GA) was done by Widyawati et al. (2020) method with slight modification. About 150 µL samples ~~contained~~ containing 100 µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH 7) were reacted with 50 µL α-glycosidase 2 mM (0.0833 unit/mL), and then the mixture was incubated at 37 ºC for 15 min. ~~Finally, the~~The reaction was stopped with the addition of 1000 µL sodium carbonate 0.2 M. The residue of this enzyme hydrolyzed p-nitrophenyl-α-D-glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The inhibition~~or~~ activity of ~~steeping~~the ~~*Pluchea* tea~~ infusion ~~to enzyme~~ was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at λ 405 nm. The inhibition percentage of α-glycosidase was calculated using formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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, Ab is the absorbance of test sample without enzyme.

~~HPLC~~ ANALYSIS OF PHENOLICS

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The phenolic compounds of the samples were analyzed by HPLC based on Kongkiatpaiboona et al. (2018) method with modifications. Each *Pluchea* infusion was sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter syringe (Whatmann, 0.2 µm, NYL). About 20 µL of 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 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (2 pieces) (ID 10 mm x 4.6 mm). ~~Analytical conditions: T~~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 minutes; then 100 % B was maintained for 20 minutes. Next the column was re-equilibrated with 10 % B in A maintained for 10 minutes before analysis of the next sample. The sample flow rate was set at 1.0 ml/min with a controlled temperature at 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 distillated water and prepared similar to the samples before 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 ~~time~~period (B). *Pluchea* leaf blades were

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subjected to 4 steeping temperatures, namely 60 ºC (T1), 70 ºC (T2), 80 ºC (T3), and 95 ºC (T4), and the storage ~~time~~period of 0 year /~~fresh~~ un-stored (B1), and 5 year/stored (B2).~~.~~ ~~The research resulted~~resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic was repeated two ~~time~~periods. The data of samples were analyzed by ANOVA at $\alpha \leq 0.05$, and continued analysis using a paired T test at $\alpha \leq 0.05$. treatment means of specific phenolic compounds that were identified were expressed as the mean ± SD. The analysis used SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

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RESULTS AND DISCUSSIONS

Pluchea leaf infusion is produced by young *Pluchea* leaf from 1-6 level on each branch the shoot (Widyawati et al., 2022), that is steeped at 95 ºC 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 *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols, cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2 g/100 mL steeping *Pluchea* 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 related to the composition of phytochemical compounds in *Pluchea* leaves, such as phenolic acids such as chlorogenic acids, caffeic acids, 3-*O*-caffeoylquinic acids, 4-*O*-caffeoylquinic acids, 5-*O*-caffeoylquinic acids, 3,4-

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di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin; β-carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Chan et al., 2022; Widyawati et al., 2022). Presence of phytochemical compounds in herbal product were 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 of 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). Therefore, this study was conducted to determine the effect of steeping temperature and storage ~~time~~period of *Pluchea* tea on levels of the bioactive compounds, antioxidant and antidiabetic properties and phenolic compound profile.

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BIOACTIVE COMPOUNDS

Phenolics Compounds

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The bioactive compounds are active compounds in plants that are essential to protect a 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).

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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 based on paired ~~T~~ t- test at $\alpha \leq 0.05$ (Figure 1a). Steeped and stored infusion had significantly higher amounts of phenolic compounds thant ~~t~~ the samples that were steeped and un-stored. Further, the highest total phenolic content was observed in samples infused at 95 ºC and stored for 5 years (at 71.38±4.14 mg GAE/g sample~~s~~) while the lowest was measured in the un-stored samples and infused at 60 ºC (at mg GAE/g sample). Phenolic content of stored samples ~~that were infused at different temperatures~~ that ~~then stored~~ were steeped only at 60 and 95 ºC ~~also~~ 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 the phenolic compounds of the infusions. Results also indicated that phenolic compounds were generally greater in the infusion at high steeping temperatures and long storage period .~~(Figure 1a). This could have been due to that 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 sleeping temperature also caused the greater the extracted total phenolic content. The temperature of infusion influenced total phenolic content, it could relate to~~ This could have been due tp the fact that the steeping temperature and storage period can 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~~increasing contact~~ exposure~~between~~of phenolic compounds to water (Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al.

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(2022). . Su et al. (2019) reported that temperature treatment can stimulate the release of phenolic compounds of lychee juice stored at different temperatures of 4 and 45 ºC and different long storage (fresh and 72 hours).

. this compounds and water. The same phenomena also occurred in Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al. (2022).

This occurrence showed that steeping temperature and storage period caused the process of degradation and oxidation of phenolic 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 ºC and different long storage (fresh and 72 hours). Hydrogen bonding is affected by Ttemperature treatment because thedegrades (or hdrolyzes) the hydrogen bond between phenolic compounds and proteins can be degraded that the measured levels resulting in an increase of phenolic compounds when exposed toare higher temperatures. The phenomena were supported by (Ali et al. (2018); Jayani et al. (2022), and 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. Besides that, 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 that the phenolic compounds in *Pluchea* infusion are degraded due to oxidation and hydrolysis because of temperature and storage timeperiod and can be easily extracted during

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steeping, thusresulting in the increased amount of ing thethe phenolic content compounds as the at higher steeping temperature and longer storage increaseperiod.

Based on using of a reference standard could be informed thatSimple phenolic compounds identified in steeped and stored ing *Pluchea* leaf infusion, includeing 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 was showed in Table 1. The treatment effectsresults of statistical analysis using a paired T test at $\alpha \leq 0.05$ showed that gallic acid and kaempferol contents of *Pluchea* infusion were insignificantly different at various steeping temperature and long storage periods. Nevertheless, the The concentration of quercetin and 3,5-di-O-caffeoylquinic acid of the un-stored and stored *Pluchea* infusion was significantly different from the rest of the samples between of two treatments except at 70 ºC. The while (+)-catechin concentration of *Pluchea* infusion was only significantly different at 95 ºC, but Tthe myricetin content was significantly different different concentration at 80 and 95 ºC. The 3,4-di-O-caffeoylquinic acid content showed significace difference at 60, 80 and 95 ºC and while 4,5-O-dicaffeoylquinic acid compounds content from *Pluchea* infusion werewas only significantly different at 60 ºC, however the concentration of 3,4-dicaffeoylquinic acid was also significantly different at 80 and 95 ºC.

Based on the analysis of concentration ofResults further showed simple phenolic compounds showed that gallic acids and kaempferol were relatively stable phenolic acid because ofas reflected by the insignificant changes when exposed no changes atto the different steeping temperature and storage timeperiod. 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.

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~~However, myricetin~~Myricetin, (+)-catechin and 3,4-di-*O*-caffeoylquinic acid showed a drastic ~~increasing~~ increase at higher steeping temperature and longer storage period ~~.~~implying ~~It's meant~~ that these compounds tended to be relatively labile. Quercetin, 3,5-di-*O*-cafffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid underwent moderate changes ~~compared to the other two groups of phenolic acids~~.~~.~~ T~~t~~herefore, myricetin, (+)-catechin and 3,4-di-*O*-caffeoylquinic acid were easier to dissolve or degraded to form simple phenolic compounds at higher steeping temperature and storage ~~time~~period. ~~can cause macromolecules of three phenolic acids in herbal tea convenient degradable to form simple phenolic compounds for storage, as explained by (~~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.

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 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 α= 0.05 showed that total flavonoid content of *Pluchea* infusion was significantly different between ~~two treatments~~the steeped un-stored and steeped stored samples

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(Figure 1b). The highest total flavonoid content was exhibited by ~~fresh~~ the un-stored samples steeped at 95 ºC at about 147.42±14.03 mg CE/g sample~~s~~. Total flavonoid content was significantly lower in the stored samples ~~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.

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Tannin Content (TTC)

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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 the samples infused at 60 ºC at about 4.81±0.58 to 17.42±1.04 mg TAE/g samples. ~~–~~which ~~is~~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 ºC 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 ºC about 9.22 ± 1.48 mg TAE/g samples. i~~I~~ndicating that the tannin content was primarily affected by ~~both high steeping temperature and~~ long storage period than high steeping temperature~~and that the presence of high tannin content was primarily brought about by long storage period. Kowalska et al. (2021) informed that T~~t~~he condensation of catechins to tannins ~~of polyphenolic compounds~~ is a dominant process ~~occurred~~ occurring in tea leaves that is accelerated during maceration of raw ~~material~~tea leaves (Kowalska et al~~.~~

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(2021) could have had contributed to the observed increase in the tannin content in the treated samples. However, the high temperature can degrade polyphenolic compounds to form simple phenolic 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 concentration 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,

Although, high temperature and long storage period can cause the degradation of tannins to catechins, Rusita et al. (2019) emphasized that tannins are a polar thermostable complex compounds, that is are resistant to heating, indicating that even with the exposure to high temperature, the tannins still remained high in the treated samples.as a result the tannin content in *Pluchea* tea increases with increasing steeping temperature and storage timeperiod, this is caused tannins are thermostable complex compounds.

ANTIOXIDANT ACTIVITY

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Antioxidant activity is capability of compounds to inhibit the oxidation of macromolecules from biological target that involve 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 antioxidant that have antioxidant

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

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DPPH Free Radical Scavengincg Asctivity

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DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate antioxidant activity because this method is simple that is suitable to measure the donating hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of DPPH to change to yellow color (Munteanu and Apetrei, 2021; Baliyan et al., 2022). Figure 2a shows that the free radical scavenging property of the stored and steeped samples were significantly higher than the un-stored steeped samples.~~The result of DPPH assay~~ It can also be observed ~~indicates~~ that the free radical scavenging property ~~DPPH values accrued~~ was significantly different among the stored and steeped samples but insignificant among the un-stored and steeped samples.~~at higher steeping temperature and longer storage time~~period.~~Statistical analysis by ANOVA using a paired T test at $\alpha \leq 0.05$ 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~~. *Pluchea* infusion stored at

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room temperature for 5 years resulted in the high ~~DPPH~~ free radical scavenging activity by~~.~~ more than 100 %~~. S~~teeping ~~at higher~~ temperatures significantly increased the ~~DPPH~~ free radical scavenging activity in stored *Pluchea* infusion by around 15 to 25 %. ~~Steeping at 80-95 °C in stored *Pluchea* infusion insignificantly affected the free radical scavenging property of the bioactive compounds (Figure 2a).~~ This implies that that the higher free radical scavenging property was primarily affected by the storage period than steeping temperature. During the storage process it is possible to form complex phenolic compounds which provide a high ability to scavenge ~~DPPH~~ free radicals (Thanajiruschaya et al., 2010)

~~Scavenging~~ The scavenging activity ~~of DPPH free radicals~~ of the the samples was strongly and positively correlated ~~with total~~ with total phenolic and tannin contents~~levels~~, but inversely ~~to~~ with total flavonoid levels. ~~Based on Pearson correlation at Table 2, the correlated coefficient values (r) between DPPH and TPC, TTC and TFC were 0.993, 0.942, and -0.940, respectively. During the storage process it is possible to form complex phenolic compounds which provide a high ability to scavenge DPPH free radicals (Thanajiruschaya et al., 2010)~~. This ~~research~~ study also demonstrated that longer storage ~~time~~period and higher infusion temperature produced many simple phenolic compounds with free hydroxyl groups capable to donor hydrogen atom to DPPH free radical. Many phenolic acids, such as gallic acids, (+)-catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 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 donor hydrogen atom depends on chemical structure, number and position of hydroxyl groups attached to a

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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 donor hydrogen atom is determined by O-H bond dissociation energy.

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

Ferric Reducing Antioxidant Power (FRAP)

FRAP is an analysis of antioxidant power of the phytochemical compounds ~~based on the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a color complex, that can be measured at λ 700 nm (Fu et al., 2011; Al-Temimi and Choudhary, 2013). The principle of the assay measures~~ that is based of the ability of antioxidant compounds to reduce iron ions of potassium ferrocyanide (Fe^{3+}) to be 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 ferric reducing antioxidant power (FRAP) increased ~~with~~ at higher steeping temperature and longer storage ~~time~~period. The lowest FRAP was observed in the un-stored samples which was steeped at 60 ^{o}C at 3.95 ± 0.17 mg gallic acid equivalents (GAE)/g samples, and the highest was ~~owned~~ exhibited ~~by~~ in *Pluchea*

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infusion which was stored for 5 years at 95 ºC at 48.63 ±10.83 mg gallic acid equivalents (GAE)/g samples (Figure 2b). FRAP increased significantly as steeping temperature was increased. FRAP of the samples stored for 5 years was also significantly higher than the un-stored samples at $\alpha \leq 0.05$. ~~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) between FRAP and DPPH, TPC, TTC and TFC were 0.956, 0.953, 0.948 and -0.826, respectively~~.

This ~~case was~~ is in contrast ~~to~~ with the study on the antioxidant activity of DPPH and FRAP ~~on~~ of matcha., ~~because The~~the longer storage ~~time~~period reduces the levels of catechin content ~~due to the catechins,~~ such as epigallocatechin gallat (EGCG), epicatechin gallat (ECG), epigallocatechin (EGC), and epicatechin (EC) which are bioactive compounds that have high antioxidant activity (Kim et al. 2020)., ~~and also the case of the effect of temperature and storage time in betel (*Piper bettle* 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). Thanajiruschaya et al. (2010) revealed that the antioxidant activity of rice stored at high temperatures is greater than that stored at low temperatures.~~ The ferric reducing capability of ***Pluchea*** could have due ~~infusion corresponded~~to the presence ~~to~~ of simple phenolic acid ~~values~~ that have the ability to transfer electron from their free hydroxyl groups of~~, presence of them in samples could accrue antioxidant activity because of ability of the electron transfer from free hydroxyl groups of phenolic acids~~.

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The FRAP of *Pluchea* infusion was strongly and positively significant correlated with the DPPH, TPC and TTC, but inversely to TFC.

ANTIDIABETIC ACTIVITY

α-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 2-degestive enzymes i.e., α-amylase and α-glucosidase, which are involved 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 α-amylase and α-glucosidase enzymes (Martinez-Solis et al., 2022) resulting in the reduced activity of the enzymes. The results showed~~,~~ that ~~the~~ lower steeping ***Pluchea*** leaf infusion was able to inhibit the action of the α-amylase enzymes (Figure 3a). The ***Pluchea*** infusion ~~had very good activity,~~exhibited a good α-amylase enzyme inhibition activity of more than 50 % and even almost 100 % ~~for fresh~~in the un-stored ***Pluchea*** infusion ~~which~~ steeped ~~was brewed~~ at 60, 70 and 80 ºC with highest at 60 ºC, and in stored *Pluchea* leaf infusion which was steeped at 60 ºC. ~~Whereas~~ The stored ~~fresh~~ ***Pluchea*** leaf infusion steeped at 70, 80 and 95 ºC for 5 minutes had lower enzyme inhibition activity~~an activity of~~ of ~~less than 50 % with lowest at~~ 95 ºC.~~inhibiting the α-amylase enzyme of less than 50 %, which was equal to 40.08±1.12~~ %. Widyawati et al. (2017) ~~detected~~ found that the ability to inhibit the α-amylase enzyme ~~from~~ in ~~fresh~~ un-stored ***Pluchea*** infusion steeped at 95 ºC for 5 minutes ~~by~~ was also low at 28.79 %. Increasing the steeping temperature and storage ~~time~~period reduced the ability ~~to~~ of the phytochemicals in the *Pluchea* infusions to inhibit the α-amylase enzyme activity. ~~The~~

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~~results of the analysis based on a paired T test at α ≤ 0.05 showed, that the steeping temperature and storage time~~period ~~had a significant effect on the ability to inhibit the α-amylase enzyme~~. ~~Based on Pearson correlation, the~~Table 2 further shows that the AA of *Pluchea* infusion was strongly and negatively significant correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively significant correlated with TFC. ~~The correlated coefficient values (r) between AA and TPC, TTC, DPPH, FRAP and TFC were -0.708, -0.857, -0.696, -0.806 and 0.429, respectively.~~

This inhibitory activity was thought to be contributed by other bioactive compounds, besides phenolics which are sensitive to steeping temperature and storage ~~time~~period. Li et al. (2018) stated that there are flavonoid compounds that contribute to the ability to inhibit the α-amylase enzyme. ~~Flavonoid compounds with a hydroxyl structure at C-4' in ring B are more effective than C-6 in ring A.~~ Akah et al. (2011) ~~informed~~ reported that the phytochemical compounds, such as terpenoids, saponins, flavonoids, glycosides and carbohydrate, and alkaloids are good antidiabetic metabolites or α-amylase enzyme activity inhibitor. Sangeetha and Vedasree (2012) explained, that the ability to inhibit the α-amylase enzyme was determined by the content of the phenolic compound and protein. The α-amylase inhibitor enzyme present in *Pluchea* infusion may be proteinaceous in nature. Aleixandre et al. (2022) informed that phenolic acids have inhibition activity to α-amylase enzyme depending their structures. Besides that, capability of phenolic acids to inhibit α-amylase enzyme was determined by low half-maximum inhibitory concentration (IC_{50}). There are C=C double bond conjugated with a carbonyl group of phenolic structures that stabilizes the binding forces to the active site of the α-amylase. The hydroxyl groups of them are able to bind by non-covalent interaction, such as hydrogen

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binding, cation-π interactions, salt bridge interactions, ionic interactions or electrostatic forces with amino acid residue at the active site in α-amylase enzyme. Elevated steeping temperature and longer storage period ~~The steeping temperature and storage time~~ can easily cause the removal of the ~~e~~ hydroxyl groups of phenolic compounds that can reduce their ~~ir~~ ability of enzyme inhibition. The phenolic acids with a greater number of hydroxyl groups ~~are~~ exhibits stronger capability~~le~~ to obstruct the α-amylase enzyme.

α-Glucosidase enzyme inhibition activity (GA)

Alpha~~α~~-glucosidase is an important enzyme in carbohydrates digestion, that catalysis the hydrolysis of 1,4-α-bonds of the unabsorbed oligo- and disaccharides, and converts them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et al., 2014; Proenca et al., 2017). The ability of bioactive compounds to inhibit the α-glucosidase enzyme is used to determine their antidiabetic~~s~~ 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 brewing fresh *Pluchea* infusion at 95 ºC for 5 minutes has an inhibitory effect on the α-glucosidase enzyme of 67.857 %.

~~The results showed,~~Figure 3b shows that the ability of the Pluchea leaf infusion to inhibit the α-glucosidase enzyme decreased with increasing steeping temperature and storage ~~time~~period. Steeping at 95 ºC ~~for fresh~~of the un-stored *Pluchea* leaf infusion ~~(un-stored)~~ obtained the lowest inhibitory ability, i.e., 48.32 ± 1.27 %, and the highest inhibitory activity was f~~ound~~ at 70 ºC ~~steeping temperature for fresh *Pluchea* infusion, which was~~at 95.11 ± 0.70%. ~~(Figure 3b).~~ The results of a paired T test showed that GA of *Pluchea* infusion was significantly different ~~at both~~between steeping temperature and long storage. ~~The antidiabetic activity of *Pluchea* infusion~~Figure 3 further ~~showed~~ shows that the ability

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of *Pulchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-amylase enzyme. Li et al. (2018) informed that flavonoid compounds have the ability to inhibit the action of the α-amylase and α-glucosidase enzymes. This is due to the total flavonoids in steeped *Pluchea* infusion which tended to have the same pattern as the ability to inhibit the activity of the α-amylase and α-glucosidase enzymes. The statistical analysis using Pearson correlation showed that GA of *Pluchea* infusion was strongly and negatively correlated with TPC, TTC, DPPH and FRAP ~~, with r was -0.555, -0,715, -0.527 and -0.560, respectively.~~ However, GA was moderately and positively correlated to TFC~~, with r was 0.350 and strongly~~ and positively correlated to AA~~, with r was 0.725~~. Flavonoid compounds, such as rutin, myricetin, kaempferol, and quercetin ~~which~~ 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 and the number of double bonds in rings A and B and the heterocyclic ring in ring C. The ability to inhibit the α-glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping temperature and long storage. The capability of *Pluchea* infusion to obstruct the α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of two enzymes was different, according to the opinion of McCue et al. (2005). Widyawati et al. (2017) informed that phenolic and non-phenolic compounds determine the inhibitory activity of the α-glucosidase enzyme. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, that may be occurred in *Pluchea* infusion during storage, affects the structure and profile of phenolic and non-phenolic compounds. Asriningtyas et al. (2014) claimed

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that *Pluchea* leaves contain 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. Quinic acid is methyl esterified with the number of caffeic groups in the molecule that determines the activity of inhibiting the α-glucosidase enzyme. Analysis of caffeoylquinic acids in *Pluchea* infusion was obtained that the higher steeping temperature and long storage caused increased concentration of them, but the α-glucosidase inhibition activity of them was reduced. Aleixandre et al. (2022) reported that the simple phenolic acids forming a dipole-dipole interaction of active site from α-glucosidase enzyme are effectively inhibiting the enzyme.

This study ~~was obtained information~~showed that the increasing of steeping temperature and storage ~~time~~period caused a degradation reaction 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, supported the results of total phenolic content and total tannin content assays. Increased concentration of simple phenolic compounds determined the ability of these compounds as antioxidant agents, but reduced their capability as antidiabetic agents.

CONCLUSION

The steeping temperature and storage ~~time~~ period of *Pluchea* infusion significantly influenced bioactive contents, antioxidant and antidiabetic activities. TPC, TTC, and TFC were significantly different at various steeping temperature and storage period based on statistical analysis using a paired ~~T~~ t-test at $\alpha \leq 0.05$. ~~There was the difference of t~~The

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1) 'Figure 3b shows that the ability of the Pluchea leaf infusion to inhibit the α-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 α-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 α-glucosidase enzyme was greater than the α-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 α-amylase and α-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 α-glucosidase enzyme. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. (May also be incorporated in 1).

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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

phenolic compound profile in ~~fresh~~ the unstored and stored ~~of~~ *Pluchea* infusion ~~and~~ at various steeping temperature~~.~~ ~~The~~ included simple phenolic compounds ~~were detected in *Pluchea* infusion including~~such as gallic acid, (+)-catechin, quercetin, myricetin, kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoilquinic acid, and 4,5-di-*O*-caffeoilquinic acid. The results of statistical analysis using a paired ~~T~~ t-test at $\alpha \leq 0.05$ showed that gallic acid and kaempferol of *Pluchea* infusion were insignificantly different at various steeping temperature and long storage. ~~Nevertheless, T~~the concentration of quercetin and 3,5-dicaffeoylquinic acid of *Pluchea* infusion was significantly different of two treatments except at 70 ^{o}C. The (+)-catechin concentration of *Pluchea* infusion was significantly different at 95 ^{o}C, but the myricetin was different concentration at 80 and 95 ^{o}C. The 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid compounds from *Pluchea* infusion were significantly different at 60 ^{o}C, however the concentration of 3,4-dicaffeoylquinic acid was also significantly different at 80 and 95 ^{o}C. TPC, TTC and TFC of *Pluchea* infusion were significantly different at various steeping temperature and storage period. TPC and TTC significantly increased with increasing steeping temperature and long storage, but TFC significantly increased at various steeping temperature and significantly decreased at long storage. The bioactive compounds of *Pluchea* infusion influenced antioxidant activities (DPPH and FRAP) and antidiabetic activity (AA and GA). The DPPH was strongly and positively correlated with TPC and TTC, but it was strongly and negatively correlated with TFC, with coefficient r 0.993, 0.942, and -0.940, respectively. The correlated pattern between FRAP and bioactive contents of *Pluchea* infusion was similar to it between DPPH and bioactive contents. The correlated coefficient values (r) between FRAP and TPC, TTC and TFC were 0.953, 0.948

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and -0.826, respectively. The AA and GA were strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively significant correlated with TFC. Between the antioxidant activity of DPPH and FRAP and the antidiabetic activity of AA and GA of Pluchea infusion were strongly and positively correlated with correlation coefficient (r) values of 0.956 and 0.725, respectively.

DATA AVAILABILITY

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

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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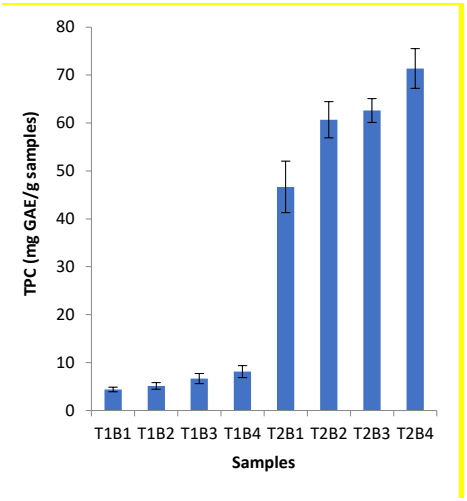

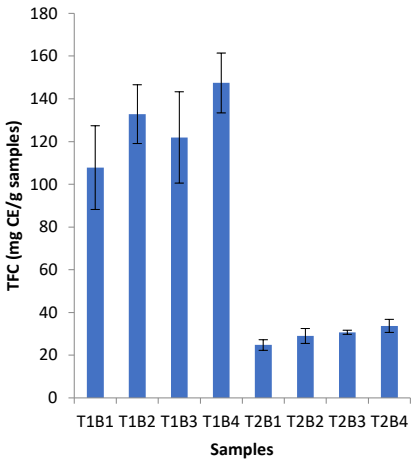

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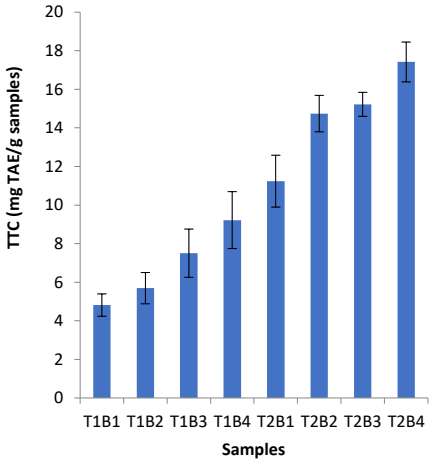

(c)

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 ±standard deviation (n=6). Samples : T1B1-steeped at 60 ºC, un-stored; T1B2-steeped at 70 ºC, un-stored; T1B3-steeped at 80 ºC, un-

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stored; T1B4-steeped at 95 ºC, un-stored; T2B1-steeped at 60 ºC, stored for 5 years; T3B2-steeped at 70 ºC, stored for 5 years; T3B3-steeped at 80 ºC, stored for 5 years; T3B4-steeped at 95 ºC, 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 $\alpha \leq 0.05$.

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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	-1.1735±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

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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

Note : Data were expressed as mean ±standard deviation (n=2). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}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 $\alpha \leq 0.05$. * $\alpha \leq 0.05$.

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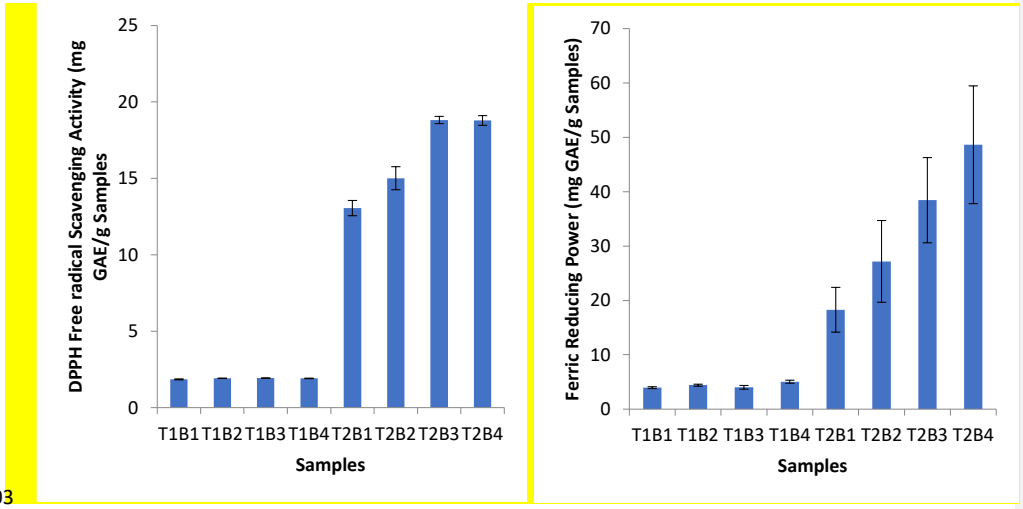

(a) (b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage ~~time~~period (a) DPPH (b) FRAP. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ºC, un-stored; T1B2-steeped at 70 ºC, un-stored; T1B3-steeped at 80 ºC, un-stored; T1B4-steeped at 95 ºC, un-stored; T2B1-steeped at 60 ºC, stored for 5 years; T3B2-steeped at 70 ºC, stored for 5 years; T3B3-steeped at 80 ºC, stored for 5 years; T3B4-steeped at 95 ºC, 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 $\alpha \leq 0.05$~~

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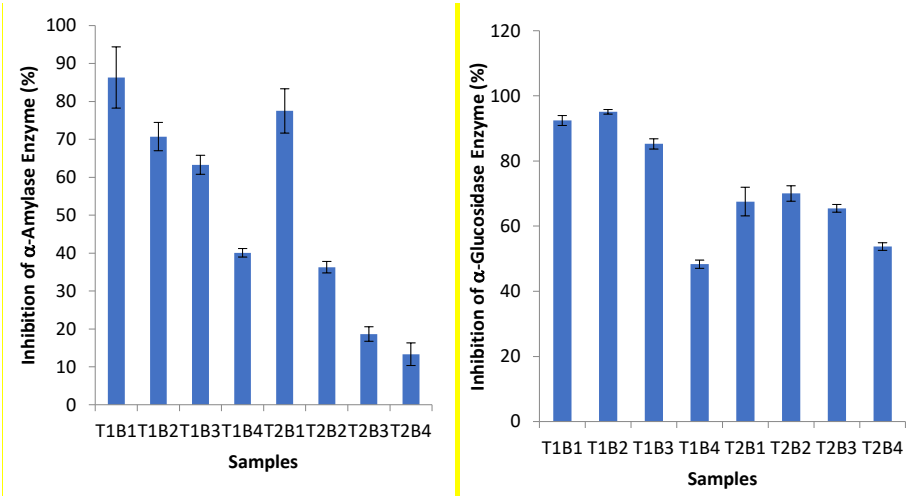

(a) (b)

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage ~~time~~period (a) α-amylase (b) α-glucosidase. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}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 $\alpha \leq 0.05$

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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

Note: *Correlation Ssignificant at the 0.05 level (2-tailed)

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

Paini Sri Widyawati <paini@ukwms.ac.id> Sat, Jan 20, 2024 at 5:30 PM
To: Caesar Saloma <caesar.saloma@gmail.com>

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
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Effect of Brewing Temperature and Storage Time on Antioxidant and Antidiabetic Properties of Pluchea Tea-Final Revision-2.docx
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Effect of Steeping Temperature and Storage Period on the Bioactive Compounds, Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea Indica* Less

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Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea indica* Less, storage period

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ABSTRACT

This study was done to determine the effects of steeping temperature and storage period on the bioactive contents, 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 4 steeping temperatures of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) ^{o}C with a storage period of 0 (B1) and 5 (B2) years resulting in 8 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), total flavonoid (TFC)], antioxidant [(DPPH scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)] potential and antidiabetic [(α-amylase (AA) and α-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 GA and AA were significantly decreased for the storage period and the steeping temperatures. The antioxidant activities of the *Pluchea* infusion were significantly determined by TPC and TTC with correlated values (r) 0.9928 of DPPH and 0.9533 of FRAP. The antidiabetic activities of samples were not influenced by the TPC and TTC but were weakly and positively correlated with TFC, r=0.3499 of GA and 0.4294 of AA. 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,

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kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-caffeoylquinic acid, 3,5- di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid

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, β-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, β-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 infusion by hot or boiling water for a few minutes (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 ^{o}C for 5 minutes exhibits total phenolic and total flavonoid contents, the ability to scavenge DPPH free radicals, and the capability to reduce ferric ions at 9.3 mg

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gallic acid equivalent (GAE)/g sample, 22.0 mg gallic acid equivalent (GAE)/g sample, 27.2 mg gallic acid equivalent (GAE)/g sample, 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 ^{o}C for 5 minutes 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 several factors, i.e., storage and temperature. The polyphenol profile and antioxidant properties of herbal tea infusion decline with an increase in steeping/brewing and storage temperatures and longer exposure periods.

Several studies have mentioned the effect of steeping temperature on the bioactive compound contents and antioxidant activity, such as some white and green teas are effective with hot water at 90 ^{o}C for 7 min (Castiglioni et al., 2015), on roseship tea is effective at infusion period around 6-8 min at temperatures of 84-86 ^{o}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 ^{o}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

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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, antioxidant and antidiabetic activities, i.e., juice from *Momordica charantia* L. (Lin et al., 2020), dried *Piper betlle* extracts (Ali et al., 2018), white tea (Xu et al., 2019), kinnow-amla beverages (Purewal et al., 2022), 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), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP)], and antidiabetic activities [(α-amylase (AA) and α-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,

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111 washed, and dried to get a moisture content of around 11.16 ± 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 ^{o}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) ^{o}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 [(α-amylase (AA) and α-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, α-amylase, α-glucosidase, pNPG (p-nitrophenyl-α-
129 glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic
130 acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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

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purchased from Merck (Kenilworth, NJ, USA). All reagents used were of analytical grade except for distillated water which was purchased from PT Aqua Industry Surabaya.

METHODOLOGY

ANALYSIS OF THE BIOACTIVE COMPOUNDS

TOTAL PHENOLIC CONTENT ANALYSIS

Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the technique by Gao et al. (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. Then 2 mL Na_2CO_3 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 λ 760 nm with gallic acid as the reference standard. The total phenolic content was calculated using the formula: $y=0.00009x+0.008$ with $R^2=0.9941$. The results were expressed as mg gallic acid equivalent (GAE)/g samples.

TOTAL FLAVONOID CONTENT ASSAY

The total flavonoid content (TFC) of the samples was measured based on the reaction between $AlCl_3$ and $NaNO_2$ with the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim et al., 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted in a yellow solution. About 30 µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5 % in 10 mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 10 % for 5 min. Then, 2 mL NaOH 1 M and distilled water were added to 10 mL volume. Then, the red solution was produced after NaOH

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solution addition that was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ 510 nm with (+)-catechin as the reference standard compound, and the results were expressed as mg catechin equivalents (CE)/g samples using the formula: y=0.00008x-0.0023 with R^2= 0.9980.

TOTAL TANNIN CONTENT ANALYSIS

Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method (Chandran and Indira, 2016). Approximately 10 µL *Pluchea* infusion was added 1 mL Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flash and incubated for 5 min. Then, the mixture was added with 2 mL Na_2CO_3 7.5 % and filled up to 10 mL volume with distilled water. The blue dark color solution was measured UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at λ 760 nm with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/g samples using the 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 µL *Pluchea* leaf infusion was poured into the reaction tube into which was added 3 mL DPPH solution (4 mg/100 mL). After incubation for 15 min in a dark

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room, the absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ. 517 nm. The reference standard compound was gallic acid and the results of the analysis were expressed as mg gallic acid equivalents (GAE)/g samples that were calculated using the formula: y=0.146x+1.7896 with R^2=0.9975.

FERRIC REDUCING POWER ANALYSIS

Ferric-reducing power (FRAP) was determined following the method used by Widyawati et al. (2014) method. Approximately 10 µL 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 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 was added 2.5 mL distilled water, 0.5 mL ferric chloride 0.1% w/v, and 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 λ 700 nm. The intensity of the blue color indicated a higher reducing capacity. The reducing power expressed as mg gallic acid equivalent (GAE)/g samples was calculated using the formula: y=0.0002x+0,0256 with R^2=0,9906.

ANALYSIS OF THE ANTIDIABETIC PROPERTIES

α-AMYLASE ENZYME INHIBITION CAPACITY ASSAY

In vitro, inhibition of α-amylase enzyme (AA) followed the procedure as described by Widyawati et al. (2020). Each 500 µL of samples, was mixed with starch 1 % (w/v) and sodium acetate buffer pH 5 into a 250 µL of the mixture was added an α-amylase solution

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(0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in 50 mL of 0.2 M sodium acetate pH 5. The mixture was shaken into which was and added 2 mL sodium hydroxide 1M. Before the analysis, this mixture was incubated at 37 ^{o}C for 10 min. Then, the capacity of the α-amylase enzyme to hydrolyze the starch to release glucose was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ 540 nm. The inhibition percentage of α-amylase was assessed using the formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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, Ab is the absorbance of test sample without enzyme.

α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

The analysis of the α-glycosidase inhibitor activity (GA) was done by Widyawati et al. (2020) method with slight modification. About 150 µL samples containing 100 µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH 7) were reacted with 50 µL α-glycosidase 2 mM (0.0833 unit/mL), and then the mixture was incubated at 37 ^{o}C for 15 min. The reaction was stopped with the addition of 1000 µL sodium carbonate 0.2 M. The amount of these enzymes that didn't react with bioactive compounds of *Pluchea* infusion hydrolyzed p-nitrophenyl-α-D-glucopyranoside (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 λ 405 nm. The inhibition percentage of α-glycosidase was calculated using the formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 100 %. Where ACb is the absorbance

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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, Ab is the absorbance of test sample without enzyme.

ANALYSIS OF PHENOLICS

The phenolic compounds of the samples were analyzed by HPLC based on the Kongkiatpaiboona et al. (2018) method with modifications. Each *Pluchea* infusion was sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter syringe (Whatmann, 0.2 µm, NYL). About 20 µL 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 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (2 pieces) (ID 10 mm x 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 minutes; then 100 % B was maintained for 20 minutes. Next the column was re-equilibrated with 10 % B in A maintained for 10 minutes 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

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4,5-dicaffeoylquinic acid. All of the reference standards were dissolved in distilled water and prepared similar 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 ^{o}C (T1), 70 ^{o}C (T2), 80 ^{o}C (T3), and 95 ^{o}C (T4), and the storage period of 0 year /fresh (B1), and 5 year/stored (B2) resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2).

The data analysis of samples was repeated for six periods. The data were analyzed by ANOVA at $\alpha \leq 0.05$, and continued analysis using a paired t-test at $\alpha \leq 0.05$ that was expressed as the mean ± SD. The analysis used SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSIONS

Pluchea leaf infusion is produced by young *Pluchea* leaf from 1-6 levels on each branch of the shoot (Widyawati et al., 2022), that is steeped at 95 ^{o}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 *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols, cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2 g/100 mL steeping *Pluchea* 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,

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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 composition of phytochemical compounds in *Pluchea* leaves, such as phenolic acids such as chlorogenic acids, caffeic acids, 3-*O*-caffeoylquinic acids, 4-*O*-caffeoylquinic acids, 5-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin; β-carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Chan et al., 2022; Widyawati et al., 2022). 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).

BIOACTIVE COMPOUNDS

Phenolics Compounds

The bioactive compounds are active compounds in plants that are essential to protecting a body's 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

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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 ^{o}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 ^{o}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 ^{o}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 ^{o}C and different long storage (fresh and 72 hours).

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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 ^{o}C while (+)-catechin concentration of *Pluchea* infusion
337 was only significantly different at 95 ^{o}C. The myricetin content was significantly different
338 at 80 and 95 ^{o}C. The 3,4-di-O-caffeoylquinic acid content showed significant difference

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339 at 60, 80, and 95 ^{o}C while 4,5-di-O-caffeoylquinic acid content was only significantly
340 different at 60 ^{o}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*-cafffeoylquinic 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 α= 0.05 showed that the total flavonoid content of *Pluchea* infusion was significantly

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different between the steeped un-stored and steeped stored samples (Figure 1b). The highest total flavonoid content was significantly lower in the stored samples than those of the un-stored 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 ^{o}C about 4.81±0.58 to 17.42±1.04 mg TAE/g samples which were 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 ^{o}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 ^{o}C about 9.22 ± 1.48 mg TAE/g samples. Indicating that the tannin content was primarily affected by a longer storage period than high steeping temperatures. 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.

However, high temperatures and long storage periods can cause the degradation of tannins to catechins. Rusita et al. (2019) emphasized that tannins are polar

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thermostable complex compounds that are resistant to heating, indicating that even with exposure to high temperatures, the tannin 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 active antioxidants that have antioxidant capability depending 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 higher antioxidant power of phenolic compounds is caused by the weaker O-H phenolic bond (Kruk et al., 2022). The mechanism of phenolic compounds as antioxidants depends on their ability to donate hydrogen atoms and transfer electrons, and as reducing agents and singlet oxygen quenchers (Ali et al., 2005; Huang et al. 2005).

DPPH Free Radical Scavenging Activity

DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate antioxidant activity because this method is simple and is suitable to measure 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 un-stored steeped samples. It can also be

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observed that the free radical scavenging property was significantly different among the stored and steeped samples but insignificant among the un-stored and steeped sample period. *Pluchea* 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 *Pluchea* infusion by around 15 to 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 which 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 phenol and tannin contents, but inversely with total flavonoid levels. The study also demonstrated that longer storage periods and higher infusion temperatures produced many simple phenolic compounds with free hydroxyl groups capable of donating 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, 4,5-di-*O*-caffeoylquinic acids have established potential antioxidant activity (Kumar and Goel, 2019) (Table 1). Kruk et al (2022) stated that the capability of phenolic compounds to donor hydrogen atoms depends on 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 compound donor hydrogen atoms is determined by O-H bond dissociation energy.

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The free radical scavenging property observed in the study was not inconsistent with the results of the study by Moraes-de-Souza et al. (2008). The research shows that the total phenolic content of herbal infusion is lowly correlated with DPPH free radical scavenging activity. However, Dobrinas et al. (2021) stated that the total phenolic content of tea infusion 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 ferric-reducing antioxidant power (FRAP) increased at higher steeping temperatures and longer storage periods. The lowest FRAP was observed in the un-stored samples which were steeped at 60 ^{o}C at 3.95 ± 0.17 mg gallic acid equivalents (GAE)/g samples, and the highest was exhibited in *Pluchea* infusion which was stored for 5 years at 95 ^{o}C at 48.63 ±10.83 mg gallic acid equivalents (GAE)/g samples (Figure 2b). FRAP increased significantly as the steeping temperature was increased. FRAP of the samples stored for 5 years was also significantly higher than the un-stored 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

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catechins, such as epigallocatechin gallat (EGCG), epicatechin gallat (ECG), epigallocatechin (EGC), and epicatechin (EC) 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 acids that can transfer electrons from their free hydroxyl groups. The FRAP of *Pluchea* infusion was strongly and positively significantly correlated with the DPPH, TPC, and TTC, but inversely to TFC.

ANTIDIABETIC ACTIVITY

α-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 2-digestive enzymes i.e., α-amylase and α-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 can bind with the protein component of α-amylase and α-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 α-amylase enzymes (Figure 3a). The *Pluchea* infusion exhibited a good α-amylase enzyme inhibition activity, more than 50 % and even almost 100 % in un-stored *Pluchea* infusion steeped at 60, 70, and 80 ^{o}C with the highest at 60 ^{o}C, and in stored *Pluchea* leaf infusion which was steeped at 60 ^{o}C. The stored *Pluchea* leaf infusion steeped at 70, 80, and 95 ^{o}C for 5 minutes had lower enzyme inhibition activity of less than 50 % with the lowest at 95 ^{o}C around 13 %. Widyawati et al. (2017) found that the ability to inhibit the α-amylase enzyme in un-stored *Pluchea* infusion steeped at 95 ^{o}C for

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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 α-
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 α-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 α-amylase enzyme activity inhibitors. Sangeetha and
486 Vedasree (2012) explained, that the ability to inhibit the α-amylase enzyme was
487 determined by the content of the phenolic compound and protein. The α-amylase inhibitor
488 enzyme present in herbal infusion may be proteinaceous or nonproteinaceous in nature.
489 It means that the α-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 α-amylase enzyme depending on their structures. Besides that, the
492 capability of phenolic acids to inhibit α-amylase enzyme was determined by low half-
493 maximum inhibitory concentration (IC_{50}). 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 α-amylase. The hydroxyl groups can bind by non-covalent interaction, such as
496 hydrogen bonding, cation-π interactions, salt bridge interactions, ionic interactions, or
497 electrostatic forces with amino acid residue at the active site in the α-amylase enzyme.

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Elevated steeping temperature and longer storage periods can easily cause the removal of the hydroxyl groups of phenolic compounds which can reduce their ability of enzyme inhibition. The phenolic acids with a greater number of hydroxyl groups exhibit a stronger capability to obstruct the α-amylase enzyme.

α-Glucosidase enzyme inhibition activity (GA)

α-glucosidase is an important enzyme in carbohydrate digestion, that catalysis the hydrolysis of 1,4-α-bonds of the unabsorbed oligo- and disaccharides, and converts them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et al., 2014; Proenca et al., 2017). The ability of bioactive compounds to inhibit the α-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 fresh *Pluchea* infusion at 95 ^{o}C for 5 minutes has an inhibitory effect on the α-glucosidase enzyme of 67.857 %.

Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme decreased with increasing steeping temperature and storage period. Steeping at 95 ^{o}C for the un-stored *Pluchea* infusion obtained the lowest inhibitory ability, i.e., 48.32 ± 1.27 %, and the highest inhibitory activity was at 70 ^{o}C at 95.11 ± 0.70%. The results of a paired t-test showed that the 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 α-glucosidase enzyme tended to be higher than the ability to inhibit the α-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

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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 α-amylase and α-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 the flavonoid compounds of samples significantly inhibit the α-glucosidase enzyme activity.

The ability to inhibit the α-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 α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of the two enzymes was different, according to the opinion of McCue et al. (2005). The mechanism of the α-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 α-amylase enzyme inhibitor includes blocking carbohydrates, limiting the digestibility and absorption of carbohydrates, and 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 of the α-glucosidase enzyme activity. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence

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of polymerization and degradation reactions, that may be occurred in *Pluchea* 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-*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 α-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 un-stored *Pluchea* infusion, and the concentrations of the simple phenolic compounds were increased at higher steeping temperature, but the α-glucosidase inhibition activity of them was reduced. It means that the methyl-esterified quinic acid with the caffeic groups had more potential to inhibit α-glucosidase enzyme than free caffeoylquinic acid.

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 *Pluchea* leaf infusion caused higher antioxidant activity and lower antidiabetic activity.

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CONCLUSION

The Total Phenol (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 un-stored. TPC was highest in the stored and steeped at 95^{o}C and lowest in the un-stored and steeped at 60^{o}C. Un-stored steeped samples exhibited significantly higher flavonoid content than the stored steeped samples. The highest total flavonoid content was exhibited by the un-stored samples steeped at 95^{o}C. The total tannin content of *Pluchea* leaf infusion significantly increased with increasing steeping temperature and storage period. Among the un-stored steeped samples, the tannin content was significantly lowest in the samples steeped at 60^{o}C and highest in the samples steeped at 95^{o}C.

The free radical scavenging property (DPPH) of the stored and steeped *Pluchea* leaf infusion was significantly higher than the un-stored steeped samples. The free radical scavenging property was highest in the stored samples steeped at 80 and 95^{o}C. 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 ferric-reducing antioxidant power (FRAP) significantly increased with increasing steeping temperature and longer storage periods. The lowest FRAP was found in the un-stored samples which were steeped at 60^{o}C and the highest was exhibited in *Pluchea* stored samples which were stored for 5 years and steeped at 95^{o}C. The FRAP of *Pluchea* leaf infusion was significantly strong and positively correlated with the free radical scavenging property, total phenolic, and total tannin content, but inversely with total flavonoid content. The

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inhibition of the α-amylase activity was generally found to be higher at lower steeping temperatures of the un-stored *Pluchea* leaf infusion than at higher steeping temperatures of the stored sample. The α-amylase enzyme inhibition 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 α-glucosidase enzyme decreased at high steeping temperatures and long storage periods. The highest inhibitory activity was obtained in the un-stored *Pluchea* leaf infusion that was steeped at 70^{o}C while the lowest was obtained in the un-stored sample that was steeped at 95^{o}C. The ability of *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-amylase enzyme. The inhibition of the α-glucosidase enzyme activity 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, 4,5-di-*O*-caffeoylquinic acids.

DATA AVAILABILITY

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

CONFLICT OF INTEREST

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The authors declare no conflict of interest.

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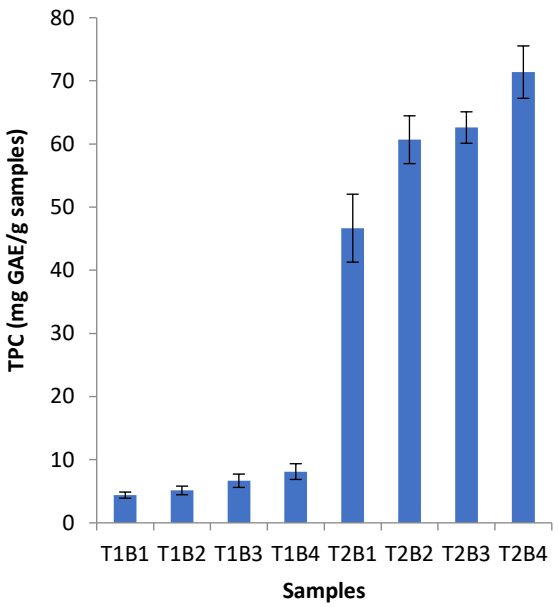
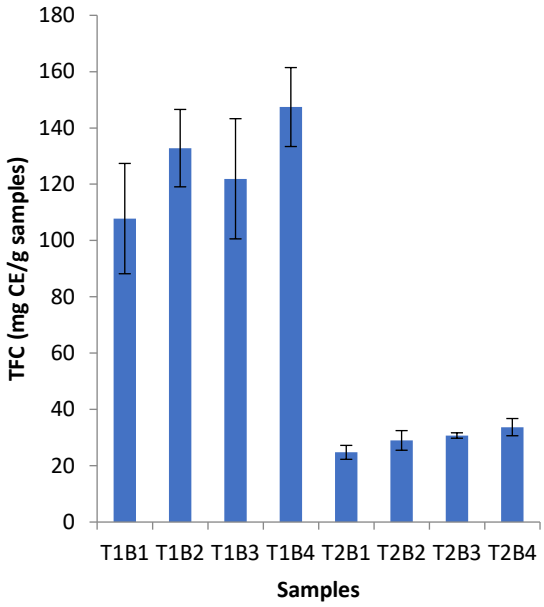


(a)



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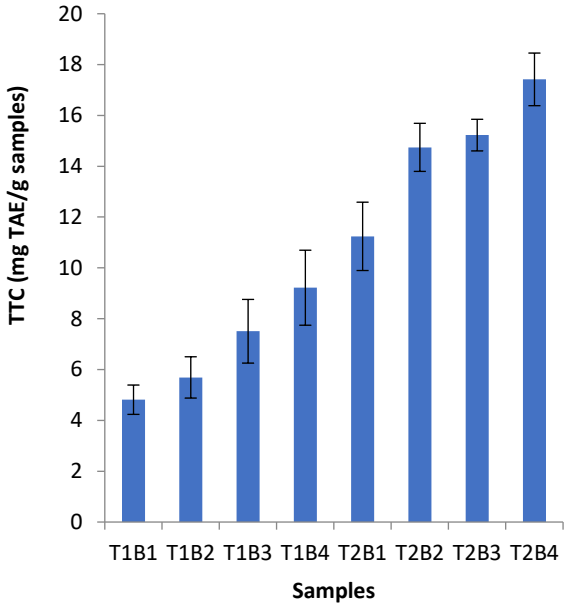

(c)

Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping temperature and storage period (a) Total phenolic content (b) Total flavonoid content (c) Total tannin content. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paited t-test at $\alpha \leq 0.05$. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored;

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T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}C, stored for 5 years.

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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

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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 paited t-test at $\alpha \leq 0.05$. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}C, stored for 5 years.

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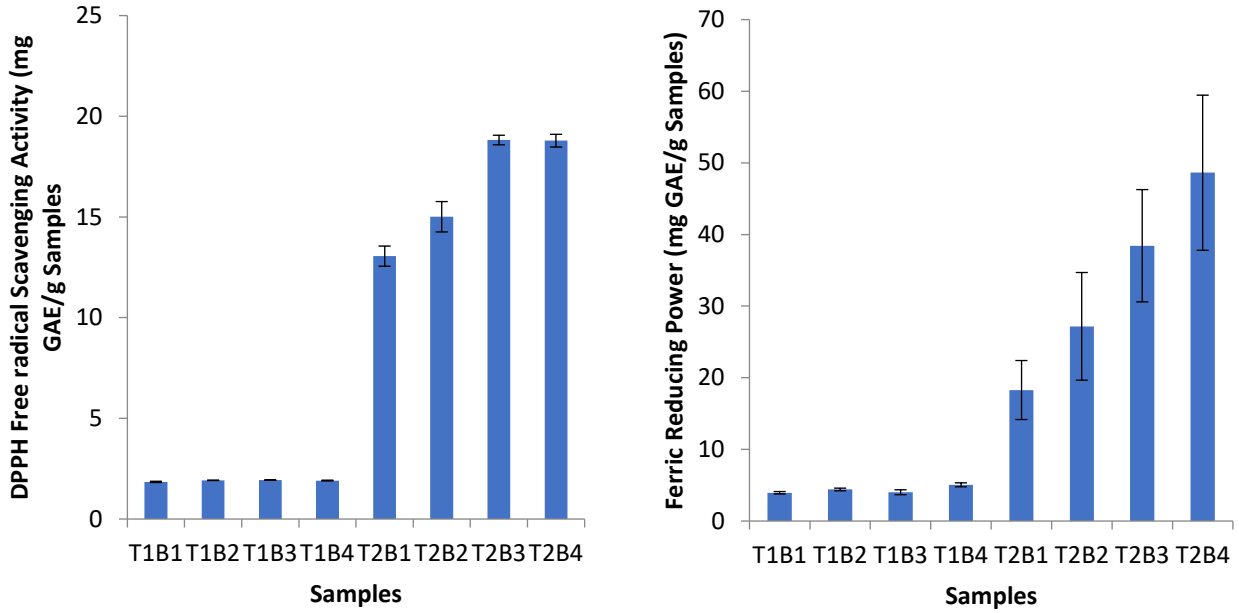

(a) (b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage period (a) DPPH (b) FRAP. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paited t-test at $\alpha \leq 0.05$. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}C, stored for 5 years.

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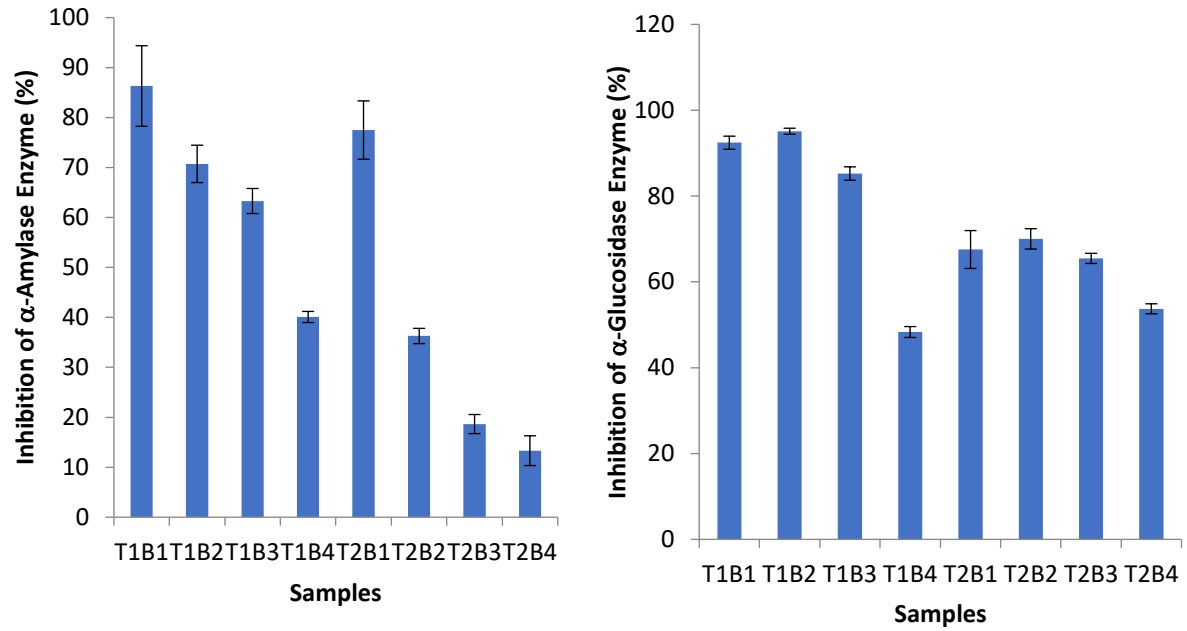

(a) (b)

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage period (a) α-amylase (b) α-glucosidase. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paited t-test at $\alpha \leq 0.05$. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}C, stored for 5 years.

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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 (2-tailed)

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

Paini Sri Widyawati <paini@ukwms.ac.id> Tue, Feb 27, 2024 at 4:57 AM
To: Caesar Saloma <caesar.saloma@gmail.com>

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]

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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> Tue, Apr 16, 2024 at 1:49 AM
To: Caesar Saloma <caesar.saloma@gmail.com>

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]

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

Paini Sri Widyawati[1*)], Yufita Ratnasari Wilianto[2)]

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Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea indica* Less, storage ~~time~~period

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ABSTRACT

This study was done to determine the effects of steeping temperature and storage ~~time~~period on the bioactive contents, 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 ~~time~~period (B). The ~~variety of the~~ *Pluchea* leaf blades were exposed to 4 steeping temperatures ~~included~~ of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) ~~(~~ºC~~)~~ with the storage ~~time~~period~~–~~period of 0 (B1) and 5 (B2) ~~(~~year~~)~~. ~~The research resulted~~resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). Statistical analysis using a paired t~~-T~~ test at $\alpha \leq 0.05$ showed that treatments significantly affected ~~influenced~~ the bioactive contents (total phenol (TPC), total tannin (TTC), total flavonoid (TFC)), antioxidant [(DPPH scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)]~~)~~ potential ~~–~~and antidiabetic [(α-amylase (AA) and α-glucosidase (GA) ~~inhibitors~~inhibition)] ~~activities~~ properties of the *Pluchea* leaf infusion~~samples~~. TFC decreased during storage period but significantly increased at higher steeping temperature. ~~The AA and GA of *Pluchea* infusion increased until 70 ºC of the steeping temperature, but deceased until 95 ºC. The bioactive contents influenced antioxidant and antidiabetic activities. TFC was decreased for storage time and significant increased at higher steeping temperature. The AA and GA of *Pluchea* infusion increased until 70 ºC of the steeping temperature, but deceased until 95 ºC.~~ The AA ~~–~~and GA were strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively correlated with TFC. ~~Between~~ The antioxidant activity ~~of DPPH and FRAP~~ and the antidiabetic activity ~~of AA and GA~~ of Pluchea infusion were strongly and positively correlated. ~~with correlation coefficient (r) values of 0.956 and~~

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0.725, respectively. The treatments gave different effect of simple phenolic compounds derived from *Pluchea* leaf infusion at different steeping temperatures and storage included, such as 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. of *Pluchea* infusion at different steeping temperature and long storage. To obtain high antioxidant activity, *Pluchea* infusion selected was stored and steeped at high temperature, however high antidiabetic activity obtained was fresh *Pluchea* infusion and steeped at low temperature.

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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 an herbal plant that has been widely used for traditional medicine and food (Chan et al., 2022). *Pluchea* leaves are composed 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, β-carotene, and vitamin C, whereas bioactive compounds is 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, β-carotene, and total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et al., 2022, Chan et al., 2022).

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Steeping process of *Pluchea* leaves can be performed with fresh or dry leaves ~~infusion by~~in hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et al., 2020; Jayani et al., 2022). In Asia~~n area~~, especially in Indonesia~~n~~, people usually consume the *Pluchea* infusion ~~with brewing of~~by steeping 2 g of powdered *Pluchea* leaves in tea bag ~~by~~in 100 mL of hot ~~water~~ or boiling water. ~~Each tea bag contained 2 g of *Pluchea* leaf powder is steeped with 100 mL hot water or boiling water.~~ Widyawati et al. (2016) claimed that steeping of 2 g of *Pluchea* leaf powder at 95 ^{o}C for 5 minutes ~~results~~ rexhibits total phenolic ~~content,~~and total flavonoid content~~s~~, the ability to scavenge DPPH free radicals, and the capability ~~of~~ to reduce ferric ions at 9.3 mg gallic acid equivalent (GAE)/g sample~~s~~, 22.0 mg gallic acid equivalent (GAE)/g sample~~s~~, 27.2 mg gallic acid equivalent (GAE)/g sample~~s~~, and 10.2 mg gallic acid equivalent (GAE)/g sample~~s~~, respectively. Werdani and Widyawati (2018) reported that drinking of *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 ^{o}C for 5 minutes certainly determines the stability and amount of extracted bioactive compounds~~,~~ that influences the biological activity~~,~~ especially antioxidant and antidiabetic activities. Silva-Ramirez et al. (2020) reported that the infusion process can influence the~~ir~~ content and composition of the bioactive compounds and antioxidant activity of tea. Acar et al. (2022) informed that infusion quality of herbal tea extract depends on several factors, i.e., ~~time~~ storage and temperature. Polyphenol profile and antioxidant properties of herbal tea infusion decline with an increase in steeping/brewing and storage temperatures, and longer exposure ~~time~~period~~s~~.

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Several studies have mentioned the effect of steeping temperature ~~to~~ on the bioactive compound contents and antioxidant activity, such as some white and green teas are effective with hot water at 90 ºC for 7 min (Castiglioni et al., 2015), on roseship tea is effective~~ly~~ at infusion ~~time~~period around 6-8 min at temperatures of 84-86 ºC (Ilyasoglu and Arpa, 2017), on the caffeine content extracted ~~the coffee~~at the brewing temperature of coffee~~influences the caffeine content extracted~~ (Zarwinda and Sartika, 2018), and ~~the steeping~~ the high total phenol content and antioxidant activity of dark tea at 92 ºC for 27 min ~~results the highest total phenol content and antioxidant activity~~ (Wang et al., 2022). The study of the effect of steeping temperature to ***Pluchea*** infusion was carried out to afford information about the most efficient preparation of powdered ***Pluchea*** leaves ~~most efficiently~~ to get higher ~~the~~ bioactive compounds, antioxidant and antidiabetic activities.

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

Therefore, this research studied the effect of steeping temperature and storage ~~time~~period on the bioactive compounds [(total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging

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activity (DPPH), ferric reducing antioxidant power (FRAP)] and antidiabetic activities [(α-amylase (AA) and α-glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea* leaves.~~- 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 antioxidant power (FRAP), α-amylase (AA) and α-glycosidase (GA) inhibition activities,~~ 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 *Asteraceae* family with specification according to the GBIF taxon ID number database:3132728 (Ferraris, 2023). *Pluchea* leaves at 1-6 level of each branch ~~of~~from the shoot were collected, sorted, washed and dried to ~~get a~~moisture content of around 11.16 ± 0.09 % dry bas~~is~~ (Widyawati et al., 2022). The ~~powdering of~~ dried *Pluchea* leaves was ~~done~~ pulverized to ~~get~~ a 45-mesh size powder. ~~And then, the heating of T~~the *Pluchea* leaf powder was ~~done using a drying~~dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 °C for 10 min to reduce microbial organisms. ~~and~~ Then, 2 g of the powder were packed ~~using~~ into a paper filter infusion bag.~~ that made from paper filter around 2 g/bag~~. ~~And then all of samples called~~Packed samples were ~~*Pluchea* herbal tea was~~ stored for 0 (un-stored) and 5 (stored)years in standing pouch before analysis.

In the research, the one tea bag of *Pluchea* herbal tea that stored 0 (B1) and 5 (B2) year, was steeped with 100 mL hot water at various temperatures, including 60 (T1),

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70 (T2), 80 (T3), and 95 (T4) ºC for 5 min with infusion method ~~that obtained~~obtaining 8 treatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2. After the temperature of *Pluchea* infusion similar to ambient temperature was analyzed further.

REAGENTS

The ~~compounds~~reagents used ~~to analyze~~in the analyses ~~including~~ include 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid, α-amylase, α-glucosidase, pNPG (p-nitrophenyl-α-glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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 distillated water which was purchased from PT Aqua Industry Surabaya.

METHODOLOGY

ANALYSIS OF THE BIOACTIVE COMPOUNDS

TOTAL PHENOLIC CONTENT ANALYSIS

Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the technique by Gao et al. (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. And

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then 2 mL Na_2CO_3 7.5 % was ~~entered~~ added and filled up to 10 mL volume with distilled water.~~and distillated water was added until 10 mL volume~~. The color intensity of solution was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at λ 760 nm with gallic acid as the reference standard. The total phenolic content was calculated using the formula: y=0.00009x+0.008 with R^2=0.9941. The results were expressed as mg gallic acid equivalent (GAE)/g samples.

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TOTAL FLAVONOID CONTENT ASSAY

Total flavonoid content (TFC) of the samples was measured based on the reaction between $AlCl_3$ and $NaNO_2$ with ~~an~~ the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim et al., 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted in a yellow solution. About 30 µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5 % in 10 mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 10 % for 5 min. And then, 2 mL NaOH 1 M and distillated water were added until 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 λ 510 nm with (+)-catechin as the reference standard compound, and the results were expressed as mg catechin equivalents (CE)/g samples using the formula: y=0.00008x-0.0023 with R^2= 0.9980.

TOTAL TANNIN CONTENT ANALYSIS

Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method (Chandran and Indira, 2016). Approximately 10 µL *Pluchea* infusion was added with 1 mL

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Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flash and incubated for 5 min. Then, the mixture was added with 2 mL Na_2CO_3 7.5 % and filled up to 10 mL volume with distillated water. ~~was added until 10 mL volume~~. The blue dark color solution ~~that~~ was measured in UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at λ 760 nm with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/g samples used the formula: y=0.00009x+0.0021 with R^2=0.9993

ANALYSIS OF THE ANTIOXIDANT POTENTIAL

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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 ~~antioxidant activity of~~in the *Pluchea* leaf infusion to ~~donor~~ donate hydrogen atom to the nitrogen atom in DPPH resulting in the formation of –DPPH-H compound ~~with~~ exhibiting a yellow-colored solution. About 25 µL *Pluchea* leaf infusion was ~~entered~~ poured into reaction tube ~~and~~ into which was added added 3 mL DPPH solution (4 mg/100 mL). ~~And then the solution was~~After incubationed for 15 min in a dark room , the ~~and~~ absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ. 517 nm. The reference standard compound was gallic acid and the results of analysis were expressed as mg gallic acid equivalents (GAE)/g samples that calculated using formula: y=0.146x+1.7896 with R^2=0.9975.

FERRIC REDUCING POWER ANALYSIS

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

α-AMYLASE ENZYME INHIBITION CAPACITY ASSAY

In vitro inhibition of α-amylase enzyme (AA) followed the procedure as described by Widyawati et al. (2020). Each 500 µL of samples, was mixed with starch 1 % (w/v) and sodium acetate buffer pH 5.~~ were mixed~~. ~~Then,~~ Into a~~each~~ 250 µL of the mixture ~~and~~ was added an α-amylase solution (0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in 50 mL of 0.2 M sodium acetate pH 5~~)~~. Mixture was shaken ~~and~~ into which was added 2 mL sodium hydroxide 1M. Before the analysis, this mixture was incubated at 37 ºC for 10 min. Then, the capacity of the α-amylase enzyme to hydrolyze~~d~~ the starch to release glucose was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) ~~that could be analyzed based on absorbance~~ at λ 540 nm. The

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inhibition percentage of α-amylase was assessed using the formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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 test sample with enzyme, Ab is absorbance of test sample without enzyme.

α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

The analysis of the α-glycosidase inhibitor activity (GA) was done by Widyawati et al. (2020) method with slight modification. About 150 µL samples ~~contained~~ containing 100 µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH 7) were reacted with 50 µL α-glycosidase 2 mM (0.0833 unit/mL), and then the mixture was incubated at 37 ºC for 15 min. ~~Finally, the~~The reaction was stopped with the addition of 1000 µL sodium carbonate 0.2 M. The residue of this enzyme hydrolyzed p-nitrophenyl-α-D-glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The inhibition~~or~~ activity of ~~steeping~~the ~~*Pluchea* tea~~ infusion ~~to enzyme~~ was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at λ 405 nm. The inhibition percentage of α-glycosidase was calculated using formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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, Ab is the absorbance of test sample without enzyme.

~~HPLC~~ ANALYSIS OF PHENOLICS

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The phenolic compounds of the samples were analyzed by HPLC based on Kongkiatpaiboona et al. (2018) method with modifications. Each *Pluchea* infusion was sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter syringe (Whatmann, 0.2 µm, NYL). About 20 µL of 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 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (2 pieces) (ID 10 mm x 4.6 mm). ~~Analytical conditions: T~~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 minutes; then 100 % B was maintained for 20 minutes. Next the column was re-equilibrated with 10 % B in A maintained for 10 minutes before analysis of the next sample. The sample flow rate was set at 1.0 ml/min with a controlled temperature at 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 distillated water and prepared similar to the samples before 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 ~~time~~period (B). *Pluchea* leaf blades were

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subjected to 4 steeping temperatures, namely 60 ºC (T1), 70 ºC (T2), 80 ºC (T3), and 95 ºC (T4), and the storage ~~time~~period of 0 year /~~fresh~~ un-stored (B1), and 5 year/stored (B2).~~.~~ ~~The research resulted~~resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic was repeated two ~~time~~periods. The data of samples were analyzed by ANOVA at $\alpha \leq 0.05$, and continued analysis using a paired T test at $\alpha \leq 0.05$. treatment means of specific phenolic compounds that were identified were expressed as the mean ± SD. The analysis used SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

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RESULTS AND DISCUSSIONS

Pluchea leaf infusion is produced by young *Pluchea* leaf from 1-6 level on each branch the shoot (Widyawati et al., 2022), that is steeped at 95 ºC 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 *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols, cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2 g/100 mL steeping *Pluchea* 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 related to the composition of phytochemical compounds in *Pluchea* leaves, such as phenolic acids such as chlorogenic acids, caffeic acids, 3-*O*-caffeoylquinic acids, 4-*O*-caffeoylquinic acids, 5-*O*-caffeoylquinic acids, 3,4-

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di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin; β-carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Chan et al., 2022; Widyawati et al., 2022). Presence of phytochemical compounds in herbal product were 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 of 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). Therefore, this study was conducted to determine the effect of steeping temperature and storage ~~time~~period of *Pluchea* tea on levels of the bioactive compounds, antioxidant and antidiabetic properties and phenolic compound profile.

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BIOACTIVE COMPOUNDS

Phenolics Compounds

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The bioactive compounds are active compounds in plants that are essential to protect a 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).

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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 based on paired ~~T~~ t- test at $\alpha \leq 0.05$ (Figure 1a). Steeped and stored infusion had significantly higher amounts of phenolic compounds thant ~~t~~ the samples that were steeped and un-stored. Further, the highest total phenolic content was observed in samples infused at 95 ºC and stored for 5 years (at 71.38±4.14 mg GAE/g sample~~s~~) while the lowest was measured in the un-stored samples and infused at 60 ºC (at mg GAE/g sample). Phenolic content of stored samples ~~that were infused at different temperatures~~ that ~~then stored~~ were steeped only at 60 and 95 ºC ~~also~~ 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 the phenolic compounds of the infusions. Results also indicated that phenolic compounds were generally greater in the infusion at high steeping temperatures and long storage period .~~(Figure 1a). This could have been due to that 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 sleeping temperature also caused the greater the extracted total phenolic content. The temperature of infusion influenced total phenolic content, it could relate to~~ This could have been due tp the fact that the steeping temperature and storage period can 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~~increasing contact~~ exposure~~between~~of phenolic compounds to water (Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al.

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(2022). . Su et al. (2019) reported that temperature treatment can stimulate the release of phenolic compounds of lychee juice stored at different temperatures of 4 and 45 ºC and different long storage (fresh and 72 hours).

. this compounds and water. The same phenomena also occurred in Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al. (2022).

This occurrence showed that steeping temperature and storage period caused the process of degradation and oxidation of phenolic 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 ºC and different long storage (fresh and 72 hours). Hydrogen bonding is affected by Ttemperature treatment because thedegrades (or hdrolyzes) the hydrogen bond between phenolic compounds and proteins can be degraded that the measured levels resulting in an increase of phenolic compounds when exposed toare higher temperatures. The phenomena were supported by (Ali et al. (2018); Jayani et al. (2022), and 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. Besides that, 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 that the phenolic compounds in *Pluchea* infusion are degraded due to oxidation and hydrolysis because of temperature and storage timeperiod and can be easily extracted during

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steeping, thusresulting in the increased amount of ing thethe phenolic content compounds as the at higher steeping temperature and longer storage increaseperiod.

Based on using of a reference standard could be informed thatSimple phenolic compounds identified in steeped and stored ing *Pluchea* leaf infusion, includeing 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 was showed in Table 1. The treatment effectsresults of statistical analysis using a paired T test at $\alpha \leq 0.05$ showed that gallic acid and kaempferol contents of *Pluchea* infusion were insignificantly different at various steeping temperature and long storage periods. Nevertheless, the The concentration of quercetin and 3,5-di-O-caffeoylquinic acid of the un-stored and stored *Pluchea* infusion was significantly different from the rest of the samples between of two treatments except at 70 ºC. The while (+)-catechin concentration of *Pluchea* infusion was only significantly different at 95 ºC, but Tthe myricetin content was significantly different different concentration at 80 and 95 ºC. The 3,4-di-O-caffeoylquinic acid content showed significace difference at 60, 80 and 95 ºC and while 4,5-O-dicaffeoylquinic acid compounds content from *Pluchea* infusion werewas only significantly different at 60 ºC, however the concentration of 3,4-dicaffeoylquinic acid was also significantly different at 80 and 95 ºC.

Based on the analysis of concentration ofResults further showed simple phenolic compounds showed that gallic acids and kaempferol were relatively stable phenolic acid because ofas reflected by the insignificant changes when exposed no changes atto the different steeping temperature and storage timeperiod. 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.

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~~However, myricetin~~Myricetin, (+)-catechin and 3,4-di-*O*-caffeoylquinic acid showed a drastic ~~increasing~~ increase at higher steeping temperature and longer storage period ~~.~~implying ~~It's meant~~ that these compounds tended to be relatively labile. Quercetin, 3,5-di-*O*-cafffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid underwent moderate changes ~~compared to the other two groups of phenolic acids~~.~~.~~ T~~t~~herefore, myricetin, (+)-catechin and 3,4-di-*O*-caffeoylquinic acid were easier to dissolve or degraded to form simple phenolic compounds at higher steeping temperature and storage ~~time~~period. ~~can cause macromolecules of three phenolic acids in herbal tea convenient degradable to form simple phenolic compounds for storage, as explained by (~~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.

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 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 α= 0.05 showed that total flavonoid content of *Pluchea* infusion was significantly different between ~~two treatments~~the steeped un-stored and steeped stored samples

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(Figure 1b). The highest total flavonoid content was exhibited by ~~fresh~~ the un-stored samples steeped at 95 ºC at about 147.42±14.03 mg CE/g sample~~s~~. Total flavonoid content was significantly lower in the stored samples ~~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.

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Tannin Content (TTC)

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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 the samples infused at 60 ºC at about 4.81±0.58 to 17.42±1.04 mg TAE/g samples. ~~–~~which ~~is~~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 ºC 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 ºC about 9.22 ± 1.48 mg TAE/g samples. i~~I~~ndicating that the tannin content was primarily affected by ~~both high steeping temperature and~~ long storage period than high steeping temperature~~and that the presence of high tannin content was primarily brought about by long storage period. Kowalska et al. (2021) informed that T~~t~~he condensation of catechins to tannins ~~of polyphenolic compounds~~ is a dominant process ~~occurred~~ occurring in tea leaves that is accelerated during maceration of raw ~~material~~tea leaves (Kowalska et al~~.~~

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(2021) could have had contributed to the observed increase in the tannin content in the treated samples. However, the high temperature can degrade polyphenolic compounds to form simple phenolic 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 concentration 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,

Although, high temperature and long storage period can cause the degradation of tannins to catechins, Rusita et al. (2019) emphasized that tannins are a polar thermostable complex compounds, that is are resistant to heating, indicating that even with the exposure to high temperature, the tannins still remained high in the treated samples.as a result the tannin content in *Pluchea* tea increases with increasing steeping temperature and storage timeperiod, this is caused tannins are thermostable complex compounds.

ANTIOXIDANT ACTIVITY

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Antioxidant activity is capability of compounds to inhibit the oxidation of macromolecules from biological target that involve 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 antioxidant that have antioxidant

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

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DPPH Free Radical Scavengincg Asctivity

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DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate antioxidant activity because this method is simple that is suitable to measure the donating hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of DPPH to change to yellow color (Munteanu and Apetrei, 2021; Baliyan et al., 2022). Figure 2a shows that the free radical scavenging property of the stored and steeped samples were significantly higher than the un-stored steeped samples.~~The result of DPPH assay~~ It can also be observed ~~indicates~~ that the free radical scavenging property ~~DPPH values accrued~~ was significantly different among the stored and steeped samples but insignificant among the un-stored and steeped samples.~~at higher steeping temperature and longer storage time~~period.~~Statistical analysis by ANOVA using a paired T test at $\alpha \leq 0.05$ 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~~. *Pluchea* infusion stored at

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room temperature for 5 years resulted in the high ~~DPPH~~ free radical scavenging activity by~~.~~ more than 100 %~~. S~~teeping ~~at higher~~ temperatures significantly increased the ~~DPPH~~ free radical scavenging activity in stored *Pluchea* infusion by around 15 to 25 %. ~~Steeping at 80-95 °C in stored *Pluchea* infusion insignificantly affected the free radical scavenging property of the bioactive compounds (Figure 2a).~~ This implies that that the higher free radical scavenging property was primarily affected by the storage period than steeping temperature. During the storage process it is possible to form complex phenolic compounds which provide a high ability to scavenge ~~DPPH~~ free radicals (Thanajiruschaya et al., 2010)

~~Scavenging~~ The scavenging activity ~~of DPPH free radicals~~ of the the samples was strongly and positively correlated ~~with total~~ with total phenolic and tannin contents~~levels~~, but inversely ~~to~~ with total flavonoid levels. ~~Based on Pearson correlation at Table 2, the correlated coefficient values (r) between DPPH and TPC, TTC and TFC were 0.993, 0.942, and -0.940, respectively. During the storage process it is possible to form complex phenolic compounds which provide a high ability to scavenge DPPH free radicals (Thanajiruschaya et al., 2010)~~. This ~~research~~ study also demonstrated that longer storage ~~time~~period and higher infusion temperature produced many simple phenolic compounds with free hydroxyl groups capable to donor hydrogen atom to DPPH free radical. Many phenolic acids, such as gallic acids, (+)-catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 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 donor hydrogen atom depends on chemical structure, number and position of hydroxyl groups attached to a

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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 %

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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 donor hydrogen atom is determined by O-H bond dissociation energy.

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

Ferric Reducing Antioxidant Power (FRAP)

FRAP is an analysis of antioxidant power of the phytochemical compounds ~~based on the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a color complex, that can be measured at λ 700 nm (Fu et al., 2011; Al-Temimi and Choudhary, 2013). The principle of the assay measures~~ that is based of the ability of antioxidant compounds to reduce iron ions of potassium ferrocyanide (Fe^{3+}) to be 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 ferric reducing antioxidant power (FRAP) increased ~~with~~ at higher steeping temperature and longer storage ~~time~~period. The lowest FRAP was observed in the un-stored samples which was steeped at 60 ^{o}C at 3.95 ± 0.17 mg gallic acid equivalents (GAE)/g samples, and the highest was ~~owned~~ exhibited ~~by~~ in *Pluchea*

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infusion which was stored for 5 years at 95 ºC at 48.63 ±10.83 mg gallic acid equivalents (GAE)/g samples (Figure 2b). FRAP increased significantly as steeping temperature was increased. FRAP of the samples stored for 5 years was also significantly higher than the un-stored samples at $\alpha \leq 0.05$. ~~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) between FRAP and DPPH, TPC, TTC and TFC were 0.956, 0.953, 0.948 and -0.826, respectively~~.

This ~~case was~~ is in contrast ~~to~~ with the study on the antioxidant activity of DPPH and FRAP ~~on~~ of matcha., ~~because The~~the longer storage ~~time~~period reduces the levels of catechin content ~~due to the catechins,~~ such as epigallocatechin gallat (EGCG), epicatechin gallat (ECG), epigallocatechin (EGC), and epicatechin (EC) which are bioactive compounds that have high antioxidant activity (Kim et al. 2020)., ~~and also the case of the effect of temperature and storage time in betel (*Piper bettle* 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). Thanajiruschaya et al. (2010) revealed that the antioxidant activity of rice stored at high temperatures is greater than that stored at low temperatures.~~ The ferric reducing capability of ***Pluchea*** could have due ~~infusion corresponded~~to the presence ~~to~~ of simple phenolic acid ~~values~~ that have the ability to transfer electron from their free hydroxyl groups of~~, presence of them in samples could accrue antioxidant activity because of ability of the electron transfer from free hydroxyl groups of phenolic acids~~.

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The FRAP of *Pluchea* infusion was strongly and positively significant correlated with the DPPH, TPC and TTC, but inversely to TFC.

ANTIDIABETIC ACTIVITY

α-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 2-degestive enzymes i.e., α-amylase and α-glucosidase, which are involved 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 α-amylase and α-glucosidase enzymes (Martinez-Solis et al., 2022) resulting in the reduced activity of the enzymes. The results showed~~,~~ that ~~the~~ lower steeping ***Pluchea*** leaf infusion was able to inhibit the action of the α-amylase enzymes (Figure 3a). The ***Pluchea*** infusion ~~had very good activity,~~exhibited a good α-amylase enzyme inhibition activity of more than 50 % and even almost 100 % ~~for fresh~~in the un-stored ***Pluchea*** infusion ~~which~~ steeped ~~was brewed~~ at 60, 70 and 80 ºC with highest at 60 ºC, and in stored *Pluchea* leaf infusion which was steeped at 60 ºC. ~~Whereas~~ The stored ~~fresh~~ ***Pluchea*** leaf infusion steeped at 70, 80 and 95 ºC for 5 minutes had lower enzyme inhibition activity~~an activity of~~ of ~~less than 50 % with lowest at~~ 95 ºC.~~inhibiting the α-amylase enzyme of less than 50 %, which was equal to 40.08±1.12~~ %. Widyawati et al. (2017) ~~detected~~ found that the ability to inhibit the α-amylase enzyme ~~from~~ in ~~fresh~~ un-stored ***Pluchea*** infusion steeped at 95 ºC for 5 minutes ~~by~~ was also low at 28.79 %. Increasing the steeping temperature and storage ~~time~~period reduced the ability ~~to~~ of the phytochemicals in the *Pluchea* infusions to inhibit the α-amylase enzyme activity. ~~The~~

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~~results of the analysis based on a paired T test at α ≤ 0.05 showed, that the steeping temperature and storage time~~period ~~had a significant effect on the ability to inhibit the α-amylase enzyme~~. ~~Based on Pearson correlation, the~~Table 2 further shows that the AA of *Pluchea* infusion was strongly and negatively significant correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively significant correlated with TFC. ~~The correlated coefficient values (r) between AA and TPC, TTC, DPPH, FRAP and TFC were -0.708, -0.857, -0.696, -0.806 and 0.429, respectively.~~

This inhibitory activity was thought to be contributed by other bioactive compounds, besides phenolics which are sensitive to steeping temperature and storage ~~time~~period. Li et al. (2018) stated that there are flavonoid compounds that contribute to the ability to inhibit the α-amylase enzyme. ~~Flavonoid compounds with a hydroxyl structure at C-4' in ring B are more effective than C-6 in ring A.~~ Akah et al. (2011) ~~informed~~ reported that the phytochemical compounds, such as terpenoids, saponins, flavonoids, glycosides and carbohydrate, and alkaloids are good antidiabetic metabolites or α-amylase enzyme activity inhibitor. Sangeetha and Vedasree (2012) explained, that the ability to inhibit the α-amylase enzyme was determined by the content of the phenolic compound and protein. The α-amylase inhibitor enzyme present in *Pluchea* infusion may be proteinaceous in nature. Aleixandre et al. (2022) informed that phenolic acids have inhibition activity to α-amylase enzyme depending their structures. Besides that, capability of phenolic acids to inhibit α-amylase enzyme was determined by low half-maximum inhibitory concentration (IC_{50}). There are C=C double bond conjugated with a carbonyl group of phenolic structures that stabilizes the binding forces to the active site of the α-amylase. The hydroxyl groups of them are able to bind by non-covalent interaction, such as hydrogen

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binding, cation-π interactions, salt bridge interactions, ionic interactions or electrostatic forces with amino acid residue at the active site in α-amylase enzyme. Elevated steeping temperature and longer storage period ~~The steeping temperature and storage time~~ can easily cause the removal of the ~~e~~ hydroxyl groups of phenolic compounds that can reduce their ~~ir~~ ability of enzyme inhibition. The phenolic acids with a greater number of hydroxyl groups ~~are~~ exhibits stronger capability~~le~~ to obstruct the α-amylase enzyme.

α-Glucosidase enzyme inhibition activity (GA)

Alpha~~α~~-glucosidase is an important enzyme in carbohydrates digestion, that catalysis the hydrolysis of 1,4-α-bonds of the unabsorbed oligo- and disaccharides, and converts them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et al., 2014; Proenca et al., 2017). The ability of bioactive compounds to inhibit the α-glucosidase enzyme is used to determine their antidiabetic~~s~~ 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 brewing fresh *Pluchea* infusion at 95 ºC for 5 minutes has an inhibitory effect on the α-glucosidase enzyme of 67.857 %.

~~The results showed,~~Figure 3b shows that the ability of the Pluchea leaf infusion to inhibit the α-glucosidase enzyme decreased with increasing steeping temperature and storage ~~time~~period. Steeping at 95 ºC ~~for fresh~~of the un-stored *Pluchea* leaf infusion ~~(un-stored)~~ obtained the lowest inhibitory ability, i.e., 48.32 ± 1.27 %, and the highest inhibitory activity was f~~ound~~ at 70 ºC ~~steeping temperature for fresh *Pluchea* infusion, which was~~at 95.11 ± 0.70%. ~~(Figure 3b).~~ The results of a paired T test showed that GA of *Pluchea* infusion was significantly different ~~at both~~between steeping temperature and long storage. ~~The antidiabetic activity of *Pluchea* infusion~~Figure 3 further ~~showed~~ shows that the ability

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of *Pulchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-amylase enzyme. Li et al. (2018) informed that flavonoid compounds have the ability to inhibit the action of the α-amylase and α-glucosidase enzymes. This is due to the total flavonoids in steeped *Pluchea* infusion which tended to have the same pattern as the ability to inhibit the activity of the α-amylase and α-glucosidase enzymes. The statistical analysis using Pearson correlation showed that GA of *Pluchea* infusion was strongly and negatively correlated with TPC, TTC, DPPH and FRAP ~~, with r was -0.555, -0,715, -0.527 and -0.560, respectively.~~ However, GA was moderately and positively correlated to TFC~~, with r was 0.350 and strongly~~ and positively correlated to AA~~, with r was 0.725~~. Flavonoid compounds, such as rutin, myricetin, kaempferol, and quercetin ~~which~~ 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 and the number of double bonds in rings A and B and the heterocyclic ring in ring C. The ability to inhibit the α-glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping temperature and long storage. The capability of *Pluchea* infusion to obstruct the α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of two enzymes was different, according to the opinion of McCue et al. (2005). Widyawati et al. (2017) informed that phenolic and non-phenolic compounds determine the inhibitory activity of the α-glucosidase enzyme. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, that may be occurred in *Pluchea* infusion during storage, affects the structure and profile of phenolic and non-phenolic compounds. Asriningtyas et al. (2014) claimed

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that *Pluchea* leaves contain 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. Quinic acid is methyl esterified with the number of caffeic groups in the molecule that determines the activity of inhibiting the α-glucosidase enzyme. Analysis of caffeoylquinic acids in *Pluchea* infusion was obtained that the higher steeping temperature and long storage caused increased concentration of them, but the α-glucosidase inhibition activity of them was reduced. Aleixandre et al. (2022) reported that the simple phenolic acids forming a dipole-dipole interaction of active site from α-glucosidase enzyme are effectively inhibiting the enzyme.

This study ~~was obtained information~~showed that the increasing of steeping temperature and storage ~~time~~period caused a degradation reaction 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, supported the results of total phenolic content and total tannin content assays. Increased concentration of simple phenolic compounds determined the ability of these compounds as antioxidant agents, but reduced their capability as antidiabetic agents.

CONCLUSION

The steeping temperature and storage ~~time~~ period of *Pluchea* infusion significantly influenced bioactive contents, antioxidant and antidiabetic activities. TPC, TTC, and TFC were significantly different at various steeping temperature and storage period based on statistical analysis using a paired ~~T~~ t-test at $\alpha \leq 0.05$. ~~There was the difference of t~~The

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1) 'Figure 3b shows that the ability of the Pluchea leaf infusion to inhibit the α-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 α-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 α-glucosidase enzyme was greater than the α-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 α-amylase and α-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 α-glucosidase enzyme. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. (May also be incorporated in 1).

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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

phenolic compound profile in ~~fresh~~ the unstored and stored ~~of~~ *Pluchea* infusion ~~and~~ at various steeping temperature~~.~~ ~~The~~ included simple phenolic compounds ~~were detected in *Pluchea* infusion including~~such as gallic acid, (+)-catechin, quercetin, myricetin, kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoilquinic acid, and 4,5-di-*O*-caffeoilquinic acid. The results of statistical analysis using a paired ~~T~~ t-test at $\alpha \leq 0.05$ showed that gallic acid and kaempferol of *Pluchea* infusion were insignificantly different at various steeping temperature and long storage. ~~Nevertheless, T~~the concentration of quercetin and 3,5-dicaffeoylquinic acid of *Pluchea* infusion was significantly different of two treatments except at 70 ^{o}C. The (+)-catechin concentration of *Pluchea* infusion was significantly different at 95 ^{o}C, but the myricetin was different concentration at 80 and 95 ^{o}C. The 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid compounds from *Pluchea* infusion were significantly different at 60 ^{o}C, however the concentration of 3,4-dicaffeoylquinic acid was also significantly different at 80 and 95 ^{o}C. TPC, TTC and TFC of *Pluchea* infusion were significantly different at various steeping temperature and storage period. TPC and TTC significantly increased with increasing steeping temperature and long storage, but TFC significantly increased at various steeping temperature and significantly decreased at long storage. The bioactive compounds of *Pluchea* infusion influenced antioxidant activities (DPPH and FRAP) and antidiabetic activity (AA and GA). The DPPH was strongly and positively correlated with TPC and TTC, but it was strongly and negatively correlated with TFC, with coefficient r 0.993, 0.942, and -0.940, respectively. The correlated pattern between FRAP and bioactive contents of *Pluchea* infusion was similar to it between DPPH and bioactive contents. The correlated coefficient values (r) between FRAP and TPC, TTC and TFC were 0.953, 0.948

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and -0.826, respectively. The AA and GA were strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively significant correlated with TFC. Between the antioxidant activity of DPPH and FRAP and the antidiabetic activity of AA and GA of Pluchea infusion were strongly and positively correlated with correlation coefficient (r) values of 0.956 and 0.725, respectively.

DATA AVAILABILITY

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

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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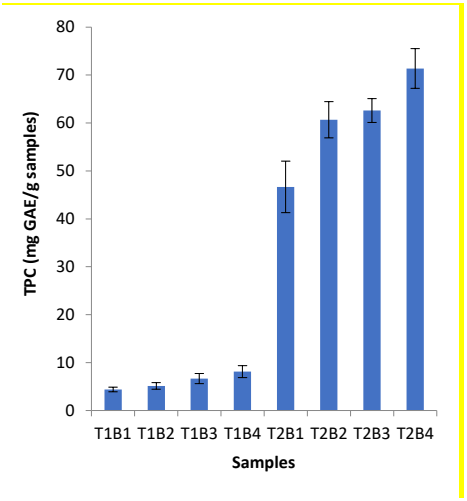

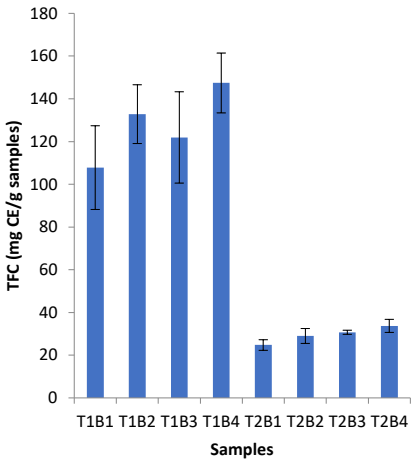

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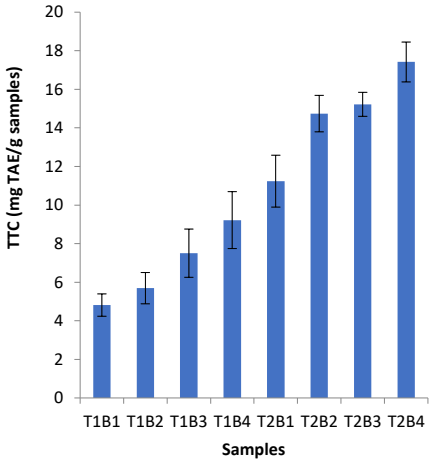

(c)

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 ±standard deviation (n=6). Samples : T1B1-steeped at 60 ºC, un-stored; T1B2-steeped at 70 ºC, un-stored; T1B3-steeped at 80 ºC, un-

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stored; T1B4-steeped at 95 ºC, un-stored; T2B1-steeped at 60 ºC, stored for 5 years; T3B2-steeped at 70 ºC, stored for 5 years; T3B3-steeped at 80 ºC, stored for 5 years; T3B4-steeped at 95 ºC, 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 $\alpha \leq 0.05$.

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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	-1.1735±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

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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

Note : Data were expressed as mean ±standard deviation (n=2). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}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 $\alpha \leq 0.05$. * $\alpha \leq 0.05$.

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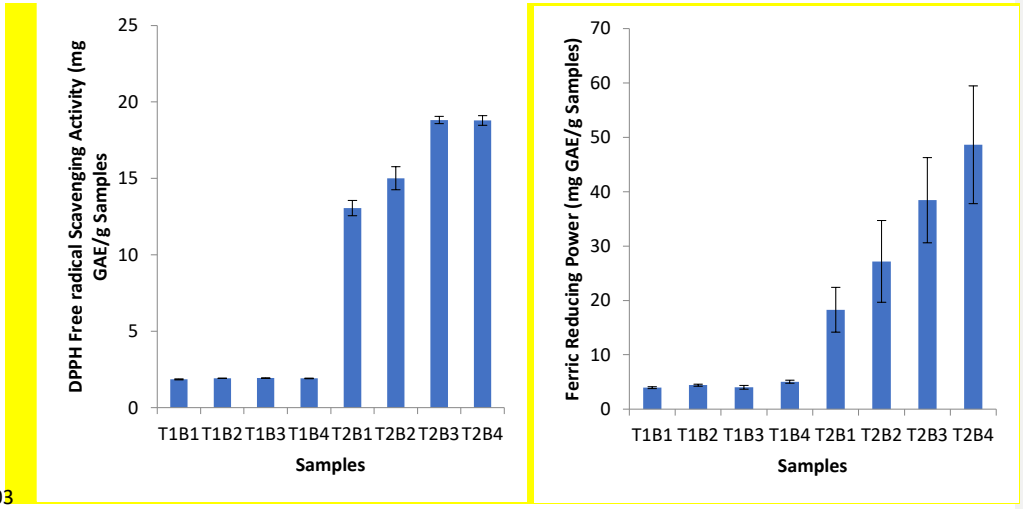

(a) (b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage ~~time~~period (a) DPPH (b) FRAP. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ºC, un-stored; T1B2-steeped at 70 ºC, un-stored; T1B3-steeped at 80 ºC, un-stored; T1B4-steeped at 95 ºC, un-stored; T2B1-steeped at 60 ºC, stored for 5 years; T3B2-steeped at 70 ºC, stored for 5 years; T3B3-steeped at 80 ºC, stored for 5 years; T3B4-steeped at 95 ºC, 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 $\alpha \leq 0.05$~~

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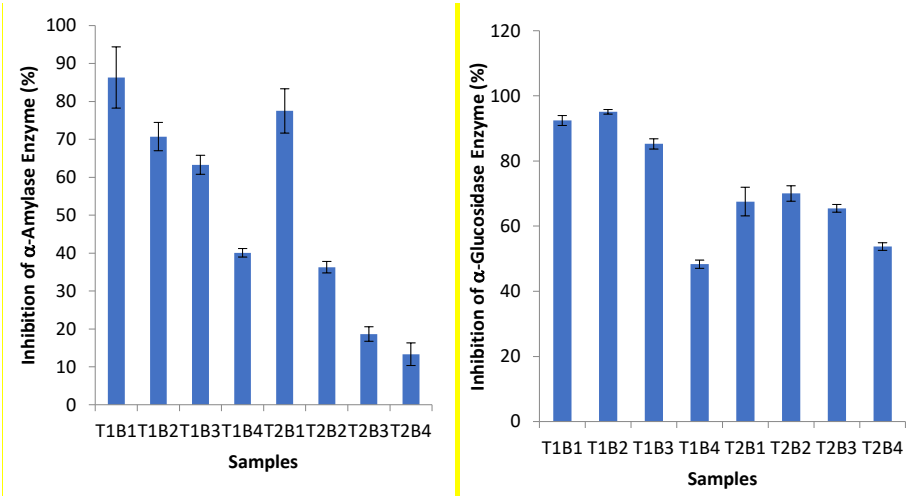

(a) (b)

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage ~~time~~period (a) α-amylase (b) α-glucosidase. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}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 $\alpha \leq 0.05$

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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

Note: *Correlation Ssignificant at the 0.05 level (2-tailed)

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

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Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea indica* Less, storage ~~time~~period

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ABSTRACT

This study was done to determine the effects of steeping temperature and storage ~~time~~period on the bioactive contents, 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 ~~time~~period (B). The ~~variety of the~~ *Pluchea* leaf blades were exposed to 4 steeping temperatures ~~included~~ of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) ~~(~~ºC~~)~~ with the storage ~~time~~period~~–~~period of 0 (B1) and 5 (B2) ~~(~~year~~)~~. ~~The research resulted~~resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). Statistical analysis using a paired t~~-T~~ test at $\alpha \leq 0.05$ showed that treatments significantly affected ~~influenced~~ the bioactive contents (total phenol (TPC), total tannin (TTC), total flavonoid (TFC)), antioxidant [(DPPH scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)]~~)~~ potential ~~–~~and antidiabetic [(α-amylase (AA) and α-glucosidase (GA) ~~inhibitors~~inhibition)] ~~activities~~ properties of the *Pluchea* leaf infusion~~samples~~. TFC decreased during storage period but significantly increased at higher steeping temperature. ~~The AA and GA of *Pluchea* infusion increased until 70 ºC of the steeping temperature, but deceased until 95 ºC. The bioactive contents influenced antioxidant and antidiabetic activities. TFC was decreased for storage time and significant increased at higher steeping temperature. The AA and GA of *Pluchea* infusion increased until 70 ºC of the steeping temperature, but deceased until 95 ºC.~~ The AA ~~–~~and GA were strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively correlated with TFC. ~~Between~~ The antioxidant activity ~~of DPPH and FRAP~~ and the antidiabetic activity ~~of AA and GA~~ of Pluchea infusion were strongly and positively correlated. ~~with correlation coefficient (r) values of 0.956 and~~

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0.725, respectively. The treatments gave different effect of simple phenolic compounds derived from *Pluchea* leaf infusion at different steeping temperatures and storage included, such as 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. of *Pluchea* infusion at different steeping temperature and long storage. To obtain high antioxidant activity, *Pluchea* infusion selected was stored and steeped at high temperature, however high antidiabetic activity obtained was fresh *Pluchea* infusion and steeped at low temperature.

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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 an herbal plant that has been widely used for traditional medicine and food (Chan et al., 2022). *Pluchea* leaves are composed 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, β-carotene, and vitamin C, whereas bioactive compounds is 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, β-carotene, and total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et al., 2022, Chan et al., 2022).

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Steeping process of *Pluchea* leaves can be performed with fresh or dry leaves ~~infusion by~~in hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et al., 2020; Jayani et al., 2022). In Asia~~n area~~, especially in Indonesia~~n~~, people usually consume the *Pluchea* infusion ~~with brewing of~~by steeping 2 g of powdered *Pluchea* leaves in tea bag ~~by~~in 100 mL of hot ~~water~~ or boiling water. ~~Each tea bag contained 2 g of *Pluchea* leaf powder is steeped with 100 mL hot water or boiling water.~~ Widyawati et al. (2016) claimed that steeping of 2 g of *Pluchea* leaf powder at 95 ^{o}C for 5 minutes ~~results~~ rexhibits total phenolic ~~content,~~and total flavonoid content~~s~~, the ability to scavenge DPPH free radicals, and the capability ~~of~~ to reduce ferric ions at 9.3 mg gallic acid equivalent (GAE)/g sample~~s~~, 22.0 mg gallic acid equivalent (GAE)/g sample~~s~~, 27.2 mg gallic acid equivalent (GAE)/g sample~~s~~, and 10.2 mg gallic acid equivalent (GAE)/g sample~~s~~, respectively. Werdani and Widyawati (2018) reported that drinking of *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 ^{o}C for 5 minutes certainly determines the stability and amount of extracted bioactive compounds~~,~~ that influences the biological activity~~,~~ especially antioxidant and antidiabetic activities. Silva-Ramirez et al. (2020) reported that the infusion process can influence the~~ir~~ content and composition of the bioactive compounds and antioxidant activity of tea. Acar et al. (2022) informed that infusion quality of herbal tea extract depends on several factors, i.e., ~~time~~ storage and temperature. Polyphenol profile and antioxidant properties of herbal tea infusion decline with an increase in steeping/brewing and storage temperatures, and longer exposure ~~time~~period~~s~~.

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Several studies have mentioned the effect of steeping temperature ~~to~~ on the bioactive compound contents and antioxidant activity, such as some white and green teas are effective with hot water at 90 ºC for 7 min (Castiglioni et al., 2015), on roseship tea is effective~~ly~~ at infusion ~~time~~period around 6-8 min at temperatures of 84-86 ºC (Ilyasoglu and Arpa, 2017), on the caffeine content extracted ~~the coffee~~at the brewing temperature of coffee~~influences the caffeine content extracted~~ (Zarwinda and Sartika, 2018), and ~~the steeping~~ the high total phenol content and antioxidant activity of dark tea at 92 ºC for 27 min ~~results the highest total phenol content and antioxidant activity~~ (Wang et al., 2022). The study of the effect of steeping temperature to ***Pluchea*** infusion was carried out to afford information about the most efficient preparation of powdered ***Pluchea*** leaves ~~most efficiently~~ to get higher ~~the~~ bioactive compounds, antioxidant and antidiabetic activities.

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

Therefore, this research studied the effect of steeping temperature and storage ~~time~~period on the bioactive compounds [(total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging

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activity (DPPH), ferric reducing antioxidant power (FRAP)] and antidiabetic activities [(α-amylase (AA) and α-glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea* leaves.~~- 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 antioxidant power (FRAP), α-amylase (AA) and α-glycosidase (GA) inhibition activities,~~ 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 *Asteraceae* family with specification according to the GBIF taxon ID number database:3132728 (Ferraris, 2023). *Pluchea* leaves at 1-6 level of each branch ~~of~~from the shoot were collected, sorted, washed and dried to ~~get a~~moisture content of around 11.16 ± 0.09 % dry bas~~is~~ (Widyawati et al., 2022). The ~~powdering of~~ dried *Pluchea* leaves was ~~done~~ pulverized to ~~get~~ a 45-mesh size powder. ~~And then, the heating of T~~the *Pluchea* leaf powder was ~~done using a drying~~dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 °C for 10 min to reduce microbial organisms. ~~and~~ Then, 2 g of the powder were packed ~~using~~ into a paper filter infusion bag.~~ that made from paper filter around 2 g/bag~~. ~~And then all of samples called~~Packed samples were ~~*Pluchea* herbal tea was~~ stored for 0 (un-stored) and 5 (stored)years in standing pouch before analysis.

In the research, the one tea bag of *Pluchea* herbal tea that stored 0 (B1) and 5 (B2) year, was steeped with 100 mL hot water at various temperatures, including 60 (T1),

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70 (T2), 80 (T3), and 95 (T4) ºC for 5 min with infusion method ~~that obtained~~obtaining 8 treatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2. After the temperature of *Pluchea* infusion similar to ambient temperature was analyzed further.

REAGENTS

The ~~compounds~~reagents used ~~to analyze~~in the analyses ~~including~~ include 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid, α-amylase, α-glucosidase, pNPG (p-nitrophenyl-α-glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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 distillated water which was purchased from PT Aqua Industry Surabaya.

METHODOLOGY

ANALYSIS OF THE BIOACTIVE COMPOUNDS

TOTAL PHENOLIC CONTENT ANALYSIS

Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the technique by Gao et al. (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. And

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then 2 mL Na_2CO_3 7.5 % was ~~entered~~ added and filled up to 10 mL volume with distilled water.~~and distillated water was added until 10 mL volume~~. The color intensity of solution was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at λ 760 nm with gallic acid as the reference standard. The total phenolic content was calculated using the formula: y=0.00009x+0.008 with R^2=0.9941. The results were expressed as mg gallic acid equivalent (GAE)/g samples.

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TOTAL FLAVONOID CONTENT ASSAY

Total flavonoid content (TFC) of the samples was measured based on the reaction between $AlCl_3$ and $NaNO_2$ with ~~an~~ the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim et al., 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted in a yellow solution. About 30 µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5 % in 10 mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 10 % for 5 min. And then, 2 mL NaOH 1 M and distillated water were added until 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 λ 510 nm with (+)-catechin as the reference standard compound, and the results were expressed as mg catechin equivalents (CE)/g samples using the formula: y=0.00008x-0.0023 with R^2= 0.9980.

TOTAL TANNIN CONTENT ANALYSIS

Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method (Chandran and Indira, 2016). Approximately 10 µL *Pluchea* infusion was added with 1 mL

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Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flash and incubated for 5 min. Then, the mixture was added with 2 mL Na_2CO_3 7.5 % and filled up to 10 mL volume with distillated water. ~~was added until 10 mL volume~~. The blue dark color solution ~~that~~ was measured in UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at λ 760 nm with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/g samples used the formula: y=0.00009x+0.0021 with R^2=0.9993

ANALYSIS OF THE ANTIOXIDANT POTENTIAL

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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 ~~antioxidant activity of~~in the *Pluchea* leaf infusion to ~~donor~~ donate hydrogen atom to the nitrogen atom in DPPH resulting in the formation of –DPPH-H compound ~~with~~ exhibiting a yellow-colored solution. About 25 µL *Pluchea* leaf infusion was ~~entered~~ poured into reaction tube ~~and~~ into which was added added 3 mL DPPH solution (4 mg/100 mL). ~~And then the solution was~~After incubationed for 15 min in a dark room , the ~~and~~ absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ. 517 nm. The reference standard compound was gallic acid and the results of analysis were expressed as mg gallic acid equivalents (GAE)/g samples that calculated using formula: y=0.146x+1.7896 with R^2=0.9975.

FERRIC REDUCING POWER ANALYSIS

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

α-AMYLASE ENZYME INHIBITION CAPACITY ASSAY

In vitro inhibition of α-amylase enzyme (AA) followed the procedure as described by Widyawati et al. (2020). Each 500 µL of samples, was mixed with starch 1 % (w/v) and sodium acetate buffer pH 5.~~ were mixed~~. ~~Then,~~ Into a~~each~~ 250 µL of the mixture ~~and~~ was added an α-amylase solution (0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in 50 mL of 0.2 M sodium acetate pH 5~~)~~. Mixture was shaken ~~and~~ into which was added 2 mL sodium hydroxide 1M. Before the analysis, this mixture was incubated at 37 ºC for 10 min. Then, the capacity of the α-amylase enzyme to hydrolyze~~d~~ the starch to release glucose was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) ~~that could be analyzed based on absorbance~~ at λ 540 nm. The

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inhibition percentage of α-amylase was assessed using the formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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 test sample with enzyme, Ab is absorbance of test sample without enzyme.

α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

The analysis of the α-glycosidase inhibitor activity (GA) was done by Widyawati et al. (2020) method with slight modification. About 150 µL samples ~~contained~~ containing 100 µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH 7) were reacted with 50 µL α-glycosidase 2 mM (0.0833 unit/mL), and then the mixture was incubated at 37 ºC for 15 min. ~~Finally, the~~The reaction was stopped with the addition of 1000 µL sodium carbonate 0.2 M. The residue of this enzyme hydrolyzed p-nitrophenyl-α-D-glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The inhibition~~or~~ activity of ~~steeping~~the ~~*Pluchea* tea~~ infusion ~~to enzyme~~ was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at λ 405 nm. The inhibition percentage of α-glycosidase was calculated using formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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, Ab is the absorbance of test sample without enzyme.

~~HPLC~~ ANALYSIS OF PHENOLICS

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The phenolic compounds of the samples were analyzed by HPLC based on Kongkiatpaiboona et al. (2018) method with modifications. Each *Pluchea* infusion was sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter syringe (Whatmann, 0.2 µm, NYL). About 20 µL of 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 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (2 pieces) (ID 10 mm x 4.6 mm). ~~Analytical conditions: T~~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 minutes; then 100 % B was maintained for 20 minutes. Next the column was re-equilibrated with 10 % B in A maintained for 10 minutes before analysis of the next sample. The sample flow rate was set at 1.0 ml/min with a controlled temperature at 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 distillated water and prepared similar to the samples before 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 ~~time~~period (B). *Pluchea* leaf blades were

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subjected to 4 steeping temperatures, namely 60 ºC (T1), 70 ºC (T2), 80 ºC (T3), and 95 ºC (T4), and the storage ~~time~~period of 0 year /~~fresh~~ un-stored (B1), and 5 year/stored (B2).~~.~~ ~~The research resulted~~resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic was repeated two ~~time~~periods. The data of samples were analyzed by ANOVA at $\alpha \leq 0.05$, and continued analysis using a paired T test at $\alpha \leq 0.05$. treatment means of specific phenolic compounds that were identified were expressed as the mean ± SD. The analysis used SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

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RESULTS AND DISCUSSIONS

Pluchea leaf infusion is produced by young *Pluchea* leaf from 1-6 level on each branch the shoot (Widyawati et al., 2022), that is steeped at 95 ºC 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 *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols, cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2 g/100 mL steeping *Pluchea* 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 related to the composition of phytochemical compounds in *Pluchea* leaves, such as phenolic acids such as chlorogenic acids, caffeic acids, 3-*O*-caffeoylquinic acids, 4-*O*-caffeoylquinic acids, 5-*O*-caffeoylquinic acids, 3,4-

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di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin; β-carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Chan et al., 2022; Widyawati et al., 2022). Presence of phytochemical compounds in herbal product were 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 of 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). Therefore, this study was conducted to determine the effect of steeping temperature and storage ~~time~~period of *Pluchea* tea on levels of the bioactive compounds, antioxidant and antidiabetic properties and phenolic compound profile.

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BIOACTIVE COMPOUNDS

Phenolics Compounds

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The bioactive compounds are active compounds in plants that are essential to protect a 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).

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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 based on paired ~~T~~ t- test at $\alpha \leq 0.05$ (Figure 1a). Steeped and stored infusion had significantly higher amounts of phenolic compounds thant ~~t~~ the samples that were steeped and un-stored. Further, the highest total phenolic content was observed in samples infused at 95 ºC and stored for 5 years (at 71.38±4.14 mg GAE/g sample~~s~~) while the lowest was measured in the un-stored samples and infused at 60 ºC (at mg GAE/g sample). Phenolic content of stored samples ~~that were infused at different temperatures~~ that ~~then stored~~ were steeped only at 60 and 95 ºC ~~also~~ 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 the phenolic compounds of the infusions. Results also indicated that phenolic compounds were generally greater in the infusion at high steeping temperatures and long storage period .~~(Figure 1a). This could have been due to that 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 sleeping temperature also caused the greater the extracted total phenolic content. The temperature of infusion influenced total phenolic content, it could relate to~~ This could have been due tp the fact that the steeping temperature and storage period can 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~~increasing contact~~ exposure~~between~~of phenolic compounds to water (Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al.

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(2022). . Su et al. (2019) reported that temperature treatment can stimulate the release of phenolic compounds of lychee juice stored at different temperatures of 4 and 45 ºC and different long storage (fresh and 72 hours).

. this compounds and water. The same phenomena also occurred in Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al. (2022).

This occurrence showed that steeping temperature and storage period caused the process of degradation and oxidation of phenolic 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 ºC and different long storage (fresh and 72 hours). Hydrogen bonding is affected by Ttemperature treatment because thedegrades (or hdrolyzes) the hydrogen bond between phenolic compounds and proteins can be degraded that the measured levels resulting in an increase of phenolic compounds when exposed toare higher temperatures. The phenomena were supported by (Ali et al. (2018); Jayani et al. (2022), and 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. Besides that, 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 that the phenolic compounds in *Pluchea* infusion are degraded due to oxidation and hydrolysis because of temperature and storage timeperiod and can be easily extracted during

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steeping, thusresulting in the increased amount of ing thethe phenolic content compounds as the at higher steeping temperature and longer storage increaseperiod.

Based on using of a reference standard could be informed thatSimple phenolic compounds identified in steeped and stored ing *Pluchea* leaf infusion, includeing 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 was showed in Table 1. The treatment effectsresults of statistical analysis using a paired T test at $\alpha \leq 0.05$ showed that gallic acid and kaempferol contents of *Pluchea* infusion were insignificantly different at various steeping temperature and long storage periods. Nevertheless, the The concentration of quercetin and 3,5-di-O-caffeoylquinic acid of the un-stored and stored *Pluchea* infusion was significantly different from the rest of the samples between of two treatments except at 70 ºC. The while (+)-catechin concentration of *Pluchea* infusion was only significantly different at 95 ºC, but Tthe myricetin content was significantly different different concentration at 80 and 95 ºC. The 3,4-di-O-caffeoylquinic acid content showed significace difference at 60, 80 and 95 ºC and while 4,5-O-dicaffeoylquinic acid compounds content from *Pluchea* infusion werewas only significantly different at 60 ºC, however the concentration of 3,4-dicaffeoylquinic acid was also significantly different at 80 and 95 ºC.

Based on the analysis of concentration ofResults further showed simple phenolic compounds showed that gallic acids and kaempferol were relatively stable phenolic acid because ofas reflected by the insignificant changes when exposed no changes atto the different steeping temperature and storage timeperiod. 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.

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~~However, myricetin~~Myricetin, (+)-catechin and 3,4-di-*O*-caffeoylquinic acid showed a drastic ~~increasing~~ increase at higher steeping temperature and longer storage period ~~.~~implying ~~It's meant~~ that these compounds tended to be relatively labile. Quercetin, 3,5-di-*O*-cafffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid underwent moderate changes ~~compared to the other two groups of phenolic acids~~.~~.~~ T~~t~~herefore, myricetin, (+)-catechin and 3,4-di-*O*-caffeoylquinic acid were easier to dissolve or degraded to form simple phenolic compounds at higher steeping temperature and storage ~~time~~period. ~~can cause macromolecules of three phenolic acids in herbal tea convenient degradable to form simple phenolic compounds for storage, as explained by (~~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.

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 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 α= 0.05 showed that total flavonoid content of *Pluchea* infusion was significantly different between ~~two treatments~~the steeped un-stored and steeped stored samples

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(Figure 1b). The highest total flavonoid content was exhibited by ~~fresh~~ the un-stored samples steeped at 95 ºC at about 147.42±14.03 mg CE/g sample~~s~~. Total flavonoid content was significantly lower in the stored samples ~~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.

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Tannin Content (TTC)

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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 the samples infused at 60 ºC at about 4.81±0.58 to 17.42±1.04 mg TAE/g samples. ~~–~~which ~~is~~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 ºC 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 ºC about 9.22 ± 1.48 mg TAE/g samples. i~~I~~ndicating that the tannin content was primarily affected by ~~both high steeping temperature and~~ long storage period than high steeping temperature~~and that the presence of high tannin content was primarily brought about by long storage period. Kowalska et al. (2021) informed that T~~t~~he condensation of catechins to tannins ~~of polyphenolic compounds~~ is a dominant process ~~occurred~~ occurring in tea leaves that is accelerated during maceration of raw ~~material~~tea leaves (Kowalska et al~~.~~

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(2021) could have had contributed to the observed increase in the tannin content in the treated samples. However, the high temperature can degrade polyphenolic compounds to form simple phenolic 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 concentration 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,

Although, high temperature and long storage period can cause the degradation of tannins to catechins, Rusita et al. (2019) emphasized that tannins are a polar thermostable complex compounds, that is are resistant to heating, indicating that even with the exposure to high temperature, the tannins still remained high in the treated samples.as a result the tannin content in *Pluchea* tea increases with increasing steeping temperature and storage timeperiod, this is caused tannins are thermostable complex compounds.

ANTIOXIDANT ACTIVITY

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Antioxidant activity is capability of compounds to inhibit the oxidation of macromolecules from biological target that involve 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 antioxidant that have antioxidant

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

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DPPH Free Radical Scavengincg Asctivity

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DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate antioxidant activity because this method is simple that is suitable to measure the donating hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of DPPH to change to yellow color (Munteanu and Apetrei, 2021; Baliyan et al., 2022). Figure 2a shows that the free radical scavenging property of the stored and steeped samples were significantly higher than the un-stored steeped samples.~~The result of DPPH assay~~ It can also be observed ~~indicates~~ that the free radical scavenging property ~~DPPH values accrued~~ was significantly different among the stored and steeped samples but insignificant among the un-stored and steeped samples.~~at higher steeping temperature and longer storage time~~period.~~Statistical analysis by ANOVA using a paired T test at $\alpha \leq 0.05$ 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~~. *Pluchea* infusion stored at

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room temperature for 5 years resulted in the high ~~DPPH~~ free radical scavenging activity by~~.~~ more than 100 %~~. S~~teeping ~~at higher~~ temperatures significantly increased the ~~DPPH~~ free radical scavenging activity in stored *Pluchea* infusion by around 15 to 25 %. ~~Steeping at 80-95 °C in stored *Pluchea* infusion insignificantly affected the free radical scavenging property of the bioactive compounds (Figure 2a).~~ This implies that that the higher free radical scavenging property was primarily affected by the storage period than steeping temperature. During the storage process it is possible to form complex phenolic compounds which provide a high ability to scavenge ~~DPPH~~ free radicals (Thanajiruschaya et al., 2010)

~~Scavenging~~ The scavenging activity ~~of DPPH free radicals~~ of the the samples was strongly and positively correlated ~~with total~~ with total phenolic and tannin contents~~levels~~, but inversely ~~to~~ with total flavonoid levels. ~~Based on Pearson correlation at Table 2, the correlated coefficient values (r) between DPPH and TPC, TTC and TFC were 0.993, 0.942, and -0.940, respectively. During the storage process it is possible to form complex phenolic compounds which provide a high ability to scavenge DPPH free radicals (Thanajiruschaya et al., 2010)~~. This ~~research~~ study also demonstrated that longer storage ~~time~~period and higher infusion temperature produced many simple phenolic compounds with free hydroxyl groups capable to donor hydrogen atom to DPPH free radical. Many phenolic acids, such as gallic acids, (+)-catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 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 donor hydrogen atom depends on chemical structure, number and position of hydroxyl groups attached to a

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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 %

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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 donor hydrogen atom is determined by O-H bond dissociation energy.

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

Ferric Reducing Antioxidant Power (FRAP)

FRAP is an analysis of antioxidant power of the phytochemical compounds ~~based on the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a color complex, that can be measured at λ 700 nm (Fu et al., 2011; Al-Temimi and Choudhary, 2013). The principle of the assay measures~~ that is based of the ability of antioxidant compounds to reduce iron ions of potassium ferrocyanide (Fe^{3+}) to be 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 ferric reducing antioxidant power (FRAP) increased ~~with~~ at higher steeping temperature and longer storage ~~time~~period. The lowest FRAP was observed in the un-stored samples which was steeped at 60 ^{o}C at 3.95 ± 0.17 mg gallic acid equivalents (GAE)/g samples, and the highest was ~~owned~~ exhibited ~~by~~ in *Pluchea*

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infusion which was stored for 5 years at 95 ºC at 48.63 ±10.83 mg gallic acid equivalents (GAE)/g samples (Figure 2b). FRAP increased significantly as steeping temperature was increased. FRAP of the samples stored for 5 years was also significantly higher than the un-stored samples at $\alpha \leq 0.05$. ~~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) between FRAP and DPPH, TPC, TTC and TFC were 0.956, 0.953, 0.948 and -0.826, respectively~~.

This ~~case was~~ is in contrast ~~to~~ with the study on the antioxidant activity of DPPH and FRAP ~~on~~ of matcha., ~~because The~~the longer storage ~~time~~period reduces the levels of catechin content ~~due to the catechins,~~ such as epigallocatechin gallat (EGCG), epicatechin gallat (ECG), epigallocatechin (EGC), and epicatechin (EC) which are bioactive compounds that have high antioxidant activity (Kim et al. 2020)., ~~and also the case of the effect of temperature and storage time in betel (*Piper bettle* 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). Thanajiruschaya et al. (2010) revealed that the antioxidant activity of rice stored at high temperatures is greater than that stored at low temperatures.~~ The ferric reducing capability of ***Pluchea*** could have due ~~infusion corresponded~~to the presence ~~to~~ of simple phenolic acid ~~values~~ that have the ability to transfer electron from their free hydroxyl groups of~~, presence of them in samples could accrue antioxidant activity because of ability of the electron transfer from free hydroxyl groups of phenolic acids~~.

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The FRAP of *Pluchea* infusion was strongly and positively significant correlated with the DPPH, TPC and TTC, but inversely to TFC.

ANTIDIABETIC ACTIVITY

α-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 2-degestive enzymes i.e., α-amylase and α-glucosidase, which are involved 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 α-amylase and α-glucosidase enzymes (Martinez-Solis et al., 2022) resulting in the reduced activity of the enzymes. The results showed~~,~~ that ~~the~~ lower steeping ***Pluchea*** leaf infusion was able to inhibit the action of the α-amylase enzymes (Figure 3a). The ***Pluchea*** infusion ~~had very good activity,~~exhibited a good α-amylase enzyme inhibition activity of more than 50 % and even almost 100 % ~~for fresh~~in the un-stored ***Pluchea*** infusion ~~which~~ steeped ~~was brewed~~ at 60, 70 and 80 ºC with highest at 60 ºC, and in stored *Pluchea* leaf infusion which was steeped at 60 ºC. ~~Whereas~~ The stored ~~fresh~~ ***Pluchea*** leaf infusion steeped at 70, 80 and 95 ºC for 5 minutes had lower enzyme inhibition activity~~an activity of~~ of ~~less than 50 % with lowest at~~ 95 ºC.~~inhibiting the α-amylase enzyme of less than 50 %, which was equal to 40.08±1.12~~ %. Widyawati et al. (2017) ~~detected~~ found that the ability to inhibit the α-amylase enzyme ~~from~~ in ~~fresh~~ un-stored ***Pluchea*** infusion steeped at 95 ºC for 5 minutes ~~by~~ was also low at 28.79 %. Increasing the steeping temperature and storage ~~time~~period reduced the ability ~~to~~ of the phytochemicals in the *Pluchea* infusions to inhibit the α-amylase enzyme activity. ~~The~~

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~~results of the analysis based on a paired T test at α ≤ 0.05 showed, that the steeping temperature and storage time~~period ~~had a significant effect on the ability to inhibit the α-amylase enzyme~~. ~~Based on Pearson correlation, the~~Table 2 further shows that the AA of *Pluchea* infusion was strongly and negatively significant correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively significant correlated with TFC. ~~The correlated coefficient values (r) between AA and TPC, TTC, DPPH, FRAP and TFC were -0.708, -0.857, -0.696, -0.806 and 0.429, respectively.~~

This inhibitory activity was thought to be contributed by other bioactive compounds, besides phenolics which are sensitive to steeping temperature and storage ~~time~~period. Li et al. (2018) stated that there are flavonoid compounds that contribute to the ability to inhibit the α-amylase enzyme. ~~Flavonoid compounds with a hydroxyl structure at C-4' in ring B are more effective than C-6 in ring A.~~ Akah et al. (2011) ~~informed~~ reported that the phytochemical compounds, such as terpenoids, saponins, flavonoids, glycosides and carbohydrate, and alkaloids are good antidiabetic metabolites or α-amylase enzyme activity inhibitor. Sangeetha and Vedasree (2012) explained, that the ability to inhibit the α-amylase enzyme was determined by the content of the phenolic compound and protein. The α-amylase inhibitor enzyme present in *Pluchea* infusion may be proteinaceous in nature. Aleixandre et al. (2022) informed that phenolic acids have inhibition activity to α-amylase enzyme depending their structures. Besides that, capability of phenolic acids to inhibit α-amylase enzyme was determined by low half-maximum inhibitory concentration (IC_{50}). There are C=C double bond conjugated with a carbonyl group of phenolic structures that stabilizes the binding forces to the active site of the α-amylase. The hydroxyl groups of them are able to bind by non-covalent interaction, such as hydrogen

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binding, cation-π interactions, salt bridge interactions, ionic interactions or electrostatic forces with amino acid residue at the active site in α-amylase enzyme. Elevated steeping temperature and longer storage period ~~The steeping temperature and storage time~~ can easily cause the removal of the ~~e~~ hydroxyl groups of phenolic compounds that can reduce their ~~ir~~ ability of enzyme inhibition. The phenolic acids with a greater number of hydroxyl groups ~~are~~ exhibits stronger capability~~le~~ to obstruct the α-amylase enzyme.

α-Glucosidase enzyme inhibition activity (GA)

Alpha~~α~~-glucosidase is an important enzyme in carbohydrates digestion, that catalysis the hydrolysis of 1,4-α-bonds of the unabsorbed oligo- and disaccharides, and converts them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et al., 2014; Proenca et al., 2017). The ability of bioactive compounds to inhibit the α-glucosidase enzyme is used to determine their antidiabetic~~s~~ 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 brewing fresh *Pluchea* infusion at 95 ºC for 5 minutes has an inhibitory effect on the α-glucosidase enzyme of 67.857 %.

~~The results showed,~~Figure 3b shows that the ability of the Pluchea leaf infusion to inhibit the α-glucosidase enzyme decreased with increasing steeping temperature and storage ~~time~~period. Steeping at 95 ºC ~~for fresh~~of the un-stored *Pluchea* leaf infusion ~~(un-stored)~~ obtained the lowest inhibitory ability, i.e., 48.32 ± 1.27 %, and the highest inhibitory activity was f~~ound~~ at 70 ºC ~~steeping temperature for fresh *Pluchea* infusion, which was~~at 95.11 ± 0.70%. ~~(Figure 3b).~~ The results of a paired T test showed that GA of *Pluchea* infusion was significantly different ~~at both~~between steeping temperature and long storage. ~~The antidiabetic activity of *Pluchea* infusion~~Figure 3 further ~~showed~~ shows that the ability

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of *Pulchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-amylase enzyme. Li et al. (2018) informed that flavonoid compounds have the ability to inhibit the action of the α-amylase and α-glucosidase enzymes. This is due to the total flavonoids in steeped *Pluchea* infusion which tended to have the same pattern as the ability to inhibit the activity of the α-amylase and α-glucosidase enzymes. The statistical analysis using Pearson correlation showed that GA of *Pluchea* infusion was strongly and negatively correlated with TPC, TTC, DPPH and FRAP ~~, with r was -0.555, -0,715, -0.527 and -0.560, respectively.~~ However, GA was moderately and positively correlated to TFC~~, with r was 0.350 and strongly~~ and positively correlated to AA~~, with r was 0.725~~. Flavonoid compounds, such as rutin, myricetin, kaempferol, and quercetin ~~which~~ 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 and the number of double bonds in rings A and B and the heterocyclic ring in ring C. The ability to inhibit the α-glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping temperature and long storage. The capability of *Pluchea* infusion to obstruct the α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of two enzymes was different, according to the opinion of McCue et al. (2005). Widyawati et al. (2017) informed that phenolic and non-phenolic compounds determine the inhibitory activity of the α-glucosidase enzyme. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, that may be occurred in *Pluchea* infusion during storage, affects the structure and profile of phenolic and non-phenolic compounds. Asriningtyas et al. (2014) claimed

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that *Pluchea* leaves contain 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. Quinic acid is methyl esterified with the number of caffeic groups in the molecule that determines the activity of inhibiting the α-glucosidase enzyme. Analysis of caffeoylquinic acids in *Pluchea* infusion was obtained that the higher steeping temperature and long storage caused increased concentration of them, but the α-glucosidase inhibition activity of them was reduced. Aleixandre et al. (2022) reported that the simple phenolic acids forming a dipole-dipole interaction of active site from α-glucosidase enzyme are effectively inhibiting the enzyme.

This study ~~was obtained information~~showed that the increasing of steeping temperature and storage ~~time~~period caused a degradation reaction 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, supported the results of total phenolic content and total tannin content assays. Increased concentration of simple phenolic compounds determined the ability of these compounds as antioxidant agents, but reduced their capability as antidiabetic agents.

CONCLUSION

The steeping temperature and storage ~~time~~ period of *Pluchea* infusion significantly influenced bioactive contents, antioxidant and antidiabetic activities. TPC, TTC, and TFC were significantly different at various steeping temperature and storage period based on statistical analysis using a paired ~~T~~ t-test at $\alpha \leq 0.05$. ~~There was the difference of t~~The

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1) 'Figure 3b shows that the ability of the Pluchea leaf infusion to inhibit the α-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 α-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 α-glucosidase enzyme was greater than the α-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 α-amylase and α-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 α-glucosidase enzyme. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. (May also be incorporated in 1).

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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

phenolic compound profile in ~~fresh~~ the unstored and stored ~~of~~ *Pluchea* infusion ~~and~~ at various steeping temperature~~.~~ ~~The~~ included simple phenolic compounds ~~were detected in *Pluchea* infusion including~~such as gallic acid, (+)-catechin, quercetin, myricetin, kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoilquinic acid, and 4,5-di-*O*-caffeoilquinic acid. The results of statistical analysis using a paired ~~T~~ t-test at $\alpha \leq 0.05$ showed that gallic acid and kaempferol of *Pluchea* infusion were insignificantly different at various steeping temperature and long storage. ~~Nevertheless, T~~the concentration of quercetin and 3,5-dicaffeoylquinic acid of *Pluchea* infusion was significantly different of two treatments except at 70 ^{o}C. The (+)-catechin concentration of *Pluchea* infusion was significantly different at 95 ^{o}C, but the myricetin was different concentration at 80 and 95 ^{o}C. The 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid compounds from *Pluchea* infusion were significantly different at 60 ^{o}C, however the concentration of 3,4-dicaffeoylquinic acid was also significantly different at 80 and 95 ^{o}C. TPC, TTC and TFC of *Pluchea* infusion were significantly different at various steeping temperature and storage period. TPC and TTC significantly increased with increasing steeping temperature and long storage, but TFC significantly increased at various steeping temperature and significantly decreased at long storage. The bioactive compounds of *Pluchea* infusion influenced antioxidant activities (DPPH and FRAP) and antidiabetic activity (AA and GA). The DPPH was strongly and positively correlated with TPC and TTC, but it was strongly and negatively correlated with TFC, with coefficient r 0.993, 0.942, and -0.940, respectively. The correlated pattern between FRAP and bioactive contents of *Pluchea* infusion was similar to it between DPPH and bioactive contents. The correlated coefficient values (r) between FRAP and TPC, TTC and TFC were 0.953, 0.948

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and -0.826, respectively. The AA and GA were strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively significant correlated with TFC. Between the antioxidant activity of DPPH and FRAP and the antidiabetic activity of AA and GA of Pluchea infusion were strongly and positively correlated with correlation coefficient (r) values of 0.956 and 0.725, respectively.

DATA AVAILABILITY

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

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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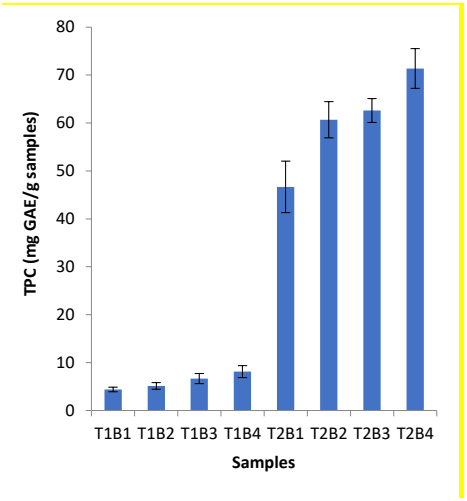

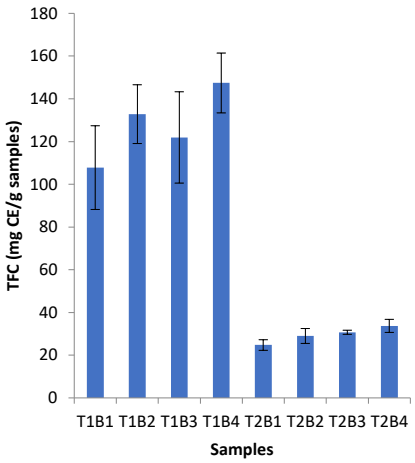

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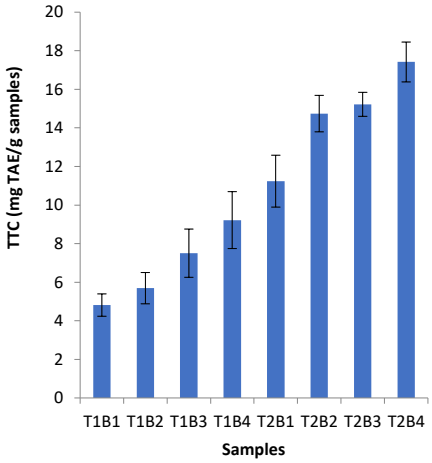

(c)

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 ±standard deviation (n=6). Samples : T1B1-steeped at 60 ºC, un-stored; T1B2-steeped at 70 ºC, un-stored; T1B3-steeped at 80 ºC, un-

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stored; T1B4-steeped at 95 ºC, un-stored; T2B1-steeped at 60 ºC, stored for 5 years; T3B2-steeped at 70 ºC, stored for 5 years; T3B3-steeped at 80 ºC, stored for 5 years; T3B4-steeped at 95 ºC, 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 $\alpha \leq 0.05$.

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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	-1.1735±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

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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

Note : Data were expressed as mean ±standard deviation (n=2). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}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 $\alpha \leq 0.05$. * $\alpha \leq 0.05$.

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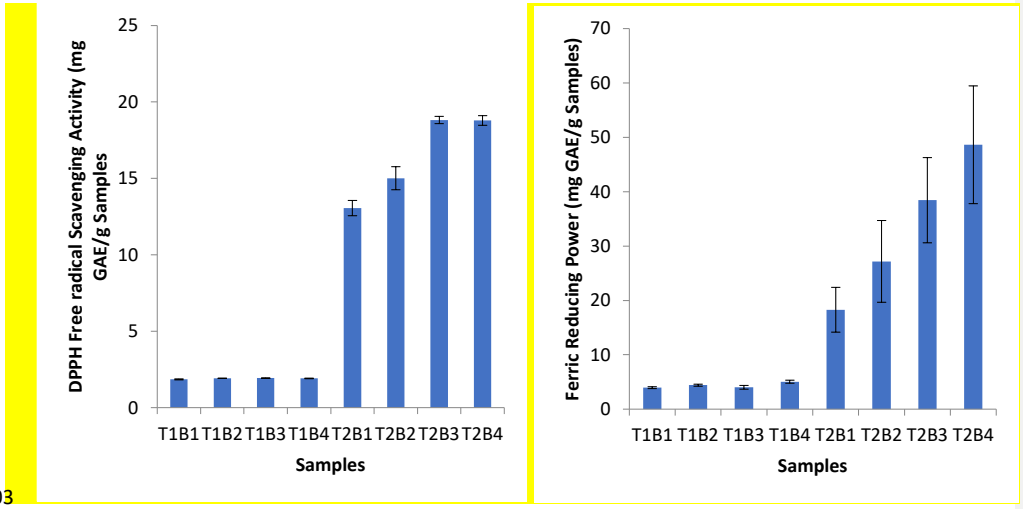

(a) (b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage ~~time~~period (a) DPPH (b) FRAP. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ºC, un-stored; T1B2-steeped at 70 ºC, un-stored; T1B3-steeped at 80 ºC, un-stored; T1B4-steeped at 95 ºC, un-stored; T2B1-steeped at 60 ºC, stored for 5 years; T3B2-steeped at 70 ºC, stored for 5 years; T3B3-steeped at 80 ºC, stored for 5 years; T3B4-steeped at 95 ºC, 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 $\alpha \leq 0.05$~~

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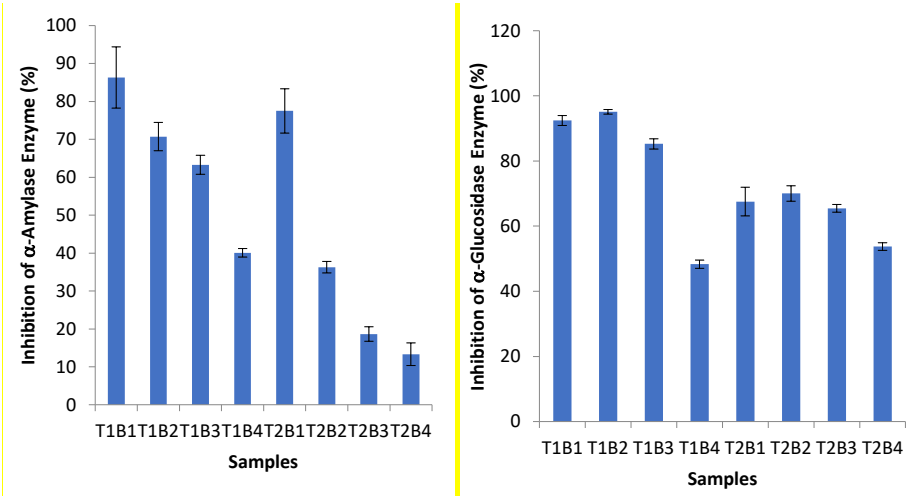

(a) (b)

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage ~~time~~period (a) α-amylase (b) α-glucosidase. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}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 $\alpha \leq 0.05$

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Effect of Brewing .

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

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Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea indica* Less, storage ~~time~~period

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ABSTRACT

This study was done to determine the effects of steeping temperature and storage ~~time~~period on the bioactive contents, 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 ~~time~~period (B). The ~~variety of the~~ *Pluchea* leaf blades were exposed to 4 steeping temperatures ~~included~~ of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) ~~(~~ºC~~)~~ with the storage ~~time~~period~~–~~period of 0 (B1) and 5 (B2) ~~(~~year~~)~~. ~~The research resulted~~resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). Statistical analysis using a paired t~~-T~~ test at $\alpha \leq 0.05$ showed that treatments significantly affected ~~influenced~~ the bioactive contents (total phenol (TPC), total tannin (TTC), total flavonoid (TFC)), antioxidant [(DPPH scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)]~~)~~ potential ~~–~~and antidiabetic [(α-amylase (AA) and α-glucosidase (GA) ~~inhibitors~~inhibition)] ~~activities~~ properties of the *Pluchea* leaf infusion~~samples~~. TFC decreased during storage period but significantly increased at higher steeping temperature. ~~The AA and GA of *Pluchea* infusion increased until 70 ºC of the steeping temperature, but deceased until 95 ºC. The bioactive contents influenced antioxidant and antidiabetic activities. TFC was decreased for storage time and significant increased at higher steeping temperature. The AA and GA of *Pluchea* infusion increased until 70 ºC of the steeping temperature, but deceased until 95 ºC.~~ The AA ~~–~~and GA were strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively correlated with TFC. ~~Between~~ The antioxidant activity ~~of DPPH and FRAP~~ and the antidiabetic activity ~~of AA and GA~~ of Pluchea infusion were strongly and positively correlated. ~~with correlation coefficient (r) values of 0.956 and~~

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0.725, respectively. The treatments gave different effect of simple phenolic compounds derived from *Pluchea* leaf infusion at different steeping temperatures and storage included, such as 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. of *Pluchea* infusion at different steeping temperature and long storage. To obtain high antioxidant activity, *Pluchea* infusion selected was stored and steeped at high temperature, however high antidiabetic activity obtained was fresh *Pluchea* infusion and steeped at low temperature.

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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 an herbal plant that has been widely used for traditional medicine and food (Chan et al., 2022). *Pluchea* leaves are composed 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, β-carotene, and vitamin C, whereas bioactive compounds is 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, β-carotene, and total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et al., 2022, Chan et al., 2022).

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Steeping process of *Pluchea* leaves can be performed with fresh or dry leaves ~~infusion by~~in hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et al., 2020; Jayani et al., 2022). In Asia~~n area~~, especially in Indonesia~~n~~, people usually consume the *Pluchea* infusion ~~with brewing of~~by steeping 2 g of powdered *Pluchea* leaves in tea bag ~~by~~in 100 mL of hot ~~water~~ or boiling water. ~~Each tea bag contained 2 g of *Pluchea* leaf powder is steeped with 100 mL hot water or boiling water.~~ Widyawati et al. (2016) claimed that steeping of 2 g of *Pluchea* leaf powder at 95 ^{o}C for 5 minutes ~~results~~ rexhibits total phenolic ~~content,~~and total flavonoid content~~s~~, the ability to scavenge DPPH free radicals, and the capability ~~of~~ to reduce ferric ions at 9.3 mg gallic acid equivalent (GAE)/g sample~~s~~, 22.0 mg gallic acid equivalent (GAE)/g sample~~s~~, 27.2 mg gallic acid equivalent (GAE)/g sample~~s~~, and 10.2 mg gallic acid equivalent (GAE)/g sample~~s~~, respectively. Werdani and Widyawati (2018) reported that drinking of *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 ^{o}C for 5 minutes certainly determines the stability and amount of extracted bioactive compounds~~,~~ that influences the biological activity~~,~~ especially antioxidant and antidiabetic activities. Silva-Ramirez et al. (2020) reported that the infusion process can influence the~~ir~~ content and composition of the bioactive compounds and antioxidant activity of tea. Acar et al. (2022) informed that infusion quality of herbal tea extract depends on several factors, i.e., ~~time~~ storage and temperature. Polyphenol profile and antioxidant properties of herbal tea infusion decline with an increase in steeping/brewing and storage temperatures, and longer exposure ~~time~~period~~s~~.

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Several studies have mentioned the effect of steeping temperature ~~to~~ on the bioactive compound contents and antioxidant activity, such as some white and green teas are effective with hot water at 90 ºC for 7 min (Castiglioni et al., 2015), on roseship tea is effective~~ly~~ at infusion ~~time~~period around 6-8 min at temperatures of 84-86 ºC (Ilyasoglu and Arpa, 2017), on the caffeine content extracted ~~the coffee~~at the brewing temperature of coffee~~influences the caffeine content extracted~~ (Zarwinda and Sartika, 2018), and ~~the steeping~~ the high total phenol content and antioxidant activity of dark tea at 92 ºC for 27 min ~~results the highest total phenol content and antioxidant activity~~ (Wang et al., 2022). The study of the effect of steeping temperature to ***Pluchea*** infusion was carried out to afford information about the most efficient preparation of powdered ***Pluchea*** leaves ~~most efficiently~~ to get higher ~~the~~ bioactive compounds, antioxidant and antidiabetic activities.

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

Therefore, this research studied the effect of steeping temperature and storage ~~time~~period on the bioactive compounds [(total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging

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activity (DPPH), ferric reducing antioxidant power (FRAP)] and antidiabetic activities [(α-amylase (AA) and α-glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea* leaves.~~- 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 antioxidant power (FRAP), α-amylase (AA) and α-glycosidase (GA) inhibition activities,~~ 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 *Asteraceae* family with specification according to the GBIF taxon ID number database:3132728 (Ferraris, 2023). *Pluchea* leaves at 1-6 level of each branch ~~of~~from the shoot were collected, sorted, washed and dried to ~~get a~~moisture content of around 11.16 ± 0.09 % dry bas~~is~~ (Widyawati et al., 2022). The ~~powdering of~~ dried *Pluchea* leaves was ~~done~~ pulverized to ~~get~~ a 45-mesh size powder. ~~And then, the heating of T~~the *Pluchea* leaf powder was ~~done using a drying~~dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 °C for 10 min to reduce microbial organisms. ~~and~~ Then, 2 g of the powder were packed ~~using~~ into a paper filter infusion bag.~~ that made from paper filter around 2 g/bag~~. ~~And then all of samples called~~Packed samples were ~~*Pluchea* herbal tea was~~ stored for 0 (un-stored) and 5 (stored)years in standing pouch before analysis.

In the research, the one tea bag of *Pluchea* herbal tea that stored 0 (B1) and 5 (B2) year, was steeped with 100 mL hot water at various temperatures, including 60 (T1),

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70 (T2), 80 (T3), and 95 (T4) ºC for 5 min with infusion method ~~that obtained~~obtaining 8 treatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2. After the temperature of *Pluchea* infusion similar to ambient temperature was analyzed further.

REAGENTS

The ~~compounds~~reagents used ~~to analyze~~in the analyses ~~including~~ include 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid, α-amylase, α-glucosidase, pNPG (p-nitrophenyl-α-glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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 distillated water which was purchased from PT Aqua Industry Surabaya.

METHODOLOGY

ANALYSIS OF THE BIOACTIVE COMPOUNDS

TOTAL PHENOLIC CONTENT ANALYSIS

Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the technique by Gao et al. (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. And

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then 2 mL Na_2CO_3 7.5 % was ~~entered~~ added and filled up to 10 mL volume with distilled water.~~and distillated water was added until 10 mL volume~~. The color intensity of solution was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at λ 760 nm with gallic acid as the reference standard. The total phenolic content was calculated using the formula: y=0.00009x+0.008 with R^2=0.9941. The results were expressed as mg gallic acid equivalent (GAE)/g samples.

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TOTAL FLAVONOID CONTENT ASSAY

Total flavonoid content (TFC) of the samples was measured based on the reaction between $AlCl_3$ and $NaNO_2$ with ~~an~~ the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim et al., 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted in a yellow solution. About 30 µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5 % in 10 mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 10 % for 5 min. And then, 2 mL NaOH 1 M and distillated water were added until 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 λ 510 nm with (+)-catechin as the reference standard compound, and the results were expressed as mg catechin equivalents (CE)/g samples using the formula: y=0.00008x-0.0023 with R^2= 0.9980.

TOTAL TANNIN CONTENT ANALYSIS

Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method (Chandran and Indira, 2016). Approximately 10 µL *Pluchea* infusion was added with 1 mL

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Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flash and incubated for 5 min. Then, the mixture was added with 2 mL Na_2CO_3 7.5 % and filled up to 10 mL volume with distillated water. ~~was added until 10 mL volume~~. The blue dark color solution ~~that~~ was measured in UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at λ 760 nm with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/g samples used the formula: y=0.00009x+0.0021 with R^2=0.9993

ANALYSIS OF THE ANTIOXIDANT POTENTIAL

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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 ~~antioxidant activity of~~in the *Pluchea* leaf infusion to ~~donor~~ donate hydrogen atom to the nitrogen atom in DPPH resulting in the formation of –DPPH-H compound ~~with~~ exhibiting a yellow-colored solution. About 25 µL *Pluchea* leaf infusion was ~~entered~~ poured into reaction tube ~~and~~ into which was added added 3 mL DPPH solution (4 mg/100 mL). ~~And then the solution was~~After incubationed for 15 min in a dark room , the ~~and~~ absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ. 517 nm. The reference standard compound was gallic acid and the results of analysis were expressed as mg gallic acid equivalents (GAE)/g samples that calculated using formula: y=0.146x+1.7896 with R^2=0.9975.

FERRIC REDUCING POWER ANALYSIS

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

α-AMYLASE ENZYME INHIBITION CAPACITY ASSAY

In vitro inhibition of α-amylase enzyme (AA) followed the procedure as described by Widyawati et al. (2020). Each 500 µL of samples, was mixed with starch 1 % (w/v) and sodium acetate buffer pH 5.~~ were mixed~~. ~~Then,~~ Into a~~each~~ 250 µL of the mixture ~~and~~ was added an α-amylase solution (0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in 50 mL of 0.2 M sodium acetate pH 5~~)~~. Mixture was shaken ~~and~~ into which was added 2 mL sodium hydroxide 1M. Before the analysis, this mixture was incubated at 37 ºC for 10 min. Then, the capacity of the α-amylase enzyme to hydrolyze~~d~~ the starch to release glucose was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) ~~that could be analyzed based on absorbance~~ at λ 540 nm. The

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inhibition percentage of α-amylase was assessed using the formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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 test sample with enzyme, Ab is absorbance of test sample without enzyme.

α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

The analysis of the α-glycosidase inhibitor activity (GA) was done by Widyawati et al. (2020) method with slight modification. About 150 µL samples ~~contained~~ containing 100 µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH 7) were reacted with 50 µL α-glycosidase 2 mM (0.0833 unit/mL), and then the mixture was incubated at 37 ºC for 15 min. ~~Finally, the~~The reaction was stopped with the addition of 1000 µL sodium carbonate 0.2 M. The residue of this enzyme hydrolyzed p-nitrophenyl-α-D-glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The inhibition~~or~~ activity of ~~steeping~~the ~~*Pluchea* tea~~ infusion ~~to enzyme~~ was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at λ 405 nm. The inhibition percentage of α-glycosidase was calculated using formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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, Ab is the absorbance of test sample without enzyme.

~~HPLC~~ ANALYSIS OF PHENOLICS

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The phenolic compounds of the samples were analyzed by HPLC based on Kongkiatpaiboona et al. (2018) method with modifications. Each *Pluchea* infusion was sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter syringe (Whatmann, 0.2 µm, NYL). About 20 µL of 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 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (2 pieces) (ID 10 mm x 4.6 mm). ~~Analytical conditions: T~~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 minutes; then 100 % B was maintained for 20 minutes. Next the column was re-equilibrated with 10 % B in A maintained for 10 minutes before analysis of the next sample. The sample flow rate was set at 1.0 ml/min with a controlled temperature at 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 distillated water and prepared similar to the samples before 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 ~~time~~period (B). *Pluchea* leaf blades were

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subjected to 4 steeping temperatures, namely 60 ºC (T1), 70 ºC (T2), 80 ºC (T3), and 95 ºC (T4), and the storage ~~time~~period of 0 year /~~fresh~~ un-stored (B1), and 5 year/stored (B2).~~.~~ ~~The research resulted~~resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic was repeated two ~~time~~periods. The data of samples were analyzed by ANOVA at $\alpha \leq 0.05$, and continued analysis using a paired T test at $\alpha \leq 0.05$. treatment means of specific phenolic compounds that were identified were expressed as the mean ± SD. The analysis used SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

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RESULTS AND DISCUSSIONS

Pluchea leaf infusion is produced by young *Pluchea* leaf from 1-6 level on each branch the shoot (Widyawati et al., 2022), that is steeped at 95 ºC 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 *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols, cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2 g/100 mL steeping *Pluchea* 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 related to the composition of phytochemical compounds in *Pluchea* leaves, such as phenolic acids such as chlorogenic acids, caffeic acids, 3-*O*-caffeoylquinic acids, 4-*O*-caffeoylquinic acids, 5-*O*-caffeoylquinic acids, 3,4-

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di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin; β-carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Chan et al., 2022; Widyawati et al., 2022). Presence of phytochemical compounds in herbal product were 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 of 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). Therefore, this study was conducted to determine the effect of steeping temperature and storage ~~time~~period of *Pluchea* tea on levels of the bioactive compounds, antioxidant and antidiabetic properties and phenolic compound profile.

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BIOACTIVE COMPOUNDS

Phenolics Compounds

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The bioactive compounds are active compounds in plants that are essential to protect a 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).

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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 based on paired ~~T~~ t- test at $\alpha \leq 0.05$ (Figure 1a). Steeped and stored infusion had significantly higher amounts of phenolic compounds thant ~~t~~ the samples that were steeped and un-stored. Further, the highest total phenolic content was observed in samples infused at 95 ºC and stored for 5 years (at 71.38±4.14 mg GAE/g sample~~s~~) while the lowest was measured in the un-stored samples and infused at 60 ºC (at mg GAE/g sample). Phenolic content of stored samples ~~that were infused at different temperatures~~ that ~~then stored~~ were steeped only at 60 and 95 ºC ~~also~~ 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 the phenolic compounds of the infusions. Results also indicated that phenolic compounds were generally greater in the infusion at high steeping temperatures and long storage period .~~(Figure 1a). This could have been due to that 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 sleeping temperature also caused the greater the extracted total phenolic content. The temperature of infusion influenced total phenolic content, it could relate to~~ This could have been due tp the fact that the steeping temperature and storage period can 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~~increasing contact~~ exposure~~between~~of phenolic compounds to water (Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al.

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(2022). . Su et al. (2019) reported that temperature treatment can stimulate the release of phenolic compounds of lychee juice stored at different temperatures of 4 and 45 ºC and different long storage (fresh and 72 hours).

. this compounds and water. The same phenomena also occurred in Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al. (2022).

This occurrence showed that steeping temperature and storage period caused the process of degradation and oxidation of phenolic 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 ºC and different long storage (fresh and 72 hours). Hydrogen bonding is affected by Ttemperature treatment because thedegrades (or hdrolyzes) the hydrogen bond between phenolic compounds and proteins can be degraded that the measured levels resulting in an increase of phenolic compounds when exposed toare higher temperatures. The phenomena were supported by (Ali et al. (2018); Jayani et al. (2022), and 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. Besides that, 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 that the phenolic compounds in *Pluchea* infusion are degraded due to oxidation and hydrolysis because of temperature and storage timeperiod and can be easily extracted during

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steeping, thusresulting in the increased amount of ing thethe phenolic content compounds as the at higher steeping temperature and longer storage increaseperiod.

Based on using of a reference standard could be informed thatSimple phenolic compounds identified in steeped and stored ing *Pluchea* leaf infusion, includeing 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 was showed in Table 1. The treatment effectsresults of statistical analysis using a paired T test at $\alpha \leq 0.05$ showed that gallic acid and kaempferol contents of *Pluchea* infusion were insignificantly different at various steeping temperature and long storage periods. Nevertheless, the The concentration of quercetin and 3,5-di-O-caffeoylquinic acid of the un-stored and stored *Pluchea* infusion was significantly different from the rest of the samples between of two treatments except at 70 ºC. The while (+)-catechin concentration of *Pluchea* infusion was only significantly different at 95 ºC, but Tthe myricetin content was significantly different different concentration at 80 and 95 ºC. The 3,4-di-O-caffeoylquinic acid content showed significace difference at 60, 80 and 95 ºC and while 4,5-O-dicaffeoylquinic acid compounds content from *Pluchea* infusion werewas only significantly different at 60 ºC, however the concentration of 3,4-dicaffeoylquinic acid was also significantly different at 80 and 95 ºC.

Based on the analysis of concentration ofResults further showed simple phenolic compounds showed that gallic acids and kaempferol were relatively stable phenolic acid because ofas reflected by the insignificant changes when exposed no changes atto the different steeping temperature and storage timeperiod. 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.

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~~However, myricetin~~Myricetin, (+)-catechin and 3,4-di-*O*-caffeoylquinic acid showed a drastic ~~increasing~~ increase at higher steeping temperature and longer storage period ~~.~~implying ~~It's meant~~ that these compounds tended to be relatively labile. Quercetin, 3,5-di-*O*-cafffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid underwent moderate changes ~~compared to the other two groups of phenolic acids~~.~~.~~ T~~t~~herefore, myricetin, (+)-catechin and 3,4-di-*O*-caffeoylquinic acid were easier to dissolve or degraded to form simple phenolic compounds at higher steeping temperature and storage ~~time~~period. ~~can cause macromolecules of three phenolic acids in herbal tea convenient degradable to form simple phenolic compounds for storage, as explained by (~~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.

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 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 α= 0.05 showed that total flavonoid content of *Pluchea* infusion was significantly different between ~~two treatments~~the steeped un-stored and steeped stored samples

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(Figure 1b). The highest total flavonoid content was exhibited by ~~fresh~~ the un-stored samples steeped at 95 ºC at about 147.42±14.03 mg CE/g sample~~s~~. Total flavonoid content was significantly lower in the stored samples ~~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.

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Tannin Content (TTC)

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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 the samples infused at 60 ºC at about 4.81±0.58 to 17.42±1.04 mg TAE/g samples. ~~–~~which ~~is~~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 ºC 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 ºC about 9.22 ± 1.48 mg TAE/g samples. i~~I~~ndicating that the tannin content was primarily affected by ~~both high steeping temperature and~~ long storage period than high steeping temperature~~and that the presence of high tannin content was primarily brought about by long storage period. Kowalska et al. (2021) informed that T~~t~~he condensation of catechins to tannins ~~of polyphenolic compounds~~ is a dominant process ~~occurred~~ occurring in tea leaves that is accelerated during maceration of raw ~~material~~tea leaves (Kowalska et al~~.~~

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(2021) could have had contributed to the observed increase in the tannin content in the treated samples. However, the high temperature can degrade polyphenolic compounds to form simple phenolic 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 concentration 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,

Although, high temperature and long storage period can cause the degradation of tannins to catechins, Rusita et al. (2019) emphasized that tannins are a polar thermostable complex compounds, that is are resistant to heating, indicating that even with the exposure to high temperature, the tannins still remained high in the treated samples.as a result the tannin content in *Pluchea* tea increases with increasing steeping temperature and storage timeperiod, this is caused tannins are thermostable complex compounds.

ANTIOXIDANT ACTIVITY

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Antioxidant activity is capability of compounds to inhibit the oxidation of macromolecules from biological target that involve 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 antioxidant that have antioxidant

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

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DPPH Free Radical Scavengincg Asctivity

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DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate antioxidant activity because this method is simple that is suitable to measure the donating hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of DPPH to change to yellow color (Munteanu and Apetrei, 2021; Baliyan et al., 2022). Figure 2a shows that the free radical scavenging property of the stored and steeped samples were significantly higher than the un-stored steeped samples.~~The result of DPPH assay~~ It can also be observed ~~indicates~~ that the free radical scavenging property ~~DPPH values accrued~~ was significantly different among the stored and steeped samples but insignificant among the un-stored and steeped samples.~~at higher steeping temperature and longer storage time~~period.~~Statistical analysis by ANOVA using a paired T test at $\alpha \leq 0.05$ 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~~. *Pluchea* infusion stored at

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room temperature for 5 years resulted in the high ~~DPPH~~ free radical scavenging activity by~~.~~ more than 100 %~~. S~~teeping ~~at higher~~ temperatures significantly increased the ~~DPPH~~ free radical scavenging activity in stored *Pluchea* infusion by around 15 to 25 %. ~~Steeping at 80-95 °C in stored *Pluchea* infusion insignificantly affected the free radical scavenging property of the bioactive compounds (Figure 2a).~~ This implies that that the higher free radical scavenging property was primarily affected by the storage period than steeping temperature. During the storage process it is possible to form complex phenolic compounds which provide a high ability to scavenge ~~DPPH~~ free radicals (Thanajiruschaya et al., 2010)

~~Scavenging~~ The scavenging activity ~~of DPPH free radicals~~ of the the samples was strongly and positively correlated ~~with total~~ with total phenolic and tannin contents~~levels~~, but inversely ~~to~~ with total flavonoid levels. ~~Based on Pearson correlation at Table 2, the correlated coefficient values (r) between DPPH and TPC, TTC and TFC were 0.993, 0.942, and -0.940, respectively. During the storage process it is possible to form complex phenolic compounds which provide a high ability to scavenge DPPH free radicals (Thanajiruschaya et al., 2010)~~. This ~~research~~ study also demonstrated that longer storage ~~time~~period and higher infusion temperature produced many simple phenolic compounds with free hydroxyl groups capable to donor hydrogen atom to DPPH free radical. Many phenolic acids, such as gallic acids, (+)-catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 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 donor hydrogen atom depends on chemical structure, number and position of hydroxyl groups attached to a

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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 %

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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 donor hydrogen atom is determined by O-H bond dissociation energy.

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

Ferric Reducing Antioxidant Power (FRAP)

FRAP is an analysis of antioxidant power of the phytochemical compounds ~~based on the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a color complex, that can be measured at λ 700 nm (Fu et al., 2011; Al-Temimi and Choudhary, 2013). The principle of the assay measures~~ that is based of the ability of antioxidant compounds to reduce iron ions of potassium ferrocyanide (Fe^{3+}) to be 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 ferric reducing antioxidant power (FRAP) increased ~~with~~ at higher steeping temperature and longer storage ~~time~~period. The lowest FRAP was observed in the un-stored samples which was steeped at 60 ^{o}C at 3.95 ± 0.17 mg gallic acid equivalents (GAE)/g samples, and the highest was ~~owned~~ exhibited ~~by~~ in *Pluchea*

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infusion which was stored for 5 years at 95 ºC at 48.63 ±10.83 mg gallic acid equivalents (GAE)/g samples (Figure 2b). FRAP increased significantly as steeping temperature was increased. FRAP of the samples stored for 5 years was also significantly higher than the un-stored samples at $\alpha \leq 0.05$. ~~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) between FRAP and DPPH, TPC, TTC and TFC were 0.956, 0.953, 0.948 and -0.826, respectively~~.

This ~~case was~~ is in contrast ~~to~~ with the study on the antioxidant activity of DPPH and FRAP ~~on~~ of matcha., ~~because The~~the longer storage ~~time~~period reduces the levels of catechin content ~~due to the catechins,~~ such as epigallocatechin gallat (EGCG), epicatechin gallat (ECG), epigallocatechin (EGC), and epicatechin (EC) which are bioactive compounds that have high antioxidant activity (Kim et al. 2020)., ~~and also the case of the effect of temperature and storage time in betel (*Piper bettle* 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). Thanajiruschaya et al. (2010) revealed that the antioxidant activity of rice stored at high temperatures is greater than that stored at low temperatures.~~ The ferric reducing capability of ***Pluchea*** could have due ~~infusion corresponded~~to the presence ~~to~~ of simple phenolic acid ~~values~~ that have the ability to transfer electron from their free hydroxyl groups of~~, presence of them in samples could accrue antioxidant activity because of ability of the electron transfer from free hydroxyl groups of phenolic acids~~.

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The FRAP of *Pluchea* infusion was strongly and positively significant correlated with the DPPH, TPC and TTC, but inversely to TFC.

ANTIDIABETIC ACTIVITY

α-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 2-degestive enzymes i.e., α-amylase and α-glucosidase, which are involved 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 α-amylase and α-glucosidase enzymes (Martinez-Solis et al., 2022) resulting in the reduced activity of the enzymes. The results showed~~,~~ that ~~the~~ lower steeping ***Pluchea*** leaf infusion was able to inhibit the action of the α-amylase enzymes (Figure 3a). The ***Pluchea*** infusion ~~had very good activity,~~exhibited a good α-amylase enzyme inhibition activity of more than 50 % and even almost 100 % ~~for fresh~~in the un-stored ***Pluchea*** infusion ~~which~~ steeped ~~was brewed~~ at 60, 70 and 80 ºC with highest at 60 ºC, and in stored *Pluchea* leaf infusion which was steeped at 60 ºC. ~~Whereas~~ The stored ~~fresh~~ ***Pluchea*** leaf infusion steeped at 70, 80 and 95 ºC for 5 minutes had lower enzyme inhibition activity~~an activity of~~ of ~~less than 50 % with lowest at~~ 95 ºC.~~inhibiting the α-amylase enzyme of less than 50 %, which was equal to 40.08±1.12~~ %. Widyawati et al. (2017) ~~detected~~ found that the ability to inhibit the α-amylase enzyme ~~from~~ in ~~fresh~~ un-stored ***Pluchea*** infusion steeped at 95 ºC for 5 minutes ~~by~~ was also low at 28.79 %. Increasing the steeping temperature and storage ~~time~~period reduced the ability ~~to~~ of the phytochemicals in the *Pluchea* infusions to inhibit the α-amylase enzyme activity. ~~The~~

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~~results of the analysis based on a paired T test at α ≤ 0.05 showed, that the steeping temperature and storage time~~period ~~had a significant effect on the ability to inhibit the α-amylase enzyme~~. ~~Based on Pearson correlation, the~~Table 2 further shows that the AA of *Pluchea* infusion was strongly and negatively significant correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively significant correlated with TFC. ~~The correlated coefficient values (r) between AA and TPC, TTC, DPPH, FRAP and TFC were -0.708, -0.857, -0.696, -0.806 and 0.429, respectively.~~

This inhibitory activity was thought to be contributed by other bioactive compounds, besides phenolics which are sensitive to steeping temperature and storage ~~time~~period. Li et al. (2018) stated that there are flavonoid compounds that contribute to the ability to inhibit the α-amylase enzyme. ~~Flavonoid compounds with a hydroxyl structure at C-4' in ring B are more effective than C-6 in ring A.~~ Akah et al. (2011) ~~informed~~ reported that the phytochemical compounds, such as terpenoids, saponins, flavonoids, glycosides and carbohydrate, and alkaloids are good antidiabetic metabolites or α-amylase enzyme activity inhibitor. Sangeetha and Vedasree (2012) explained, that the ability to inhibit the α-amylase enzyme was determined by the content of the phenolic compound and protein. The α-amylase inhibitor enzyme present in *Pluchea* infusion may be proteinaceous in nature. Aleixandre et al. (2022) informed that phenolic acids have inhibition activity to α-amylase enzyme depending their structures. Besides that, capability of phenolic acids to inhibit α-amylase enzyme was determined by low half-maximum inhibitory concentration (IC_{50}). There are C=C double bond conjugated with a carbonyl group of phenolic structures that stabilizes the binding forces to the active site of the α-amylase. The hydroxyl groups of them are able to bind by non-covalent interaction, such as hydrogen

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binding, cation-π interactions, salt bridge interactions, ionic interactions or electrostatic forces with amino acid residue at the active site in α-amylase enzyme. Elevated steeping temperature and longer storage period ~~The steeping temperature and storage time~~ can easily cause the removal of the ~~e~~ hydroxyl groups of phenolic compounds that can reduce their ~~ir~~ ability of enzyme inhibition. The phenolic acids with a greater number of hydroxyl groups ~~are~~ exhibits stronger capability~~le~~ to obstruct the α-amylase enzyme.

α-Glucosidase enzyme inhibition activity (GA)

Alpha~~α~~-glucosidase is an important enzyme in carbohydrates digestion, that catalysis the hydrolysis of 1,4-α-bonds of the unabsorbed oligo- and disaccharides, and converts them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et al., 2014; Proenca et al., 2017). The ability of bioactive compounds to inhibit the α-glucosidase enzyme is used to determine their antidiabetic~~s~~ 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 brewing fresh *Pluchea* infusion at 95 ºC for 5 minutes has an inhibitory effect on the α-glucosidase enzyme of 67.857 %.

~~The results showed,~~Figure 3b shows that the ability of the Pluchea leaf infusion to inhibit the α-glucosidase enzyme decreased with increasing steeping temperature and storage ~~time~~period. Steeping at 95 ºC ~~for fresh~~of the un-stored *Pluchea* leaf infusion ~~(un-stored)~~ obtained the lowest inhibitory ability, i.e., 48.32 ± 1.27 %, and the highest inhibitory activity was f~~ound~~ at 70 ºC ~~steeping temperature for fresh *Pluchea* infusion, which was~~at 95.11 ± 0.70%. ~~(Figure 3b).~~ The results of a paired T test showed that GA of *Pluchea* infusion was significantly different ~~at both~~between steeping temperature and long storage. ~~The antidiabetic activity of *Pluchea* infusion~~Figure 3 further ~~showed~~ shows that the ability

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of *Pulchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-amylase enzyme. Li et al. (2018) informed that flavonoid compounds have the ability to inhibit the action of the α-amylase and α-glucosidase enzymes. This is due to the total flavonoids in steeped *Pluchea* infusion which tended to have the same pattern as the ability to inhibit the activity of the α-amylase and α-glucosidase enzymes. The statistical analysis using Pearson correlation showed that GA of *Pluchea* infusion was strongly and negatively correlated with TPC, TTC, DPPH and FRAP ~~, with r was -0.555, -0,715, -0.527 and -0.560, respectively.~~ However, GA was moderately and positively correlated to TFC~~, with r was 0.350 and strongly~~ and positively correlated to AA~~, with r was 0.725~~. Flavonoid compounds, such as rutin, myricetin, kaempferol, and quercetin ~~which~~ 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 and the number of double bonds in rings A and B and the heterocyclic ring in ring C. The ability to inhibit the α-glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping temperature and long storage. The capability of *Pluchea* infusion to obstruct the α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of two enzymes was different, according to the opinion of McCue et al. (2005). Widyawati et al. (2017) informed that phenolic and non-phenolic compounds determine the inhibitory activity of the α-glucosidase enzyme. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, that may be occurred in *Pluchea* infusion during storage, affects the structure and profile of phenolic and non-phenolic compounds. Asriningtyas et al. (2014) claimed

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that *Pluchea* leaves contain 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. Quinic acid is methyl esterified with the number of caffeic groups in the molecule that determines the activity of inhibiting the α-glucosidase enzyme. Analysis of caffeoylquinic acids in *Pluchea* infusion was obtained that the higher steeping temperature and long storage caused increased concentration of them, but the α-glucosidase inhibition activity of them was reduced. Aleixandre et al. (2022) reported that the simple phenolic acids forming a dipole-dipole interaction of active site from α-glucosidase enzyme are effectively inhibiting the enzyme.

This study ~~was obtained information~~showed that the increasing of steeping temperature and storage ~~time~~period caused a degradation reaction 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, supported the results of total phenolic content and total tannin content assays. Increased concentration of simple phenolic compounds determined the ability of these compounds as antioxidant agents, but reduced their capability as antidiabetic agents.

CONCLUSION

The steeping temperature and storage ~~time~~ period of *Pluchea* infusion significantly influenced bioactive contents, antioxidant and antidiabetic activities. TPC, TTC, and TFC were significantly different at various steeping temperature and storage period based on statistical analysis using a paired ~~T~~ t-test at $\alpha \leq 0.05$. ~~There was the difference of t~~The

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1) 'Figure 3b shows that the ability of the Pluchea leaf infusion to inhibit the α-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 α-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 α-glucosidase enzyme was greater than the α-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 α-amylase and α-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 α-glucosidase enzyme. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. (May also be incorporated in 1).

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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

phenolic compound profile in ~~fresh~~ the unstored and stored ~~of~~ *Pluchea* infusion ~~and~~ at various steeping temperature~~.~~ ~~The~~ included simple phenolic compounds ~~were detected in *Pluchea* infusion including~~such as gallic acid, (+)-catechin, quercetin, myricetin, kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoilquinic acid, and 4,5-di-*O*-caffeoilquinic acid. The results of statistical analysis using a paired ~~T~~ t-test at $\alpha \leq 0.05$ showed that gallic acid and kaempferol of *Pluchea* infusion were insignificantly different at various steeping temperature and long storage. ~~Nevertheless, T~~the concentration of quercetin and 3,5-dicaffeoylquinic acid of *Pluchea* infusion was significantly different of two treatments except at 70 ^{o}C. The (+)-catechin concentration of *Pluchea* infusion was significantly different at 95 ^{o}C, but the myricetin was different concentration at 80 and 95 ^{o}C. The 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid compounds from *Pluchea* infusion were significantly different at 60 ^{o}C, however the concentration of 3,4-dicaffeoylquinic acid was also significantly different at 80 and 95 ^{o}C. TPC, TTC and TFC of *Pluchea* infusion were significantly different at various steeping temperature and storage period. TPC and TTC significantly increased with increasing steeping temperature and long storage, but TFC significantly increased at various steeping temperature and significantly decreased at long storage. The bioactive compounds of *Pluchea* infusion influenced antioxidant activities (DPPH and FRAP) and antidiabetic activity (AA and GA). The DPPH was strongly and positively correlated with TPC and TTC, but it was strongly and negatively correlated with TFC, with coefficient r 0.993, 0.942, and -0.940, respectively. The correlated pattern between FRAP and bioactive contents of *Pluchea* infusion was similar to it between DPPH and bioactive contents. The correlated coefficient values (r) between FRAP and TPC, TTC and TFC were 0.953, 0.948

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and -0.826, respectively. The AA and GA were strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively significant correlated with TFC. Between the antioxidant activity of DPPH and FRAP and the antidiabetic activity of AA and GA of Pluchea infusion were strongly and positively correlated with correlation coefficient (r) values of 0.956 and 0.725, respectively.

DATA AVAILABILITY

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

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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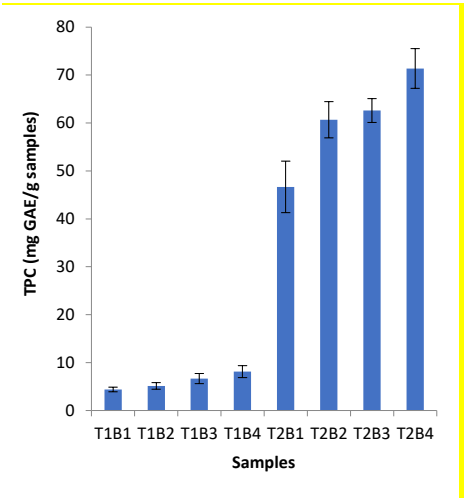
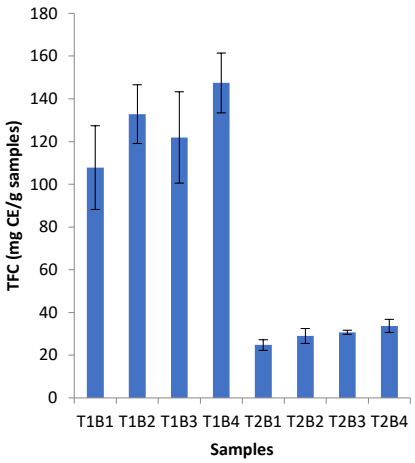
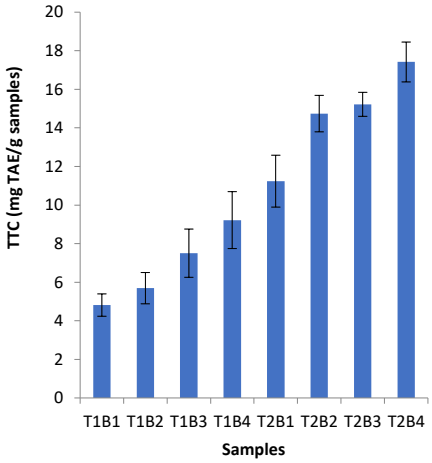




(a) (b)



(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 ±standard deviation (n=6). Samples : T1B1-steeped at 60 ºC, un-stored; T1B2-steeped at 70 ºC, un-stored; T1B3-steeped at 80 ºC, un-

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stored; T1B4-steeped at 95 ºC, un-stored; T2B1-steeped at 60 ºC, stored for 5 years; T3B2-steeped at 70 ºC, stored for 5 years; T3B3-steeped at 80 ºC, stored for 5 years; T3B4-steeped at 95 ºC, 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 $\alpha \leq 0.05$.

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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	-1.1735±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

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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

Note : Data were expressed as mean ±standard deviation (n=2). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}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 $\alpha \leq 0.05$. * $\alpha \leq 0.05$.

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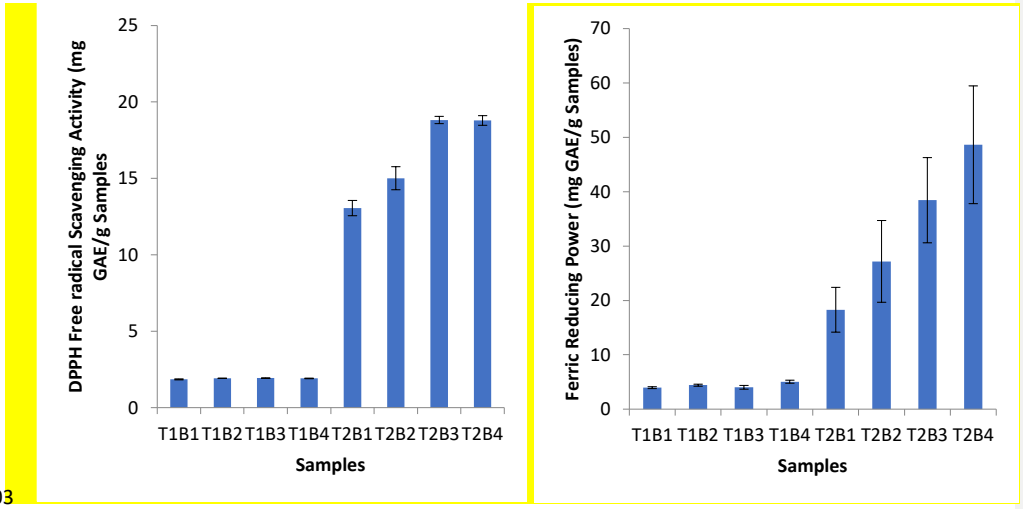

(a) (b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage ~~time~~period (a) DPPH (b) FRAP. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ºC, un-stored; T1B2-steeped at 70 ºC, un-stored; T1B3-steeped at 80 ºC, un-stored; T1B4-steeped at 95 ºC, un-stored; T2B1-steeped at 60 ºC, stored for 5 years; T3B2-steeped at 70 ºC, stored for 5 years; T3B3-steeped at 80 ºC, stored for 5 years; T3B4-steeped at 95 ºC, 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 $\alpha \leq 0.05$~~

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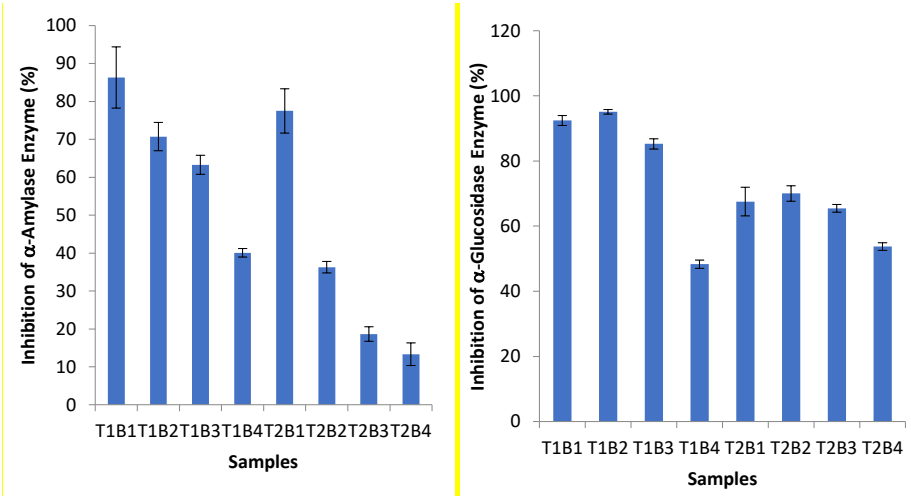

(a) (b)

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage ~~time~~period (a) α-amylase (b) α-glucosidase. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}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 $\alpha \leq 0.05$

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6. Final Decision (17-4-2024)
    -Correspondence
    -Decision Letter
    -Document

Gmail

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> Wed, Apr 17, 2024 at 2:04 PM
To: Paini Sri Widyawati <paini@ukwms.ac.id>

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
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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> Wed, Apr 17, 2024 at 2:32 PM
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

Dear Ms Caryl

Thanks for attention

Regards

Paini SW
[Quoted text hidden]

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Final Decision - PJS Paper Ms 23-158

Philippine Journal of Science <pjs@stii.dost.gov.ph> Fri, Apr 19, 2024 at 5:00 PM
To: Paini Sri Widyawati <paini@ukwms.ac.id>

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]

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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
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[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
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[1st evaluation]
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Paper should be published as a research note/short communication

[2nd evaluation]
Accept paper for publication

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The folder can be accessed through this link: https://bit.ly/3UTfrKK. Thank you!

Sincerely,
Ms. CARYL MARIA MINETTE I. ULAY
Editorial Assistant
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Fwd: Comments on PJS Paper Ms 23-158

Philippine Journal of Science <pjs@stii.dost.gov.ph> Tue, Apr 23, 2024 at 8:57 AM
To: Paini Sri Widyawati <paini@ukwms.ac.id>

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

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

Paini Sri Widyawati[1*)], Yufita Ratnasari Wilianto[2)]

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Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea indica* Less, storage ~~time~~period

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ABSTRACT

This study was done to determine the effects of steeping temperature and storage ~~time~~period on the bioactive contents, 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 ~~time~~period (B). The ~~variety of the~~ *Pluchea* leaf blades were exposed to 4 steeping temperatures ~~included~~ of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) ~~(~~ºC~~)~~ with the storage ~~time~~period ~~–~~period of 0 (B1) and 5 (B2) ~~(~~year~~)~~. ~~The research resulted~~resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). Statistical analysis using a paired t-~~T~~ test at $\alpha \leq 0.05$ showed that treatments significantly affected ~~influenced~~ the bioactive contents (total phenol (TPC), total tannin (TTC), total flavonoid (TFC)), antioxidant [(DPPH scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)]~~)~~ potential ~~—~~and antidiabetic [(α-amylase (AA) and α-glucosidase (GA) ~~inhibitors~~inhibition)] ~~activities~~ properties of the *Pluchea* leaf infusion~~samples~~. TFC decreased during storage period but significantly increased at higher steeping temperature. ~~The AA and GA of *Pluchea* infusion increased until 70 ºC of the steeping temperature, but deceased until 95 ºC. The bioactive contents influenced antioxidant and antidiabetic activities. TFC was decreased for storage time and significant increased at higher steeping temperature. The AA and GA of *Pluchea* infusion increased until 70 ºC of the steeping temperature, but deceased until 95 ºC.~~ The AA ~~-~~and GA were strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively correlated with TFC. ~~Between T~~the antioxidant activity ~~of DPPH and FRAP~~ and the antidiabetic activity ~~of AA and GA~~ of Pluchea infusion were strongly and positively correlated~~.~~ ~~with correlation~~

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coefficient (r) values of 0.956 and 0.725, respectively. The treatments gave different effect of simple phenolic compounds derived from *Pluchea* leaf infusion at different steeping temperatures and storage included, such as 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. of *Pluchea* infusion at different steeping temperature and long storage. To obtain high antioxidant activity, *Pluchea* infusion selected was stored and steeped at high temperature, however high antidiabetic activity obtained was fresh *Pluchea* infusion and steeped at low temperature.

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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 an herbal plant that has been widely used for traditional medicine and food (Chan et al., 2022). *Pluchea* leaves are composed 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, β-carotene, and vitamin C, whereas bioactive compounds is 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, β-carotene, and total carotenoid (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Widyawati et al., 2022, Chan et al., 2022).

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Steeping process of *Pluchea* leaves can be performed with fresh or dry leaves ~~infusion by~~in hot or boiling water for a few minutes (Suriyaphan, 2014; Silva-Ramirez et al., 2020; Jayani et al., 2022). In Asia~~n area~~, especially in Indonesia~~n~~, people usually consume the *Pluchea* infusion ~~with brewing of~~by steeping 2 g of powdered *Pluchea* leaves in tea bag ~~by~~in 100 mL of hot ~~water~~ or boiling water. ~~Each tea bag contained 2 g of *Pluchea* leaf powder is steeped with 100 mL hot water or boiling water.~~ Widyawati et al. (2016) claimed that steeping of 2 g of *Pluchea* leaf powder at 95 ^{o}C for 5 minutes ~~results~~ exhibits total phenolic ~~content,~~and total flavonoid content~~s~~, the ability to scavenge DPPH free radicals, and the capability ~~of~~ to reduce ferric ions at 9.3 mg gallic acid equivalent (GAE)/g sample~~s~~, 22.0 mg gallic acid equivalent (GAE)/g sample~~s~~, 27.2 mg gallic acid equivalent (GAE)/g sample~~s~~, and 10.2 mg gallic acid equivalent (GAE)/g sample~~s~~, respectively. Werdani and Widyawati (2018) reported that drinking of *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 ^{o}C for 5 minutes certainly determines the stability and amount of extracted bioactive compounds~~,~~ that influences the biological activity~~,~~ especially antioxidant and antidiabetic activities. Silva-Ramirez et al. (2020) reported that the infusion process can influence the~~ir~~ content and composition of the bioactive compounds and antioxidant activity of tea. Acar et al. (2022) informed that infusion quality of herbal tea extract depends on several factors, i.e., ~~time~~ storage and temperature. Polyphenol profile and antioxidant properties of herbal tea infusion decline with an increase in steeping/brewing and storage temperatures, and longer exposure ~~time~~period~~s~~.

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Several studies have mentioned the effect of steeping temperature ~~to~~ on the bioactive compound contents and antioxidant activity, such as some white and green teas are effective with hot water at 90 ^{o}C for 7 min (Castiglioni et al., 2015), on roseship tea is effective~~ly~~ at infusion ~~time~~period around 6-8 min at temperatures of 84-86 ^{o}C (Ilyasoglu and Arpa, 2017), on the caffeine content extracted ~~the coffee~~at the brewing temperature of coffee~~influences the caffeine content extracted~~ (Zarwinda and Sartika, 2018), and ~~the steeping~~ the high total phenol content and antioxidant activity of dark tea at 92 ^{o}C for 27 min ~~results the highest total phenol content and antioxidant activity~~ (Wang et al., 2022). The study of the effect of steeping temperature to *Pluchea* infusion was carried out to afford information about the most efficient preparation of powdered *Pluchea* leaves ~~most efficiently~~ to get higher ~~the~~ bioactive compounds, antioxidant and antidiabetic activities.

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

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Therefore, this research studied the effect of steeping temperature and storage ~~time~~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 [(α-amylase (AA) and α-glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea* leaves. ~~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 antioxidant power (FRAP), α-amylase (AA) and α-glycosidase (GA) inhibition activities,~~ 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 *Asteraceae* family with specification according to the GBIF taxon ID number database:3132728 (Ferraris, 2023). *Pluchea* leaves at 1-6 level of each branch ~~of~~from the shoot were collected, sorted, washed and dried to ~~get~~ a moisture content of around 11.16 ± 0.09 % dry bas~~is~~ (Widyawati et al., 2022). The ~~powdering of~~ dried *Pluchea* leaves was ~~done~~ pulverized to ~~get~~ a 45-mesh size powder. ~~And then, the heating of~~ T~~t~~he *Pluchea* leaf powder was ~~done using a drying~~dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 ºC for 10 min to reduce microbial organisms. ~~and~~ Then, 2 g of the powder were packed ~~using~~ into a paper filter infusion bag. ~~that made from paper filter around 2 g/bag~~. ~~And~~

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~~then all of samples called~~Packed samples were ~~*Pluchea* herbal tea was~~ stored for 0 (un-stored) and 5 (stored)years in standing pouch before analysis.

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

REAGENTS

The ~~compounds~~reagents used ~~to analyze~~in the analyses ~~including~~ include 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid, α-amylase, α-glucosidase, pNPG (p-nitrophenyl-α-glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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 distillated water which was purchased from PT Aqua Industry Surabaya.

METHODOLOGY

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 ^{o}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..

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TOTAL PHENOLIC CONTENT ANALYSIS

Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the technique by Gao et al. (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. And then 2 mL Na_2CO_3 7.5 % was ~~entered~~ added and filled up to 10 mL volume with distilled water.~~and distillated water was added until 10 mL volume~~. The color intensity of solution was measured in the spectrophotometer UV-Vis 1800 (Shimadzu, Japan) at λ 760 nm with gallic acid as the reference standard. The total phenolic content was calculated using the formula: y=0.00009x+0.008 with R^2=0.9941. The results were expressed as mg gallic acid equivalent (GAE)/g samples.

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TOTAL FLAVONOID CONTENT ASSAY

Total flavonoid content (TFC) of the samples was measured based on the reaction between $AlCl_3$ and $NaNO_2$ with ~~an~~ the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim et al., 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted in a yellow solution. About 30 µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5 % in 10 mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 10 % for 5 min. And then, 2 mL NaOH 1 M and distillated water were added until 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 λ 510 nm with (+)-catechin as the reference standard compound, and the results were expressed as mg catechin equivalents (CE)/g samples using the formula: y=0.00008x-0.0023 with R^2= 0.9980.

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TOTAL TANNIN CONTENT ANALYSIS

Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method (Chandran and Indira, 2016). Approximately 10 µL *Pluchea* infusion was added with 1 mL Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flash and incubated for 5 min. Then, the mixture was added with 2 mL Na_2CO_3 7.5 % and filled up to 10 mL volume with distillated water. ~~was added until 10 mL volume~~. The blue dark color solution ~~that~~ was measured in UV-Vis spectrophotometer 1800 (Shimadzu, Japan) at λ 760 nm with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/g samples used the formula: $y=0.00009x+0.0021$ with $R^2=0.9993$

ANALYSIS OF THE ANTIOXIDANT POTENTIAL

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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 ~~antioxidant activity of~~in the *Pluchea* leaf infusion to ~~donor~~ donate hydrogen atom to the nitrogen atom in DPPH resulting in the formation of –DPPH-H compound ~~with~~ exhibiting a yellow-colored solution. About 25 µL *Pluchea* leaf infusion was ~~entered~~ poured into reaction tube ~~and~~ into which was added added 3 mL DPPH solution (4 mg/100 mL). ~~And then the solution was~~After incubation~~ed~~ for 15 min in a dark room , the ~~and~~ absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ. 517 nm. The reference

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standard compound was gallic acid and the results of analysis were expressed as mg gallic acid equivalents (GAE)/g samples that calculated using formula: y=0.146x+1.7896 with R^2=0.9975.

FERRIC REDUCING POWER ANALYSIS

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

α-AMYLASE ENZYME INHIBITION CAPACITY ASSAY

In vitro inhibition of α-amylase enzyme (AA) followed the procedure as described by Widyawati et al. (2020). Each 500 µL of samples, was mixed with starch 1 % (w/v) and sodium acetate buffer pH 5. ~~were mixed~~. ~~Then,~~ Into a~~each~~ 250 µL of the mixture ~~and~~ was added an α-amylase solution (0.1 g of this enzyme 12.5 unit/mL) then, was

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dissolved in 50 mL of 0.2 M sodium acetate pH 5~~)~~. Mixture was shaken ~~and~~ into which was added 2 mL sodium hydroxide 1M. Before the analysis, this mixture was incubated at 37 ºC for 10 min. Then, the capacity of the α-amylase enzyme to hydrolyze~~d~~ the starch to release glucose was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) ~~that could be analyzed based on absorbance~~ at λ 540 nm. The inhibition percentage of α-amylase was assessed using the formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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 test sample with enzyme, Ab is absorbance of test sample without enzyme.

α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

The analysis of the α-glycosidase inhibitor activity (GA) was done by Widyawati et al. (2020) method with slight modification. About 150 µL samples ~~contained~~ containing 100 µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH 7) were reacted with 50 µL α-glycosidase 2 mM (0.0833 unit/mL), and then the mixture was incubated at 37 ºC for 15 min. ~~Finally, the~~The reaction was stopped with the addition of 1000 µL sodium carbonate 0.2 M. The residue of this enzyme hydrolyzed p-nitrophenyl-α-D-glucopyranoside (pNPG) as a substrate to result p-nitrophenol. The inhibition~~or~~ activity of ~~steeping~~the ~~*Pluchea* tea~~ infusion ~~to enzyme~~ was measured by UV-vis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at λ 405 nm. The inhibition percentage of α-glycosidase was calculated using formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 100 %. Where, ACb

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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, Ab is the absorbance of test sample without enzyme.

~~HPLC~~ ANALYSIS OF PHENOLICS

The phenolic compounds of the samples were analyzed by HPLC based on Kongkiatpaiboona et al. (2018) method with modifications. Each *Pluchea* infusion was sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter syringe (Whatmann, 0.2 µm, NYL). About 20 µL of 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 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (2 pieces) (ID 10 mm x 4.6 mm). ~~Analytical conditions:~~ 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 minutes; then 100 % B was maintained for 20 minutes. Next the column was re-equilibrated with 10 % B in A maintained for 10 minutes before analysis of the next sample. The sample flow rate was set at 1.0 ml/min with a controlled temperature at 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

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dissolved in distillated water and prepared similar to the samples before 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 ~~time~~period (B). *Pluchea* leaf blades were subjected to 4 steeping temperatures, namely 60 ^{o}C (T1), 70 ^{o}C (T2), 80 ^{o}C (T3), and 95 ^{o}C (T4), and the storage ~~time~~period of 0 year /~~fresh~~ un-stored (B1), and 5 year/stored (B2).~~.~~ ~~The research resulted~~resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). The HPLC analysis of phenolic was repeated two ~~time~~periods. The data of samples were analyzed by ANOVA at $\alpha \leq 0.05$, and continued analysis using a paired T test at $\alpha \leq 0.05$. treatment means of specific phenolic compounds that were identified were expressed as the mean ± SD. The analysis used SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

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RESULTS AND DISCUSSIONS

Pluchea leaf infusion is produced by young *Pluchea* leaf from 1-6 level on each branch the shoot (Widyawati et al., 2022), that is steeped at 95 ^{o}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 *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols, cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2 g/100 mL steeping *Pluchea* tea has total phenolic content 9.3 mg gallic acid

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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 related to the composition of phytochemical compounds in *Pluchea* leaves, such as phenolic acids such as chlorogenic acids, caffeic acids, 3-*O*-caffeoylquinic acids, 4-*O*-caffeoylquinic acids, 5-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin; β-carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Chan et al., 2022; Widyawati et al., 2022). Presence of phytochemical compounds in herbal product were 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 of 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). Therefore, this study was conducted to determine the effect of steeping temperature and storage ~~time~~period of *Pluchea* tea on levels of the bioactive compounds, antioxidant and antidiabetic properties and phenolic compound profile.

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BIOACTIVE COMPOUNDS

Phenolics Compounds

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The bioactive compounds are active compounds in plants that are essential to protect a 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 total phenolic content (TPC) of *Pluchea* infusion at different steeping temperature and storage period generally significantly increased with increasing steeping temperature and storage period based on paired ~~T~~ t- test at $\alpha \leq 0.05$ (Figure 1a). Steeped and stored infusion had significantly higher amounts of phenolic compounds than~~t~~ the samples that were steeped and un-stored. Further, the highest total phenolic content was observed in samples infused at 95 ºC and stored for 5 years (at 71.38±4.14 mg GAE/g sample~~s~~) while the lowest was measured in the un-stored samples and infused at 60 ºC (at mg GAE/g sample). Phenolic content of stored samples ~~that were infused at different temperatures~~ that ~~then stored~~ were steeped only at 60 and 95 ºC ~~also~~ 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 the phenolic compounds of the infusions. Results also indicated that phenolic compounds were generally greater in the infusion at high steeping temperatures and long storage period ~~(Figure 1a). This could have been due to that 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~~

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caused the greater the extracted total phenolic content. The temperature of infusion influenced total phenolic content, it could relate to This could have been due tp the fact that the steeping temperature and storage period can 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 longerincreasing contact exposurebetweenof 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 of lychee juice stored at different temperatures of 4 and 45 ºC and different long storage (fresh and 72 hours).

. this compounds and water. The same phenomena also occurred in Castiglioni et al. (2015); Kilic et al. (2017), and Acar et al. (2022).

This occurrence showed that steeping temperature and storage period caused the process of degradation and oxidation of phenolic 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 ºC and different long storage (fresh and 72 hours). Hydrogen bonding is affected by Ttemperature treatment because thedegrades (or hdrolyzes) the hydrogen bond between phenolic compounds and proteins can be degraded that the measured levels resulting in an increase of phenolic compounds when exposed toare higher temperatures. The phenomena were supported by (Ali et al. (2018); Jayani et al. (2022), and Ramphinwa et al. (2023). Zhang et al. (2021) reported that phenolic compounds

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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. ~~Besides that,~~ 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 ~~that the phenolic compounds in *Pluchea* infusion are degraded due to oxidation and hydrolysis because of temperature and storage time~~period ~~and can be easily extracted during steeping, thus~~resulting in the increased amount of ~~ing the~~the ~~phenolic content~~ compounds ~~as the~~ at higher steeping temperature and longer storage ~~increase~~period.

~~Based on using of a reference standard could be informed that~~Simple phenolic compounds identified in steeped and stored ~~ing~~ ***Pluchea*** leaf infusion~~,~~ include~~ing~~ 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 was showed in Table 1. The treatment effects~~results of statistical analysis~~ using a paired T test at $\alpha \leq 0.05$ showed that gallic acid and kaempferol contents ~~of *Pluchea* infusion~~ were insignificantly different at various steeping temperature and ~~long~~ storage periods. ~~Nevertheless, the~~ The concentration of quercetin and 3,5-di-O-caffeoylquinic acid of the un-stored and stored ***Pluchea*** infusion was significantly different from the rest of the samples between ~~of two treatments except~~ at 70 ºC~~. The~~ while (+)-catechin concentration of ***Pluchea*** infusion was only significantly different at 95 ºC~~, but T~~the myricetin content was significantly different ~~different concentration~~ at 80 and 95 ºC. The 3,4-di-O-caffeoylquinic acid content showed significace difference at 60, 80 and 95 ºC ~~and~~ while

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4,5-O-dicaffeoylquinic acid ~~compounds~~ content ~~from *Pluchea* infusion were~~was only significantly different at 60 ºC~~, however the concentration of 3,4-dicaffeoylquinic acid was also significantly different at 80 and 95 ºC.~~

~~Based on the analysis of concentration of~~Results further showed ~~simple phenolic compounds showed~~ that gallic acids and kaempferol were relatively stable ~~phenolic acid because of~~as reflected by the insignificant changes when exposed ~~no changes at~~to the different steeping temperature and storage ~~time~~period. ~~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~~Myricetin, (+)-catechin and 3,4-di-*O*-caffeoylquinic acid showed a drastic ~~increasing~~ increase at higher steeping temperature and longer storage period ~~.~~implying ~~It's meant~~ that these compounds tended to be relatively labile. Quercetin, 3,5-di-*O*-cafffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid underwent moderate changes ~~compared to the other two groups of phenolic acids.~~. T~~t~~herefore, myricetin, (+)-catechin and 3,4-di-*O*-caffeoylquinic acid were easier to dissolve or degraded to form simple phenolic compounds at higher steeping temperature and storage ~~time~~period. ~~can cause macromolecules of three phenolic acids in herbal tea convenient degradable to form simple phenolic compounds for storage, as explained by (~~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.

Flavonoid Content (TFC)

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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 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 α= 0.05 showed that total flavonoid content of *Pluchea* infusion was significantly different between ~~two treatments~~the steeped un-stored and steeped stored samples (Figure 1b). The highest total flavonoid content was exhibited by ~~fresh~~ the un-stored samples steeped at 95 ºC at about 147.42±14.03 mg CE/g sample~~s~~. Total flavonoid content was significantly lower in the stored samples ~~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.

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Tannin Content (TTC)

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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 the samples infused at 60 ºC at about 4.81±0.58 to 17.42±1.04 mg TAE/g samples. ~~which is~~was significantly different ~~lower~~ from that of the lowest tannin content of the stored samples. Among the

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stored and steeped samples, the highest tannin content was observed at samples steeped at 95 ºC 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 ºC about 9.22 ± 1.48 mg TAE/g samples. ~~i~~Indicating that the tannin content was primarily affected by ~~both high steeping temperature and~~ long storage period than high steeping temperature~~and that the presence of high tannin content was primarily brought about by long storage period.~~ ~~Kowalska et al. (2021) informed that~~ The condensation of catechins to tannins ~~of polyphenolic compounds~~ is a dominant process ~~occurred~~ occurring in tea leaves that is accelerated during maceration of raw ~~material~~tea leaves (Kowalska et al. ~~(~~2021) could have had contributed to the observed increase in the tannin content in the treated samples~~. However, the high temperature can degrade polyphenolic compounds to form simple phenolic 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 concentration 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,~~

Although, high temperature and long storage period can cause the degradation of tannins to catechins, Rusita et al. (2019) emphasized that tannins are ~~a~~ polar thermostable complex compounds, that ~~is~~ are resistant to heating, indicating that even with the exposure to high temperature, the tannins still remained high in the treated samples.~~as a result the tannin content in *Pluchea* tea increases with increasing steeping~~

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~~temperature and storage timeperiod, this is caused tannins are thermostable complex compounds.~~

ANTIOXIDANT ACTIVITY

Antioxidant activity is capability of compounds to inhibit the oxidation of macromolecules from biological target that involve 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 antioxidant that have antioxidant capability that depends on their redox properties. The structure of phenolic compounds determine the effectivity to ~~donor~~ donate hydrogen atom which is negatively correlated with the O-H phenolic bond strength. The higher antioxidant power of phenolic compounds is caused by the weaker O-H phenolic bond (Kruk et al., 2022). The mechanism of phenolic compounds ~~is involved~~ as antioxidants ~~through~~ depends on their the ability to donate hydrogen atom and~~s~~, transfer electrons, and as reducing agents and singlet oxygen quenchers (Ali et al., 2005; Huang et al. 2005).

DPPH Free Radical Scavengincg Asctivity

DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate antioxidant activity because this method is simple that is suitable to measure the donating hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of DPPH to change to yellow color (Munteanu and Apetrei, 2021; Baliyan et al., 2022). Figure 2a shows that the free radical scavenging property of the stored and

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steeped samples were significantly higher than the un-stored steeped samples.~~The result of DPPH assay~~ It can also be observed ~~indicates~~ that the free radical scavenging property ~~DPPH values accrued~~ was significantly different among the stored and steeped samples but insignificant among the un-stored and steeped samples.~~at higher steeping temperature and longer storage time~~period.~~ Statistical analysis by ANOVA using a paired T test at α ≤ 0.05 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~~. *Pluchea* infusion stored at room temperature for 5 years resulted in the high ~~DPPH~~ free radical scavenging activity by ~~—~~ more than 100 %.~~ S~~teeping ~~at higher~~ temperatures significantly increased the ~~DPPH~~ free radical scavenging activity in stored *Pluchea* infusion by around 15 to 25 %. ~~Steeping at 80-95 °C in stored *Pluchea* infusion insignificantly affected the free radical. scavenging property of the bioactive compounds (Figure 2a).~~ This implies that that the higher free radical scavenging property was primarily affected by the storage period than steeping temperature. During the storage process it is possible to form complex phenolic compounds which provide a high ability to scavenge ~~DPPH~~ free radicals (Thanajiruschaya et al., 2010)

~~Scavenging~~ The scavenging activity ~~of DPPH free radicals~~ of the the samples was strongly and positively correlated ~~with total~~ with total phenolic and tannin contents~~levels~~, but inversely ~~to~~ with total flavonoid levels~~. Based on Pearson correlation at Table 2, the correlated coefficient values (r) between DPPH and TPC, TTC and TFC were 0.993, 0.942, and -0.940, respectively. During the storage process it is possible to~~

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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 %

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~~form complex phenolic compounds which provide a high ability to scavenge DPPH free radicals (Thanajiruschaya et al., 2010)~~. This ~~research~~ study also demonstrated that longer storage ~~time~~period and higher infusion temperature produced many simple phenolic compounds with free hydroxyl groups capable to donor hydrogen atom to DPPH free radical. Many phenolic acids, such as gallic acids, (+)-catechins, myricetins, kaempferols, quercetins, 3,5-di-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 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 donor hydrogen atom depends on 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 donor hydrogen atom is determined by O-H bond dissociation energy.

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

Ferric Reducing Antioxidant Power (FRAP)

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FRAP is an analysis of antioxidant power of the phytochemical compounds ~~based on the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a color complex, that can be~~

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~~measured at λ 700 nm (Fu et al., 2011; Al-Temimi and Choudhary, 2013). The principle of the assay measures~~ that is based of the ability of antioxidant compounds to reduce iron ions of potassium ferrocyanide (Fe^{3+}) to be 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 ferric reducing antioxidant power (FRAP) increased ~~with~~ at higher steeping temperature and longer storage ~~time~~period. The lowest FRAP was observed in the un-stored samples which was steeped at 60 ºC at 3.95 ± 0.17 mg gallic acid equivalents (GAE)/g samples, and the highest was ~~owned~~ exhibited ~~by~~ in *Pluchea* infusion which was stored for 5 years at 95 ºC at 48.63 ±10.83 mg gallic acid equivalents (GAE)/g samples (Figure 2b). FRAP increased significantly as steeping temperature was increased. FRAP of the samples stored for 5 years was also significantly higher than the un-stored samples at $\alpha \leq 0.05$. ~~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) between FRAP and DPPH, TPC, TTC and TFC were 0.956, 0.953, 0.948 and 0.826, respectively~~.

This ~~case was~~ is in contrast ~~to~~ with the study on the antioxidant activity of DPPH and FRAP ~~on~~ of matcha.~~,~~ ~~because~~ The~~the~~ longer storage ~~time~~period reduces the levels of catechin content ~~due to the catechins,~~ such as epigallocatechin gallat (EGCG), epicatechin gallat (ECG), epigallocatechin (EGC), and epicatechin (EC) which are bioactive compounds that have high antioxidant activity (Kim et al. 2020).~~,~~ ~~and also the case of the effect of temperature and storage time in betel (*Piper bettle* L.) extract. Light~~

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~~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). Thanajiruschaya et al. (2010) revealed that the antioxidant activity of rice stored at high temperatures is greater than that stored at low temperatures.~~ The ferric reducing capability of *Pluchea* could have due ~~infusion corresponded~~to the presence ~~to~~ of simple phenolic acid ~~values~~ that have the ability to transfer electron from their free hydroxyl groups of~~, presence of them in samples could accrue antioxidant activity because of ability of the electron transfer from free hydroxyl groups of phenolic acids~~. The FRAP of *Pluchea* infusion was strongly and positively significant correlated with the DPPH, TPC and TTC, but inversely to TFC.

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ANTIDIABETIC ACTIVITY

α-Amylase enzyme inhibition activity (AA)

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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 2-degestive enzymes i.e., α-amylase and α-glucosidase, which are involved 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 α-amylase and α-glucosidase enzymes (Martinez-Solis et al., 2022) resulting in the reduced activity of the enzymes. The results showed~~,~~ that ~~the~~ lower steeping *Pluchea* leaf infusion was able to inhibit the action of the α-amylase enzymes (Figure 3a). The *Pluchea* infusion ~~had very good activity,~~exhibited a good α-

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amylase enzyme inhibition activity of more than 50 % and even almost 100 % ~~for fresh~~in the un-stored *Pluchea* infusion ~~which~~ steeped ~~was brewed~~ at 60, 70 and 80 ºC with highest at 60 ºC, and in stored *Pluchea* leaf infusion which was steeped at 60 ºC. ~~Whereas~~ The stored ~~fresh~~ *Pluchea* leaf infusion steeped at 70, 80 and 95 ºC for 5 minutes had lower enzyme inhibition activity~~an activity of~~ of less than 50 % with lowest at 95 ºC.~~inhibiting the α-amylase enzyme of less than 50 %, which was equal to 40.08±1.12~~ %. Widyawati et al. (2017) ~~detected~~ found that the ability to inhibit the α-amylase enzyme ~~from~~ in ~~fresh~~ un-stored *Pluchea* infusion steeped at 95 ºC for 5 minutes ~~by~~ was also low at 28.79 %. Increasing the steeping temperature and storage ~~time~~period reduced the ability ~~to~~ of the phytochemicals in the *Pluchea* infusions to inhibit the α-amylase enzyme activity. ~~The results of the analysis based on a paired T test at α ≤ 0.05 showed, that the steeping temperature and storage time~~period ~~had a significant effect on the ability to inhibit the α-amylase enzyme~~. ~~Based on Pearson correlation,~~ ~~the~~Table 2 further shows that the AA of *Pluchea* infusion was strongly and negatively significant correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively significant correlated with TFC. ~~The correlated coefficient values (r) between AA and TPC, TTC, DPPH, FRAP and TFC were -0.708, -0.857, -0.696, -0.806 and 0.429, respectively.~~

This inhibitory activity was thought to be contributed by other bioactive compounds, besides phenolics which are sensitive to steeping temperature and storage ~~time~~period. Li et al. (2018) stated that there are flavonoid compounds that contribute to the ability to inhibit the α-amylase enzyme. ~~Flavonoid compounds with a hydroxyl structure at C-4' in ring B are more effective than C-6 in ring A.~~ Akah et al. (2011)

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~~informed~~ reported that the phytochemical compounds, such as terpenoids, saponins, flavonoids, glycosides and carbohydrate, and alkaloids are good antidiabetic metabolites or α-amylase enzyme activity inhibitor. Sangeetha and Vedasree (2012) explained, that the ability to inhibit the α-amylase enzyme was determined by the content of the phenolic compound and protein. The α-amylase inhibitor enzyme present in *Pluchea* infusion may be proteinaceous in nature. Aleixandre et al. (2022) informed that phenolic acids have inhibition activity to α-amylase enzyme depending their structures. Besides that, capability of phenolic acids to inhibit α-amylase enzyme was determined by low half-maximum inhibitory concentration (IC_{50}). There are C=C double bond conjugated with a carbonyl group of phenolic structures that stabilizes the binding forces to the active site of the α-amylase. The hydroxyl groups of them are able to bind by non-covalent interaction, such as hydrogen binding, cation-π interactions, salt bridge interactions, ionic interactions or electrostatic forces with amino acid residue at the active site in α-amylase enzyme. Elevated steeping temperature and longer storage period ~~The steeping temperature and storage time~~ can easily cause the removal of the ~~e~~ hydroxyl groups of phenolic compounds that can reduce their ~~–~~ability of enzyme inhibition. The phenolic acids with a greater number of hydroxyl groups ~~are~~ exhibits stronger capability~~le~~ to obstruct the α-amylase enzyme.

α-Glucosidase enzyme inhibition activity (GA)

Alpha~~a~~-glucosidase is an important enzyme in carbohydrates digestion, that catalysis the hydrolysis of 1,4-α-bonds of the unabsorbed oligo- and disaccharides, and converts them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et al., 2014; Proenca et al., 2017). The ability of bioactive compounds to inhibit the α-

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glucosidase enzyme is used to determine their antidiabetics 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 brewing fresh *Pluchea* infusion at 95 ^{o}C for 5 minutes has an inhibitory effect on the α-glucosidase enzyme of 67.857 %.

The results showed,Figure 3b shows that the ability of the Pluchea leaf infusion to inhibit the α-glucosidase enzyme decreased with increasing steeping temperature and storage timeperiod. Steeping at 95 ^{o}C for freshof the un-stored *Pluchea* leaf infusion (un-stored) obtained the lowest inhibitory ability, i.e., 48.32 ± 1.27 %, and the highest inhibitory activity was found at 70 ^{o}C steeping temperature for fresh *Pluchea* infusion, which wasat 95.11 ± 0.70%. (Figure 3b). The results of a paired T test showed that GA of *Pluchea* infusion was significantly different at bothbetween steeping temperature and long storage. The antidiabetic activity of *Pluchea* infusionFigure 3 further showed shows that the ability of *Pulchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-amylase enzyme. Li et al. (2018) informed that flavonoid compounds have the ability to inhibit the action of the α-amylase and α-glucosidase enzymes. This is due to the total flavonoids in steeped *Pluchea* infusion which tended to have the same pattern as the ability to inhibit the activity of the α-amylase and α-glucosidase enzymes. The statistical analysis using Pearson correlation showed that GA of *Pluchea* infusion was strongly and negatively correlated with TPC, TTC, DPPH and FRAP

, with r was -0.555, -0,715, -0.527 and -0.560, respectively. However, GA was moderately and positively correlated to TFC, with r was 0.350 and strongly and positively correlated to AA. with r was 0.725. Flavonoid compounds, such as rutin,

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myricetin, kaempferol, and quercetin ~~which~~ 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 and the number of double bonds in rings A and B and the heterocyclic ring in ring C. The ability to inhibit the α-glucosidase enzyme from *Pluchea* infusion was significantly affected by the steeping temperature and long storage. The capability of *Pluchea* infusion to obstruct the α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of two enzymes was different, according to the opinion of McCue et al. (2005). Widyawati et al. (2017) informed that phenolic and non-phenolic compounds determine the inhibitory activity of the α-glucosidase enzyme. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, that may be occurred in *Pluchea* infusion during storage, affects the structure and profile of phenolic and non-phenolic compounds. Asriningtyas et al. (2014) claimed that *Pluchea* leaves contain 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. Quinic acid is methyl esterified with the number of caffeic groups in the molecule that determines the activity of inhibiting the α-glucosidase enzyme. Analysis of caffeoylquinic acids in *Pluchea* infusion was obtained that the higher steeping temperature and long storage caused increased concentration of them, but the α-glucosidase inhibition activity of them was reduced. Aleixandre et al. (2022) reported that the simple phenolic acids forming a dipole-dipole interaction of active site from α-glucosidase enzyme are effectively inhibiting the enzyme.

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This study ~~was obtained information~~showed that the increasing of steeping temperature and storage ~~time~~period caused a degradation reaction 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, supported the results of total phenolic content and total tannin content assays. Increased concentration of simple phenolic compounds determined the ability of these compounds as antioxidant agents, but reduced their capability as antidiabetic agents.

CONCLUSION

The steeping temperature and storage ~~time~~ period of *Pluchea* infusion significantly influenced bioactive contents, antioxidant and antidiabetic activities. TPC, TTC, and TFC were significantly different at various steeping temperature and storage period based on statistical analysis using a paired ~~T~~ t-test at $\alpha \leq 0.05$. ~~There was the difference of t~~The phenolic compound profile in ~~fresh~~ the unstored and stored ~~of~~ *Pluchea* infusion ~~and~~ at various steeping temperature~~.~~ ~~The~~ included simple phenolic compounds ~~were detected in *Pluchea* infusion including~~such as gallic acid, (+)-catechin, quercetin, myricetin, kaempferol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoilquinic acid, and 4,5-di-*O*-caffeoilquinic acid. The results of statistical analysis using a paired ~~T~~ t-test at $\alpha \leq 0.05$ showed that gallic acid and kaempferol of *Pluchea* infusion were insignificantly different at various steeping temperature and long storage. ~~Nevertheless,~~ ~~T~~the concentration of quercetin and 3,5-dicaffeoylquinic acid of *Pluchea* infusion was significantly different of two treatments except at 70 ^{o}C. The (+)-catechin concentration

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1) 'Figure 3b shows that the ability of the Pluchea leaf infusion to inhibit the α-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 α-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 α-glucosidase enzyme was greater than the α-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 α-amylase and α-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 α-glucosidase enzyme. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. (May also be incorporated in 1).

8) Lines 618 t0 629 into 1)

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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 ^{o}C and lowest in the un-stored and steeped at 60 ^{o}C. Un-stored steeped samples exhibited significantly higher flavonoid content than th

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of *Pluchea* infusion was significantly different at 95 ºC, but the myricetin was different concentration at 80 and 95 ºC. The 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid compounds from *Pluchea* infusion were significantly different at 60 ºC, however the concentration of 3,4-dicaffeoylquinic acid was also significantly different at 80 and 95 ºC. TPC, TTC and TFC of *Pluchea* infusion were significantly different at various steeping temperature and storage period. TPC and TTC significantly increased with increasing steeping temperature and long storage, but TFC significantly increased at various steeping temperature and significantly decreased at long storage. The bioactive compounds of *Pluchea* infusion influenced antioxidant activities (DPPH and FRAP) and antidiabetic activity (AA and GA). The DPPH was strongly and positively correlated with TPC and TTC, but it was strongly and negatively correlated with TFC., with coefficient r 0.993, 0.942, and -0.940, respectively. The correlated pattern between FRAP and bioactive contents of *Pluchea* infusion was similar to it between DPPH and bioactive contents. The correlated coefficient values (r) between FRAP and TPC, TTC and TFC were 0.953, 0.948 and -0.826, respectively. The AA and GA were strongly and negatively correlated with TPC, TTC, DPPH and FRAP, but it was moderately and negatively significant correlated with TFC. Between the antioxidant activity of DPPH and FRAP and the antidiabetic activity of AA and GA of Pluchea infusion were strongly and positively correlated with correlation coefficient (r) values of 0.956 and 0.725, respectively.

DATA AVAILABILITY

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

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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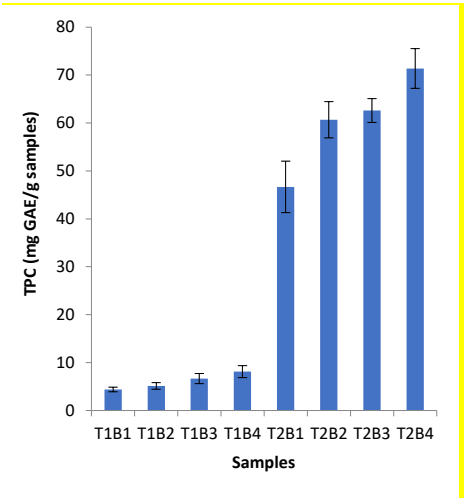

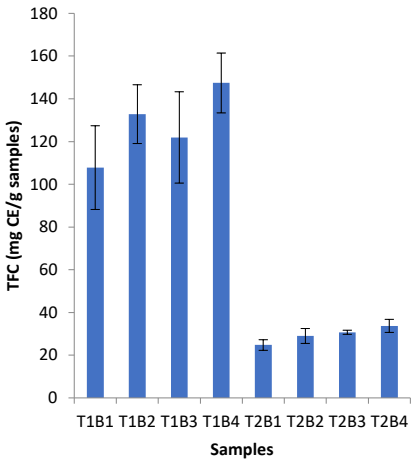

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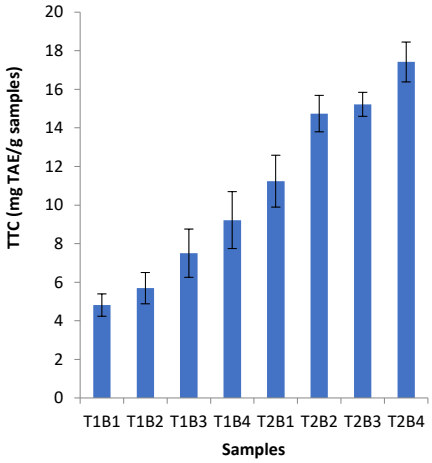

(c)

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 ±standard deviation (n=6). Samples : T1B1-steeped at 60 ºC, un-stored; T1B2-steeped at 70 ºC, un-stored; T1B3-steeped at 80 ºC, un-

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stored; T1B4-steeped at 95 ºC, un-stored; T2B1-steeped at 60 ºC, stored for 5 years; T3B2-steeped at 70 ºC, stored for 5 years; T3B3-steeped at 80 ºC, stored for 5 years; T3B4-steeped at 95 ºC, 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 $\alpha \leq 0.05$.

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909 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	-1.1735±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

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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

Note : Data were expressed as mean ±standard deviation (n=2). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}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 $\alpha \leq 0.05$. * $\alpha \leq 0.05$.

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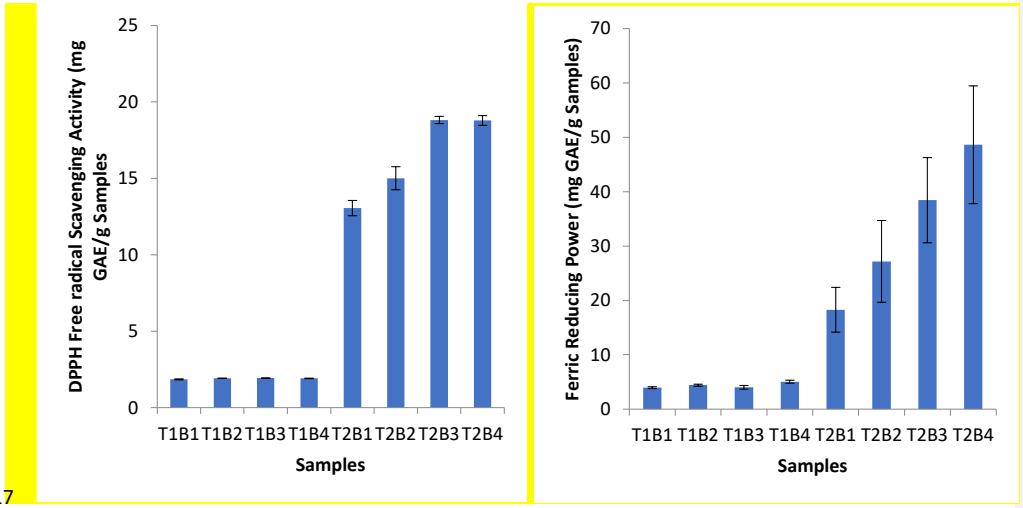

(a) (b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage ~~time~~period (a) DPPH (b) FRAP. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ºC, un-stored; T1B2-steeped at 70 ºC, un-stored; T1B3-steeped at 80 ºC, un-stored; T1B4-steeped at 95 ºC, un-stored; T2B1-steeped at 60 ºC, stored for 5 years; T3B2-steeped at 70 ºC, stored for 5 years; T3B3-steeped at 80 ºC, stored for 5 years; T3B4-steeped at 95 ºC, 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 $\alpha \leq 0.05$~~

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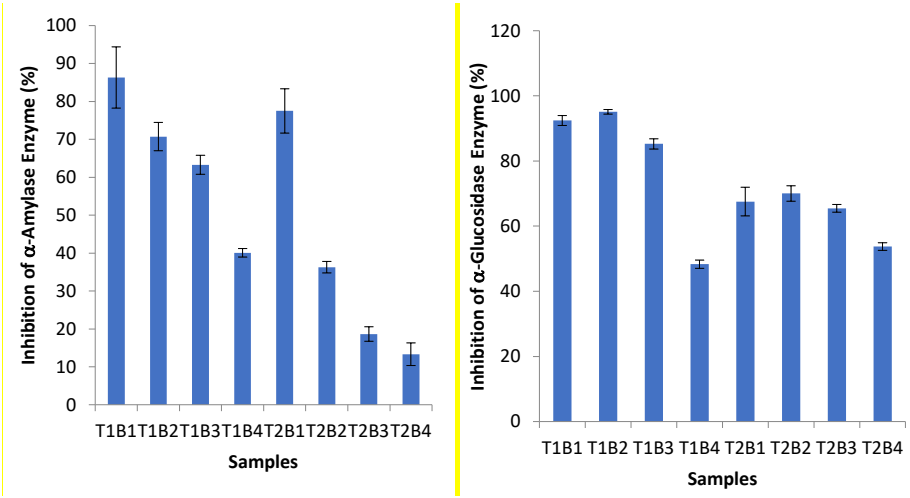

(a) (b)

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage ~~time~~period (a) α-amylase (b) α-glucosidase. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ºC, un-stored; T1B2-steeped at 70 ºC, un-stored; T1B3-steeped at 80 ºC, un-stored; T1B4-steeped at 95 ºC, un-stored; T2B1-steeped at 60 ºC, stored for 5 years; T3B2-steeped at 70 ºC, stored for 5 years; T3B3-steeped at 80 ºC, stored for 5 years; T3B4-steeped at 95 ºC, 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 $\alpha \leq 0.05$

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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

Note: *Correlation Ssignificant at the 0.05 level (2-tailed)

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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 *Pluchea* leaf blades were exposed to 4	Variety of the has been deleted and *Pluchea* 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 ^{o}C	Includes has been deleted, of inserted and ^{o}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 pluchea leaf infusion, TPC decreased during	Properties, the pluchea 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^{o}C of the steeping temperature, but decreased until 95^{o}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^{o}C of the steeping temperature, but decreased until 95^{o}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), α-amylase (AA) and α-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 [(α-amylase (AA) and α-glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea* 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

		all of samples called, pluchea herbal tea was Insert then 2 g of the powder were, into a paper filter, packed samples were , (un-stored), (stored)	tea was…have been deleted and , using, that made from paper filter around 2g/bag, and then all of samples called, pluchea herbal tea was… have been inserted
61	Line 117-122	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 [(α-amylase (AA) and α-glycosidase (GA) inhibition)] of the infusion from powdered *Pluchea* leaves, and on the phenolic compound profile.	Replace to be … .In the research, the one tea bag of *Pluchea* 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 teatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2. After the temperature of *Pluchea* infusion similar to ambient temperature was analyzed further.
62	Line 125	Delete compounds, to analysis, including Insert reagents, in the analysis include	compounds, to analysis, including … has been deleted reagents, in the analysis include … has been inserted
63	Line 137	Change center … analysis of the bioactive compounds	Analysis of the bioactive compounds … has been changed to center
64	Line 142-143	Delete entered, and distillated water was added until 10 mL volume Add color specific Insert …added and filled up to 10 mL volume with distilled water, …..the blue color intensity of	Entered, and distillated water was added until 10 mL volume …. Have been deleted 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

		the solution was measured	
65	Line 151-156	Delete …an…., distillated 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 hydrolized p-nitrophenyl-α-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 *Pluchea* infusion hydrolyzed p-nitrophenyl-α-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 distillated and insert distilled	Distillated 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 α

		test at $\alpha \leq 0.05$'? Rewrite this part of the paragraph.	≤ 0.05, treatment means of specific phenolic compounds that were identified were expressed as the mean ± SD. The analysis used SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).
78	Line 264	Delete *Pluchea* leaf infusion is produced by young *Pluchea* 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 *Pluchea* tea involve alkaloids, flavonoids, phenolics, sterols, cardiac glycosides, phenol hydroquinone, tannins, terpenoids, and saponins, where 2 g/100 mL steeping *Pluchea* 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

		composition of phytochemical compounds in *Pluchea* leaves, such as phenolic acids such as chlorogenic acids, caffeic acids, 3-*O*-caffeoylquinic acids, 4-*O*-caffeoylquinic acids, 5-*O*-caffeoylquinic acids, 3,4-di-*O*-caffeoylquinic acids, 3,5-di-*O*-caffeoylquinic acids, and 4,5-di-*O*-caffeoylquinic acids; total flavonoids which cover quercetin, kaempferol, myricetin, anthocyanin; β-carotene; and total carotenoids (Suriyaphan, 2014; Vongsak et al., 2018; Ruan et al., 2019; Chan et al., 2022; Widyawati et al., 2022). 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).	

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

		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 45oC and different long store (fresh and 72 hours) Delete hydrogen bonding is affected by Delete t Delete because the… Delete can be degraded that the measured levels Delete are the phenomena were supported by Delete and Delete besides that,	increase of phenolic compounds when exposed to higher temperatures (Ali et al. (2018); Jayani et al. (2022) and Ramphinwa et al. (2023)).
87	Line 302	Delete besides that	Besides that…has been deleted
88	Line 304-307	Delete that the phenolic compounds in pluchea infusion are degraded due to .. Delete …and can be easily extracted… Delete ing.. Delete the Delete the.. Delete content Delete as the Delete increase	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.
89	Line 308-319	Delete based on using of a reference Delete ing Delete . (dot) Delete ing Delete results of statistical analysis	Change to be… Simple phenolic compounds are identified in steeped and stored. <u>*Pluchea* leaf</u> infusion included gallic acids, (+)-catechins, myricetins, quercetins, kaempferols, 3,4-di-*O*-

		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^{o}C.	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 $\alpha \leq 0.05$ 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 un-stored and stored *Pluchea* infusion was significantly different from the rest of the samples between 70 ^{o}C while (+)-catechin concentration of *Pluchea* infusion was only significantly different at 95 ^{o}C. The myricetin content was significantly different at 80 and 95 ^{o}C. The 3,4-di-O-caffeoylquinic acid content showed significant difference at 60, 80, and 95 ^{o}C while 4,5-di-O-caffeoylquinic acid content was only significantly different at 60 ^{o}C.
90	Line 320-331	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, (,)	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*-cafffeoylquinic 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), 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.., I.., 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.., I (, ., 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.., I.., 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.., I (, ., 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 ccompounds. 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

		phenol levels and the concentrations of (+)-catechin ccompounds. 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 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. Move ..Kowalska et al. (2021)	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. …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 ..Kowalska et al. (2021)… has been moved
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

		Insert that, s, the, donate, depends on their, and, and as Rewrite: the higher antioxidant power of phenolic compounds is caused the weaker O-H phenolic bond	That, s, the, donate, depends on their, and, and as … has been inserted 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
99	Line 382	Center DPPH free radical scavenging activity	DPPH free radical scavenging activity has been centered
100	Line 387-398	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 $\alpha \leq 0.05$ 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^{o}C in stored pluchea infusion insignificantly affected the free radial, scavenging property of the, DPPH Insert figure 2a shows that the free radical savenging property of the stored and steeped samples were	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 $\alpha \leq 0.05$ 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^{o}C in stored pluchea infusion insignificantly affected the free radial, scavenging property of the, DPPH Figure 2a shows that the free radical savenging property of the stored and steeped samples were significantly higher than the un-stored steeped samples, it can also be observed, free radical

		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) 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) 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 activityin stored pluchea infusion by around 15 to 25%	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 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 Change to be… *Pluchea* 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 *Pluchea* infusion by around 15 to 25 %.
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

		Insert the scavenging, of the samples, with total, content, with, study, period, donate, table 1. Comment: explain this observation based on the data that you were able to obtain	the scavenging, of the samples, with total, content, with, study, period, donate, table 1… have been inserted explain to be … 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.
102	Line 414	Delete DPPH, DPPH, of tea infusion, inhibitor activity of DPPH Insert …In, the free radical scavenging property, of tea infusion	DPPH, DPPH, of tea infusion, inhibitor activity of DPPH have been deleted In, the free radical scavenging property, of tea infusion… have been inserted
103	Line 419	center ferric reducing antioxidant power (FRAP)	ferric reducing antioxidant power (FRAP) has been centered
104	Line 420-424	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 λ 700 nm (Fu et al., 2011; Al-Temi8ni and Choudhary, 2013). Principle of the assay measures, … Insert that is based	…based on the reaction among antioxidant compounds, potassium ferricyanide, trichloroacetic acid, and ferric chloride to produce a color complex, that can be measured at λ 700 nm (Fu et al., 2011; Al-Temi8ni and Choudhary, 2013). Principle of the assay measures, …have been deleted that is based… has been inserted
105	Line 425-432	Delete with,…time, … owned, .. by.., based on pearson correlation, the FRAP of Pluchea infusion was strongly and positively significant correlated with the	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)

		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 Insert at, er, period, exhibited, in, un-	between FRAP and DPPH, TPC, TTC and TFC were 0.956, 0.948, and -0.826, respectively… have been deleted at, er, period, exhibited, in, un-… have been inserted
106	Line 433-440	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 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	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 .. 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

		with the DPPH, TPC, and TTC but inversely to TFC.	
107	Line 443	Center α-amylase enzyme inhibition activity (AA)	α-amylase enzyme inhibition activity (AA) has been centered
108	Line 445-463		digestive… has been deleted digestive … has been inserted
		Delete,, the, had very good activity, for fresh, which, was brewed, whereas, fresh, an activity of, inhibiting the α-alylase enzyme of less than 50%, which was equal to 40.08±1.12, detected, from, fresh, by, time, to, the results of the analysis based on a paired Ttest at α≤0.05 showed, that the steeping temperature and storage time, had a significant effect on the ability to inhibit the α-amylse enzyme, besed on pearson correlation, the, the correlated coefficient..	,, the, had very good activity, for fresh, which, was brewed, whereas, fresh, an activity of, inhibiting the α-alylase enzyme of less than 50%, which was equal to 40.08±1.12, detected, from, fresh, by, time, to, the results of the analysis based on a paired Ttest at α≤0.05 showed, that the steeping temperature and storage time, had a significant effect on the ability to inhibit the α-amylse enzyme, besed on pearson correlation, the, the correlated coefficient.. have been deleted
		Pattern: clear (yellow), not highlight	… has been done
		Insert lower, leaf, exhibited a good α-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,	lower, leaf, exhibited a good α-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

		activity, period, Table 2 further shows that the, formatted	
109	Line 466-485	Insert period, reported, α-amylase enzyme activity inhibition, elevated steeping temperature and longer storage period, easily cause the, al of the, ir, exhibits, ility,	period, reported, α-amylase enzyme activity inhibition, elevated steeping temperature and longer storage period, easily cause the, al of the, ir, exhibits, ility, … have been inserted
		Delete time, flavonoid compounds with…, informed, the steeping temperature and storage time, e,	time, flavonoid compounds with…, informed, the steeping temperature and storage time, e,… have been deleted
			Delete by content and insert content
		Comment : rewrite	ability of *Threspesia populnea* extract to inhibit the α-amylase enzyme was determined of their phenolic compound content and protein. Moreover, the presence of α-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 with their protein and polyphenolic content. Aleixandre et al. (2022) also stated that phenolic acids have inhibition activity to α-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

			α-amylase. The hydroxyl groups can bind by non-covalent interaction (hydrogen bonding, cation-π interactions, salt bridge interactions, ionic interactions, or electrostatic forces) with amino acid residue at the active site in α-amylase enzyme.have been inserted
110	Line 485	center α-glucosidase enzyme inhibition activity (GA)	α-glucosidase enzyme inhibition activity (GA)… has been centered
111	Line 486-512	Delete α, 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	α, 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
		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	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
		Comment : rewrite and delete literature because unnecessary	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 α-amylase and α-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 α-glucosidase enzyme activity.
112	Line 513-548	Rewrite the statement and delete literature not support	Rewrite to be … The ability to inhibit the α-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 α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of the two enzymes was different, according to the opinion of McCue et al. (2005). The mechanism of the α-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 α-amylase enzyme inhibitor includes blocking carbohydrates,

			limiting the digestibility and absorption of carbohydrates, and 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 of the α-glucosidase enzyme activity. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, that may be occurred in *Pluchea* 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-*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 α-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 un-stored *Pluchea* infusion, and the concentrations of the simple phenolic compounds were increased at higher steeping temperature, but the α-glucosidase inhibition activity of them was reduced. It means that the methyl-esterified quinic acid with the caffeic groups had more potential to inhibit α-glucosidase enzyme than free caffeoylquinic acid.

			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 *Pluchea* leaf infusion caused higher antioxidant activity and lower antidiabetic activity.
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	Delete time, T, within group differences at unstored vs stored for 5 years at certain steeping temperatures, calculated using a paired T test at $\alpha \leq 0.05$ Insert period, t, Formatted figure and table, description of figures and tables	time, T, within group differences at unstored vs stored for 5 years at certain steeping temperatures, calculated using a paired T test at $\alpha \leq 0.05$… have been deleted period, t, … have been inserted figure and table, description of figures and tables… have been formetted

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

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Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea indica* Less, storage period

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ABSTRACT

This study was done to determine the effects of steeping temperature and storage period on the bioactive contents, 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 4 steeping temperatures of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) ^{o}C with the storage period of 0 (B1) and 5 (B2) years resulting in 8 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), total flavonoid (TFC)], antioxidant [(DPPH scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)] potential and antidiabetic [(α-amylase (AA) and α-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 ^{o}C of the steeping temperature, but decreased until 95 ^{o}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,

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quercetin, 3,4-di-O-caffeoylquinic acid, 3,5- di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid.

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, β-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, β-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 minutes (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 of 2 g of *Pluchea* leaf powder at 95 ^{o}C for 5 minutes exhibits total phenolic and total flavonoid contents, the ability to scavenge DPPH free radicals, and the capability to reduce ferric ions at 9.3 mg

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gallic acid equivalent (GAE)/g sample, 22.0 mg gallic acid equivalent (GAE)/g sample, 27.2 mg gallic acid equivalent (GAE)/g sample, 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 ^{o}C for 5 minutes certainly determines the stability and amount of extracted bioactive compounds t hat 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) informed that the infusion quality of herbal tea extract depends on several factors, i.e., storage and temperature. The polyphenol profile and antioxidant properties of herbal tea infusion decline with an increase in steeping/brewing and storage temperatures, and longer exposure periods.

Several studies have mentioned the effect of steeping temperature on the bioactive compound contents and antioxidant activity, such as some white and green teas are effective with hot water at 90 ^{o}C for 7 min (Castiglioni et al., 2015), on roseship tea is effective at infusion period around 6-8 min at temperatures of 84-86 ^{o}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 ^{o}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

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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, antioxidant and antidiabetic activities, i.e., juice from *Momordica charantia* L. (Lin et al., 2020), dried *Piper betlle* extracts (Ali et al., 2018), white tea (Xu et al., 2019), kinnow-amla beverages (Purewal et al., 2022), 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), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP)], and antidiabetic activities [(α-amylase (AA) and α-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,

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sorted, washed, and dried to get a moisture content of around 11.16 ± 0.09 % dry basis (Widyawati et al., 2022). The dried *Pluchea* leaves was pulverized to a 45-mesh size powder. The *Pluchea* leaf powder was dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 ^{o}C for 10 min to reduce microbial organisms. Then, 2 g of the powder were packed into a paper filter infusion bag. Packed samples were stored for 0 (un-stored) and 5 (stored) years in standing pouch before analysis.

In the research, the one tea bag of *Pluchea* 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) ^{o}C for 5 min with infusion method obtaining 8 teatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, 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, α-amylase, α-glucosidase, pNPG (p-nitrophenyl-α-glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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

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grade except for distillated water which was purchased from PT Aqua Industry Surabaya.

METHODOLOGY

ANALYSIS OF THE BIOACTIVE COMPOUNDS

TOTAL PHENOLIC CONTENT ANALYSIS

Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the technique by Gao et al. (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. And then 2 mL Na_2CO_3 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 λ 760 nm with gallic acid as the reference standard. The total phenolic content was calculated using the formula: y=0.00009x+0.008 with R^2=0.9941. The results were expressed as mg gallic acid equivalent (GAE)/g samples.

TOTAL FLAVONOID CONTENT ASSAY

Total flavonoid content (TFC) of the samples was measured based on the reaction between $AlCl_3$ and $NaNO_2$ with the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim et al., 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted in a yellow solution. About 30 µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5 % in 10 mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 10 % for 5 min. Then, 2 mL NaOH 1 M and

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distilled water were added until 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 λ 510 nm with (+)-catechin as the reference standard compound, and the results were expressed as mg catechin equivalents (CE)/g samples using the formula: y=0.00008x-0.0023 with R^2= 0.9980.

TOTAL TANNIN CONTENT ANALYSIS

Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method (Chandran and Indira, 2016). Approximately 10 µL *Pluchea* infusion was added with 1 mL Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flash and incubated for 5 min. Then, the mixture was added with 2 mL Na_2CO_3 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 λ 760 nm with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/g samples used the 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 atom to the nitrogen atom in DPPH resulting in the formation of DPPH-H compound exhibiting a yellow-colored solution. About 25 µL *Pluchea* leaf infusion was poured into reaction tube into

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which was added 3 mL DPPH solution (4 mg/100 mL). After incubation for 15 min in a dark room, the absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ. 517 nm. The reference standard compound was gallic acid and the results of analysis were expressed as mg gallic acid equivalents (GAE)/g samples that calculated using formula: y=0.146x+1.7896 with R^2=0.9975.

FERRIC REDUCING POWER ANALYSIS

Ferric-reducing power (FRAP) was determined following the method used by Widyawati et al. (2014) method. Approximately 10 µL 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. And then 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 was added 2.5 mL distilled water, 0.5 mL ferric chloride 0.1% w/v, and incubated for 10 min. 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 λ 700 nm. Intensity of the blue color indicated higher reducing capacity. The reducing power expressed as mg gallic acid equivalent (GAE)/g samples was calculated using the formula: y=0.0002x+0,0256 with R^2=0,9906.

ANALYSIS OF THE ANTIDIABETIC PROPERTIES

α-AMYLASE ENZYME INHIBITION CAPACITY ASSAY

In vitro inhibition of α-amylase enzyme (AA) followed the procedure as described by Widyawati et al. (2020). Each 500 µL of samples, was mixed with starch 1 % (w/v)

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and sodium acetate buffer pH 5. Into a 250 µL of the mixture was added an α-amylase solution (0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in 50 mL of 0.2 M sodium acetate pH 5. Mixture was shaken into which was and added 2 mL sodium hydroxide 1M. Before the analysis, this mixture was incubated at 37 ^{o}C for 10 min. Then, the capacity of the α-amylase enzyme to hydrolyze the starch to release glucose was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ 540 nm. The inhibition percentage of α-amylase was assessed using the formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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 test sample with enzyme, Ab is absorbance of test sample without enzyme.

α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

The analysis of the α-glycosidase inhibitor activity (GA) was done by Widyawati et al. (2020) method with slight modification. About 150 µL samples containing 100 µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH 7) were reacted with 50 µL α-glycosidase 2 mM (0.0833 unit/mL), and then the mixture was incubated at 37 ^{o}C for 15 min. The reaction was stopped with the addition of 1000 µL sodium carbonate 0.2 M. The amount of these enzymes that didn't react with bioactive compounds of *Pluchea* infusion hydrolyzed p-nitrophenyl-α-D-glucopyranoside (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 λ 405 nm. The inhibition percentage of α-glycosidase was

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calculated using formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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, Ab is the absorbance of test sample without enzyme.

ANALYSIS OF PHENOLICS

The phenolic compounds of the samples were analyzed by HPLC based on Kongkiatpaiboona et al. (2018) method with modifications. Each *Pluchea* infusion was sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter syringe (Whatmann, 0.2 µm, NYL). About 20 µL of 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 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (2 pieces) (ID 10 mm x 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 minutes; then 100 % B was maintained for 20 minutes. Next the column was re-equilibrated with 10 % B in A maintained for 10 minutes before analysis of the next sample. The sample flow rate was set at 1.0 ml/min with a controlled temperature at 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

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4,5-dicaffeoylquinic acid. All of reference standard was dissolved in distilled water and prepared similar to the samples before 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 ^{o}C (T1), 70 ^{o}C (T2), 80 ^{o}C (T3), and 95 ^{o}C (T4), and the storage period of 0 year /un-stored (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 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 ± SD. The analysis used SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSIONS

BIOACTIVE COMPOUNDS

Phenolic Compounds

The bioactive compounds are active compounds in plants that are essential to protect a 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;

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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 total phenolic content (TPC) of *Pluchea* infusion at different steeping temperature and storage period 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 un-stored. Further, the highest total phenolic content was observed in samples infused at 95 ^{o}C and stored for 5 years (at 71.38±4.14 mg GAE/g sample) while the lowest was measured in the un-stored samples and infused at 60 ^{o}C (at 4.39±0.49 mg GAE/g sample). The phenolic content of stored samples that were steeped only at 60 and 95 ^{o}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 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

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antioxidant activity of lychee juice stored at different temperatures of 4 and 45 ^{o}C and different long storage (fresh and 72 hours).

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) and 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 temperature and longer storage period.

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 was showed in Table 1. The treatment effects using a 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 un-stored and stored *Pluchea* infusion was significantly different from the rest of the samples between 70 ^{o}C while (+)-catechin concentration of *Pluchea* infusion was only significantly different at 95 ^{o}C. The myricetin content was

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317 significantly different at 80 and 95 ^{o}C. The 3,4-di-O-caffeoylquinic acid content showed
318 significant difference at 60, 80, and 95 ^{o}C while 4,5-di-O-caffeoylquinic acid content was
319 only significantly different at 60 ^{o}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 cafffeoylquinic 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

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t-test at $\alpha \leq 0.05$ showed that the total flavonoid content of *Pluchea* infusion was significantly different between the steeped un-stored and steeped stored samples (Figure 1b). The highest total flavonoid content was exhibited by the un-stored samples steeped at 95^{o}C at about 147.42±14.03 mg CE/g sample. Total flavonoid content was significantly lower in the stored samples than those of the un-stored 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 the samples infused at 60 ^{o}C at about 4.81±0.58 to 17.42±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 ^{o}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 ^{o}C about 9.22 ± 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 had contributed to the observed increase in the tannin content in the treated samples.

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Although, high temperature and long storage period 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 capability of compounds to inhibit the oxidation of macromolecules from biological target that involve 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 antioxidant that have antioxidant capability that depends on their redox properties. The structure of phenolic compounds determines the effectivity to donate hydrogen atom 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 reducing agents and singlet oxygen quenchers (Ali et al., 2005; Huang et al. 2005).

DPPH Free Radical Scavenging Activity

DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate antioxidant activity because this method is simple that is suitable to measure the donating hydrogen atoms capability of herbal infusion. This reaction can cause the

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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

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capability of phenolic compounds to donate hydrogen atom depends on 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 atom is determined by O-H bond dissociation energy.

The free radical scavenging property observed in the study was not in consistent with the results of the study by Moraes-de-Souza et al. (2008). The research shows that total phenolic content of herbal infusion is low correlated with free radical scavenging activity. However, Dobrinas et al. (2021) informed that total phenolic content 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 of 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 ferric reducing antioxidant power (FRAP) increased at higher steeping temperature and longer storage period. The lowest FRAP was observed in the un-stored samples which were steeped at 60 ^{o}C at 3.95 ± 0.17 mg gallic acid equivalents (GAE)/g samples, and the highest was exhibited in *Pluchea* infusion which was stored for 5 years at 95 ^{o}C at 48.63 ±10.83 mg gallic acid equivalents (GAE)/g samples (Figure 2b). FRAP increased significantly as the steeping temperature

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was increased. FRAP of the samples stored for 5 years was also significantly higher than the un-stored 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 gallat (EGCG), epicatechin gallat (ECG), epigallocatechin (EGC), and epicatechin (EC) 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 sample. 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 2-digestive enzymes i.e., α-amylase and α-glucosidase, which are involved 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 α-amylase and α-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 α-amylase enzymes (Figure 3a). The *Pluchea* infusion exhibited a good α-amylase enzyme inhibition activity of more than 50 % and even almost 100 % in un-stored *Pluchea* infusion steeped at 60,

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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 α-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 α-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 α-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 α-amylase enzyme activity inhibitors.
470 Sangeetha and Vedasree (2012) explained that the ability of *Threspesia populnea*
471 extract to inhibit the α-amylase enzyme was determined of their phenolic compound
472 content and protein. Moreover, the presence of α-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 α-amylase enzyme depending on their structures. There are C=C

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477 double bonds conjugated with a carbonyl group of phenolic structures that stabilize the
478 binding forces to the active site of the α-amylase. The hydroxyl groups can bind by non-
479 covalent interaction (hydrogen bonding, cation-π interactions, salt bridge interactions,
480 ionic interactions, or electrostatic forces) with amino acid residue at the active site in α-
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 α-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-α-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 α-
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 ^{o}C for 5 minutes has an inhibitory effect on the α-glucosidase enzyme of
494 67.857 %.

495 Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the α-
496 glucosidase enzyme decreased with increasing steeping temperature and storage
497 period. Steeping at 95 ^{o}C of the un-stored *Pluchea* leaf infusion obtained the lowest
498 inhibitory ability, i.e., 48.32 ± 1.27 %, and the highest inhibitory activity was at 70 ^{o}C at
499 95.11 ± 0.70%. The results of a paired t-test showed that GA of *Pluchea* infusion was

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significantly different between steeping temperature and long storage. Figure 3 further shows that the ability of *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-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 α-amylase and α-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 α-glucosidase enzyme activity.

The ability to inhibit the α-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 α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of the two enzymes was different, according to the opinion of McCue et al. (2005). The mechanism of the α-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 α-amylase enzyme inhibitor

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includes blocking carbohydrates, limiting the digestibility and absorption of carbohydrates, and 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 of the α-glucosidase enzyme activity. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, that may be occurred in *Pluchea* 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-*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 α-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 un-stored *Pluchea* infusion, and the concentrations of the simple phenolic compounds were increased at higher steeping temperature, but the α-glucosidase inhibition activity of them was reduced. It means that the methyl-esterified quinic acid with the caffeic groups had more potential to inhibit α-glucosidase enzyme than free caffeoylquinic acid.

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

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increased the total phenolic content and total tannin content. The increase in the simple phenolic concentration of the *Pluchea* leaf infusion caused higher antioxidant activity and lower antidiabetic activity.

CONCLUSION

The Total Phenol (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 un-stored. TPC was highest in the stored and steeped at 95^{o}C and lowest in the un-stored and steeped at 60^{o}C. Un-stored steeped samples exhibited significantly higher flavonoid content than the stored steeped samples. The highest total flavonoid content was exhibited by the un-stored samples steeped at 95^{o}C. The total tannin content of *Pluchea* leaf infusion significantly increased with increasing steeping temperature and storage period. Among the un-stored steeped samples, the tannin content was significantly lowest in the samples steeped at 60^{o}C and highest in the samples steeped at 95^{o}C.

The free radical scavenging property (DPPH) of the stored and steeped *Pluchea* leaf infusion was significantly higher than the un-stored steeped samples. The free radical scavenging property was highest in the stored samples steeped at 80 and 95^{o}C. 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 ferric-reducing antioxidant power (FRAP) significantly increased with increasing steeping temperature and longer storage periods. The lowest FRAP was found in the un-stored samples which were steeped at 60^{o}C and the highest was exhibited in

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Pluchea stored samples which were stored for 5 years and steeped at 95ºC. The FRAP of *Pluchea* leaf infusion was significantly strong and positively correlated with the free radical scavenging property, total phenolic, and total tannin content, but inversely with total flavonoid content. The inhibition of the α-amylase activity was generally found to be higher at lower steeping temperatures of the un-stored *Pluchea* leaf infusion than at higher steeping temperatures of the stored sample. The α-amylase enzyme inhibition 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 α-glucosidase enzyme decreased at high steeping temperatures and long storage periods. The highest inhibitory activity was obtained in the un-stored *Pluchea* leaf infusion that was steeped at 70ºC while the lowest was obtained in the un-stored sample that was steeped at 95ºC. The ability of *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-amylase enzyme. The inhibition of the α-glucosidase enzyme activity 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, 4,5-di-*O*-caffeoylquinic acids.

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DATA AVAILABILITY

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

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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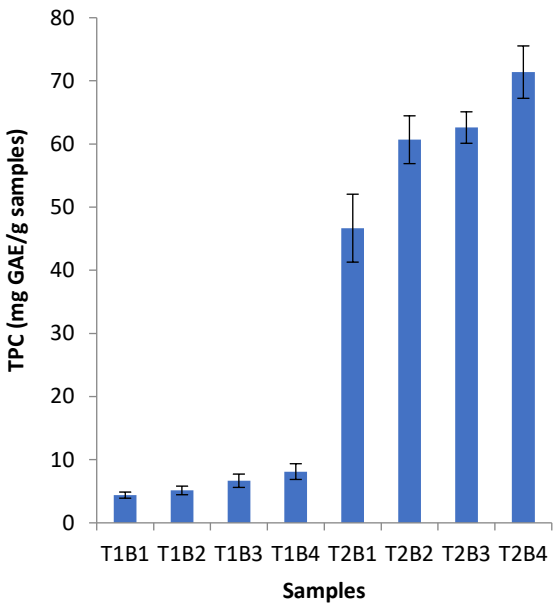

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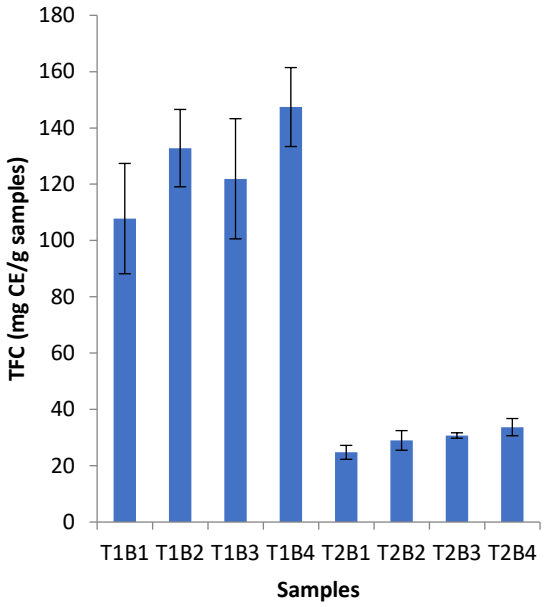

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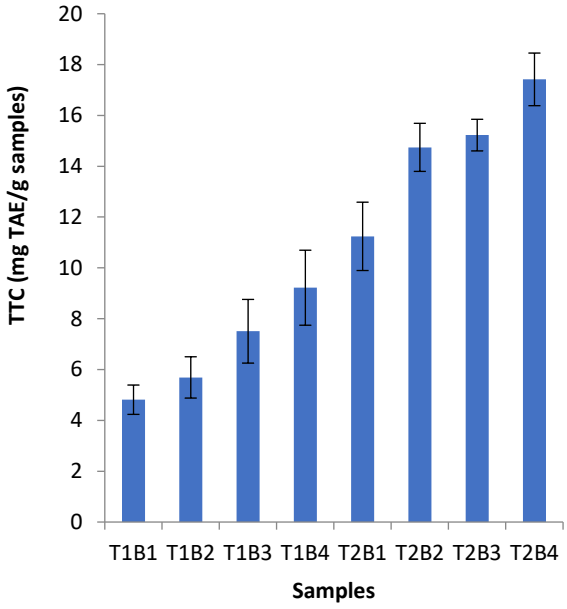

(c)

Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping temperature and storage period (a) Total phenolic content (b) Total flavonoid content (c) Total tannin content. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paited t-test at $\alpha \leq 0.05$. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-

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stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}C, stored for 5 years.

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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

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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 paited t-test at $\alpha \leq 0.05$. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}C, stored for 5 years.

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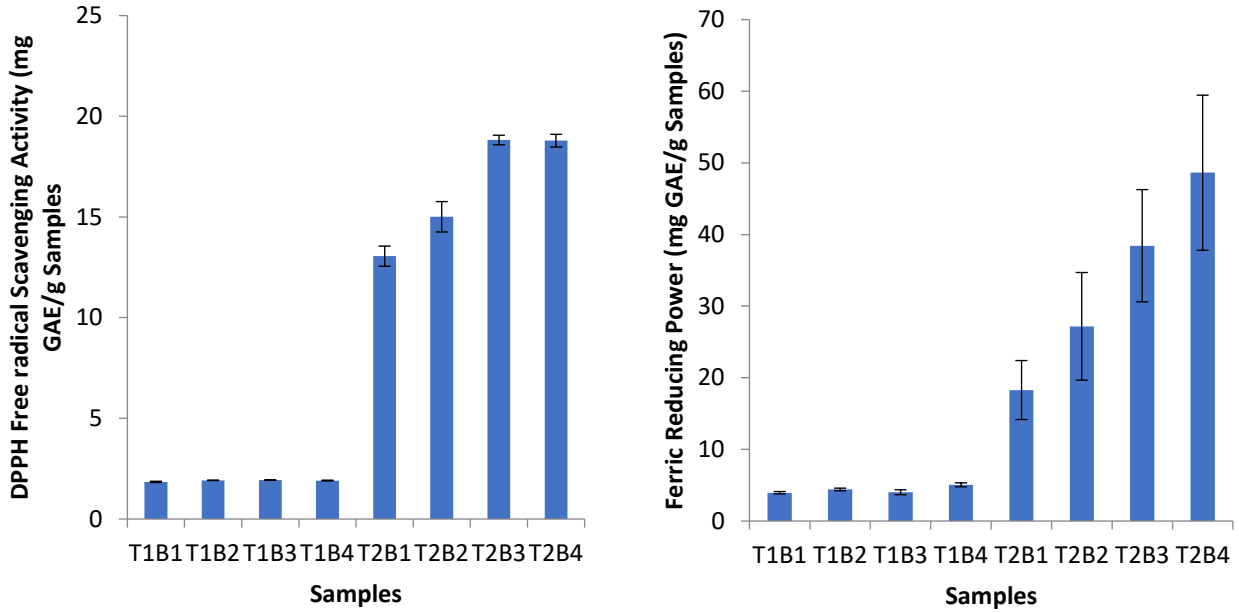

804

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 paited t-test at $\alpha \leq 0.05$. Data were expressed as
809 mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored;
810 T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-
811 steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-
812 steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5
813 years; T3B4-steeped at 95 ^{o}C, stored for 5 years.
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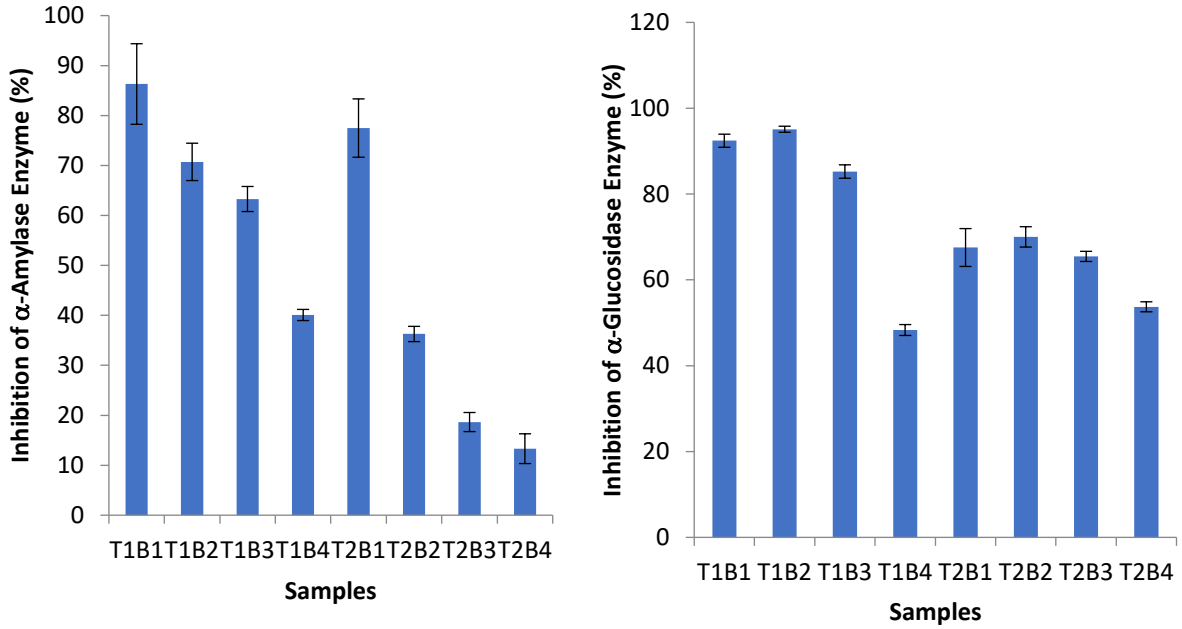

(a) (b)

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage period (a) α-amylase (b) α-glucosidase. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paited t-test at $\alpha \leq 0.05$. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}C, stored for 5 years.

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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 (2-tailed)

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Fwd: Comments on PJS Paper Ms 23-158

Philippine Journal of Science <pjs@stii.dost.gov.ph> Wed, May 8, 2024 at 3:51 PM
To: Paini Sri Widyawati <paini@ukwms.ac.id>

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]

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

From Caesar Saloma/12 May 2024/ Acceptance/ MS 23-158R3

Caesar Saloma <caesar.saloma@gmail.com> Sun, May 12, 2024 at 3:17 AM
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12 May 2024

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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.

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Dear Mr Allyster Endozo
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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

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

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Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea indica* Less, storage period

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ABSTRACT

This study was done to determine the effects of steeping temperature and storage period on the bioactive contents, 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 4 steeping temperatures of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) ^{o}C with the storage period of 0 (B1) and 5 (B2) years resulting in 8 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), total flavonoid (TFC)], antioxidant [(DPPH scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)] potential and antidiabetic [(α-amylase (AA) and α-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 ^{o}C of the steeping temperature, but decreased until 95 ^{o}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

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acid, kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-caffeoylquinic acid, 3,5- di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid.

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, β-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, β-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 minutes (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 of 2 g of *Pluchea* leaf powder at 95 ^{o}C for 5 minutes exhibits total phenolic and total flavonoid contents, the ability to scavenge DPPH free radicals, and the capability to reduce ferric ions at 9.3 mg gallic acid equivalent (GAE)/g sample, 22.0 mg gallic acid equivalent (GAE)/g sample, 27.2 mg gallic

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acid equivalent (GAE)/g sample, 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 ^{o}C for 5 minutes 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) informed that the infusion quality of herbal tea extract depends on several factors, i.e., storage and temperature. The polyphenol profile and antioxidant properties of herbal tea infusion decline with an increase in steeping/brewing and storage temperatures, and longer exposure periods.

Several studies have mentioned the effect of steeping temperature on the bioactive compound contents and antioxidant activity, such as some white and green teas are effective with hot water at 90 ^{o}C for 7 min (Castiglioni et al., 2015), on roseship tea is effective at infusion period around 6-8 min at temperatures of 84-86 ^{o}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 ^{o}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.

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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, 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), 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), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP)], and antidiabetic activities [(α-amylase (AA) and α-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 ± 0.09 % dry basis (Widyawati et al., 2022). The dried *Pluchea* leaves was pulverized to a 45-mesh size

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powder. The *Pluchea* leaf powder was dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 ^{o}C for 10 min to reduce microbial organisms. Then, 2 g of the powder were packed into a paper filter infusion bag. Packed samples were stored for 0 (un-stored) and 5 (stored) years in standing pouch before analysis.

In the research, the one tea bag of *Pluchea* 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) ^{o}C for 5 min with infusion method obtaining 8 treatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, 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, α-amylase, α-glucosidase, pNPG (p-nitrophenyl-α-glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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 distillated water which was purchased from PT Aqua Industry Surabaya.

METHODOLOGY

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ANALYSIS OF THE BIOACTIVE COMPOUNDS

TOTAL PHENOLIC CONTENT ANALYSIS

Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the technique by Gao et al. (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. And then 2 mL Na_2CO_3 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 λ 760 nm with gallic acid as the reference standard. The total phenolic content was calculated using the formula: $y=0.00009x+0.008$ with $R^2=0.9941$. The results were expressed as mg gallic acid equivalent (GAE)/g samples.

TOTAL FLAVONOID CONTENT ASSAY

Total flavonoid content (TFC) of the samples was measured based on the reaction between $AlCl_3$ and $NaNO_2$ with the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim et al., 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted in a yellow solution. About 30 µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5 % in 10 mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 10 % for 5 min. Then, 2 mL NaOH 1 M and distilled water were added until 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 λ 510 nm with (+)-catechin as the reference standard compound,

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

TOTAL TANNIN CONTENT ANALYSIS

Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method (Chandran and Indira, 2016). Approximately 10 µL *Pluchea* infusion was added with 1 mL Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flash and incubated for 5 min. Then, the mixture was added with 2 mL Na_2CO_3 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 λ 760 nm with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/g samples used the 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 atom to the nitrogen atom in DPPH resulting in the formation of DPPH-H compound exhibiting a yellow-colored solution. About 25 µL *Pluchea* leaf infusion was poured into reaction tube into which was added 3 mL DPPH solution (4 mg/100 mL). After incubation for 15 min in a dark room, the absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis

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1800, Shimadzu, Japan) at λ. 517 nm. The reference standard compound was gallic acid and the results of analysis were expressed as mg gallic acid equivalents (GAE)/g samples that calculated using formula: y=0.146x+1.7896 with R^2=0.9975.

FERRIC REDUCING POWER ANALYSIS

Ferric-reducing power (FRAP) was determined following the method used by Widyawati et al. (2014) method. Approximately 10 µL 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. And then 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 was added 2.5 mL distilled water, 0.5 mL ferric chloride 0.1% w/v, and incubated for 10 min. 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 λ 700 nm. Intensity of the blue color indicated higher reducing capacity. The reducing power expressed as mg gallic acid equivalent (GAE)/g samples was calculated using the formula: y=0.0002x+0,0256 with R^2=0,9906.

ANALYSIS OF THE ANTIDIABETIC PROPERTIES

α-AMYLASE ENZYME INHIBITION CAPACITY ASSAY

In vitro inhibition of α-amylase enzyme (AA) followed the procedure as described by Widyawati et al. (2020). Each 500 µL of samples, was mixed with starch 1 % (w/v) and sodium acetate buffer pH 5. Into a 250 µL of the mixture was added an α-amylase solution

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(0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in 50 mL of 0.2 M sodium acetate pH 5. Mixture was shaken into which was and added 2 mL sodium hydroxide 1M. Before the analysis, this mixture was incubated at 37 ^{o}C for 10 min. Then, the capacity of the α-amylase enzyme to hydrolyze the starch to release glucose was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ 540 nm. The inhibition percentage of α-amylase was assessed using the formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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 test sample with enzyme, Ab is absorbance of test sample without enzyme.

α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

The analysis of the α-glycosidase inhibitor activity (GA) was done by Widyawati et al. (2020) method with slight modification. About 150 µL samples containing 100 µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH 7) were reacted with 50 µL α-glycosidase 2 mM (0.0833 unit/mL), and then the mixture was incubated at 37 ^{o}C for 15 min. The reaction was stopped with the addition of 1000 µL sodium carbonate 0.2 M. The amount of these enzymes that didn't react with bioactive compounds of *Pluchea* infusion hydrolyzed p-nitrophenyl-α-D-glucopyranoside (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 λ 405 nm. The inhibition percentage of α-glycosidase was calculated using formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 100 %. Where, ACb is the absorbance

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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, Ab is the absorbance of test sample without enzyme.

ANALYSIS OF PHENOLICS

The phenolic compounds of the samples were analyzed by HPLC based on Kongkiatpaiboona et al. (2018) method with modifications. Each *Pluchea* infusion was sonicated for 15 minutes (Branson 1510) and then the sample was filtered using a filter syringe (Whatmann, 0.2 µm, NYL). About 20 µL of 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 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (2 pieces) (ID 10 mm x 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 minutes; then 100 % B was maintained for 20 minutes. Next the column was re-equilibrated with 10 % B in A maintained for 10 minutes before analysis of the next sample. The sample flow rate was set at 1.0 ml/min with a controlled temperature at 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

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4,5-dicaffeoylquinic acid. All of reference standard was dissolved in distilled water and prepared similar to the samples before 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 ^{o}C (T1), 70 ^{o}C (T2), 80 ^{o}C (T3), and 95 ^{o}C (T4), and the storage period of 0 year /un-stored (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 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 ± SD. The analysis used SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSIONS

BIOACTIVE COMPOUNDS

Phenolic Compounds

The bioactive compounds are active compounds in plants that are essential to protect a 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

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

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 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 un-stored. Further, the highest total phenolic content was observed in samples infused at 95 ^{o}C and stored for 5 years (at 71.38±4.14 mg GAE/g sample) while the lowest was measured in the un-stored samples and infused at 60 ^{o}C (at 4.39±0.49 mg GAE/g sample). The phenolic content of stored samples that were steeped only at 60 and 95 ^{o}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 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 ^{o}C and different long storage (fresh and 72 hours).

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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) and 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 temperature and longer storage period.

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 was showed in Table 1. The treatment effects using a 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 un-stored and stored *Pluchea* infusion was significantly different from the rest of the samples between 70 ^{o}C while (+)-catechin concentration of *Pluchea* infusion was only significantly different at 95 ^{o}C. The myricetin content was significantly different at 80 and 95 ^{o}C. The 3,4-di-O-caffeoylquinic acid content showed significant difference at 60, 80, and 95 ^{o}C while 4,5-di-O-caffeoylquinic acid content was only significantly different at 60 ^{o}C.

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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*-cafffeoylquinic 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), 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.

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 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 $\alpha \leq 0.05$ showed that the total flavonoid content of *Pluchea* infusion was significantly different between the steeped un-stored and steeped stored samples (Figure 1b). The

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highest total flavonoid content was exhibited by the un-stored samples steeped at 95^{o}C at about 147.42±14.03 mg CE/g sample. Total flavonoid content was significantly lower in the stored samples than those of the un-stored 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 the samples infused at 60 ^{o}C at about 4.81±0.58 to 17.42±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 ^{o}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 ^{o}C about 9.22 ± 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 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. Rusita et al. (2019) emphasized that tannins are polar thermostable

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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 capability of compounds to inhibit the oxidation of macromolecules from biological target that involve 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 antioxidant that have antioxidant capability that depends on their redox properties. The structure of phenolic compounds determines the effectivity to donate hydrogen atom 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 reducing agents and singlet oxygen quenchers (Ali et al., 2005; Huang et al. 2005).

DPPH Free Radical Scavenging Activity

DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate antioxidant activity because this method is simple that is suitable to measure the donating hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of DPPH to change to yellow color (Munteanu and Apetrei, 2021; Baliyan et al., 2022). Figure 2a. shows that the free radical scavenging properties of the stored and steeped

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samples were significantly higher than the un-stored 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 un-stored and steeped sample period. *Pluchea* 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 *Pluchea* infusion by around 15 to 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 which 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 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, 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 chemical structure, number and position of hydroxyl groups attached to a benzene ring, a double bond between C2 and C3 rings, and a

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carbonyl group (C=O) on the C ring at C4. The effectivity of antioxidant compounds to donate hydrogen atom is determined by O-H bond dissociation energy.

The free radical scavenging property observed in the study was not in consistent with the results of the study by Moraes-de-Souza et al. (2008). The research shows that total phenolic content of herbal infusion is low correlated with free radical scavenging activity. However, Dobrinas et al. (2021) informed that total phenolic content 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 ferric reducing antioxidant power (FRAP) increased at higher steeping temperature and longer storage period. The lowest FRAP was observed in the un-stored samples which were steeped at 60 ^{o}C at 3.95 ± 0.17 mg gallic acid equivalents (GAE)/g samples, and the highest was exhibited in ***Pluchea*** infusion which was stored for 5 years at 95 ^{o}C at 48.63 ±10.83 mg gallic acid equivalents (GAE)/g samples (Figure 2b). FRAP increased significantly as the steeping temperature was increased. FRAP of the samples stored for 5 years was also significantly higher than the un-stored samples at $\alpha \leq 0.05$.

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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., α-amylase and α-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 α-amylase and α-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 α-amylase enzymes (Figure 3a).
456 The *Pluchea* infusion exhibited a good α-amylase enzyme inhibition activity of more than
457 50 % and even almost 100 % in un-stored *Pluchea* infusion steeped at 60, 70, and 80 ^{o}C
458 with the highest at 60 ^{o}C, and in stored *Pluchea* leaf infusion which was steeped at 60 ^{o}C.

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459 The stored *Pluchea* leaf infusion steeped at 70, 80, and 95 ^{o}C for 5 minutes had lower
460 enzyme inhibition activity of less than 50 % with the lowest at 95 ^{o}C around 13 %.
461 Widyawati et al. (2017) found that the ability to inhibit the α-amylase enzyme in un-stored
462 *Pluchea* infusion steeped at 95 ^{o}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 α-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 α-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 α-amylase enzyme activity inhibitors. Sangeetha and
474 Vedasree (2012) explained that the ability of *Threspesia populnea* extract to inhibit the α-
475 amylase enzyme was determined of their phenolic compound content and protein.
476 Moreover, the presence of α-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 α-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

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to the active site of the α-amylase. The hydroxyl groups can bind by non-covalent interaction (hydrogen bonding, cation-π interactions, salt bridge interactions, ionic interactions, or electrostatic forces) with amino acid residue at the active site in α-amylase enzyme. Elevated steeping temperature and longer storage period can easily cause the removal of the hydroxyl groups of phenolic compounds that can reduce their ability of enzyme inhibition. The phenolic acids with a greater number of hydroxyl groups exhibits stronger capability to obstruct the α-amylase enzyme.

Alpha glucosidase enzyme inhibition activity (GA)

Alpha glucosidase is an important enzyme in carbohydrate digestion, that catalysis the hydrolysis of 1,4-α-bonds of the unabsorbed oligo- and disaccharides, and converts them into monosaccharides (glucose), resulting in hyperglycemia (Nurcholis et al., 2014; Proenca et al., 2017). The ability of bioactive compounds to inhibit the α-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 un-stored ***Pluchea*** infusion at 95 ^{o}C for 5 minutes has an inhibitory effect on the α-glucosidase enzyme of 67.857 %.

Figure 3b shows that the ability of the ***Pluchea*** leaf infusion to inhibit the α-glucosidase enzyme decreased with increasing steeping temperature and storage period. Steeping at 95 ^{o}C of the un-stored ***Pluchea*** leaf infusion obtained the lowest inhibitory ability, i.e., 48.32 ± 1.27 %, and the highest inhibitory activity was at 70 ^{o}C at 95.11 ± 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

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ability of *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-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 α-amylase and α-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 α-glucosidase enzyme activity.

The ability to inhibit the α-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 α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of the two enzymes was different, according to the opinion of McCue et al. (2005). The mechanism of the α-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 α-amylase enzyme inhibitor includes blocking carbohydrates,

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limiting the digestibility and absorption of carbohydrates, and 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 of the α-glucosidase enzyme activity. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, that may be occurred in *Pluchea* 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-*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 α-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 un-stored *Pluchea* infusion, and the concentrations of the simple phenolic compounds were increased at higher steeping temperature, but the α-glucosidase inhibition activity of them was reduced. It means that the methyl-esterified quinic acid with the caffeic groups had more potential to inhibit α-glucosidase enzyme than free caffeoylquinic acid.

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

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concentration of the *Pluchea* leaf infusion caused higher antioxidant activity and lower antidiabetic activity.

CONCLUSION

The Total Phenol (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 un-stored. TPC was highest in the stored and steeped at 95^{o}C and lowest in the un-stored and steeped at 60^{o}C. Un-stored steeped samples exhibited significantly higher flavonoid content than the stored steeped samples. The highest total flavonoid content was exhibited by the un-stored samples steeped at 95^{o}C. The total tannin content of *Pluchea* leaf infusion significantly increased with increasing steeping temperature and storage period. Among the un-stored steeped samples, the tannin content was significantly lowest in the samples steeped at 60^{o}C and highest in the samples steeped at 95^{o}C.

The free radical scavenging property (DPPH) of the stored and steeped *Pluchea* leaf infusion was significantly higher than the un-stored steeped samples. The free radical scavenging property was highest in the stored samples steeped at 80 and 95^{o}C. 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 ferric-reducing antioxidant power (FRAP) significantly increased with increasing steeping temperature and longer storage periods. The lowest FRAP was found in the un-stored samples which were steeped at 60^{o}C and the highest was exhibited in *Pluchea* stored samples which

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were stored for 5 years and steeped at 95ºC. The FRAP of *Pluchea* leaf infusion was significantly strong and positively correlated with the free radical scavenging property, total phenolic, and total tannin content, but inversely with total flavonoid content. The inhibition of the α-amylase activity was generally found to be higher at lower steeping temperatures of the un-stored *Pluchea* leaf infusion than at higher steeping temperatures of the stored sample. The α-amylase enzyme inhibition 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 α-glucosidase enzyme decreased at high steeping temperatures and long storage periods. The highest inhibitory activity was obtained in the un-stored *Pluchea* leaf infusion that was steeped at 70ºC while the lowest was obtained in the un-stored sample that was steeped at 95ºC. The ability of *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-amylase enzyme. The inhibition of the α-glucosidase enzyme activity 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, 4,5-di-*O*-caffeoylquinic acids.

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STATEMENT ON CONFLICT OF INTEREST

The authors declare no conflict of interest.

NOTES ON APPENDICES (if any)

The complete appendices section of the study is accessible at

http://philjournsci.dost.gov.ph

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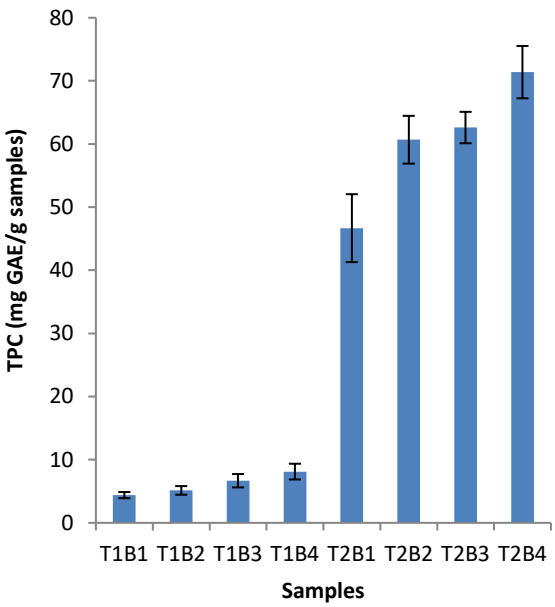

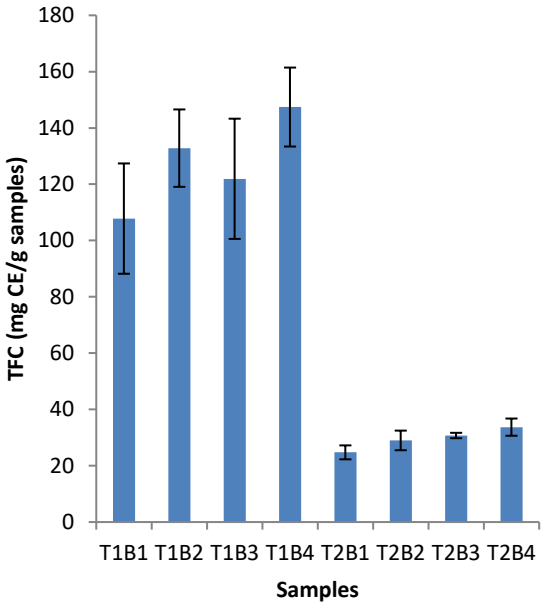

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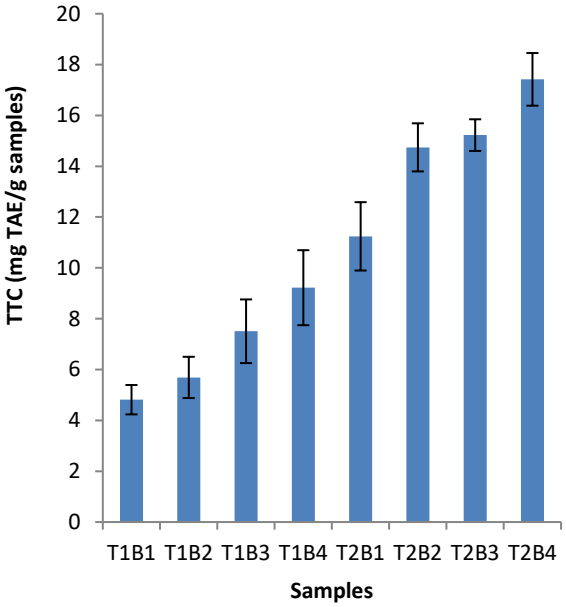
790 (a) (b)



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 paited t-test at $\alpha \leq 0.05$. Data were expressed as
797 mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored;

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T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}C, stored for 5 years.

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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

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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 paited t-test at $\alpha \leq 0.05$. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}C, stored for 5 years.

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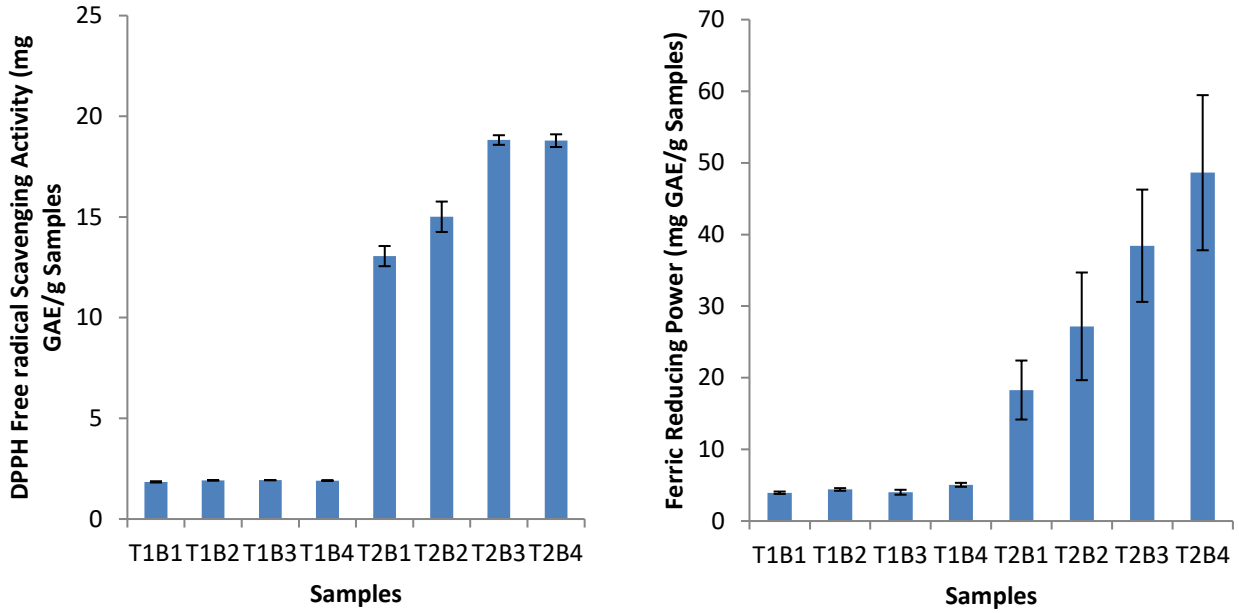

(a) (b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage period (a) DPPH (b) FRAP. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paited t-test at $\alpha \leq 0.05$. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}C, stored for 5 years.

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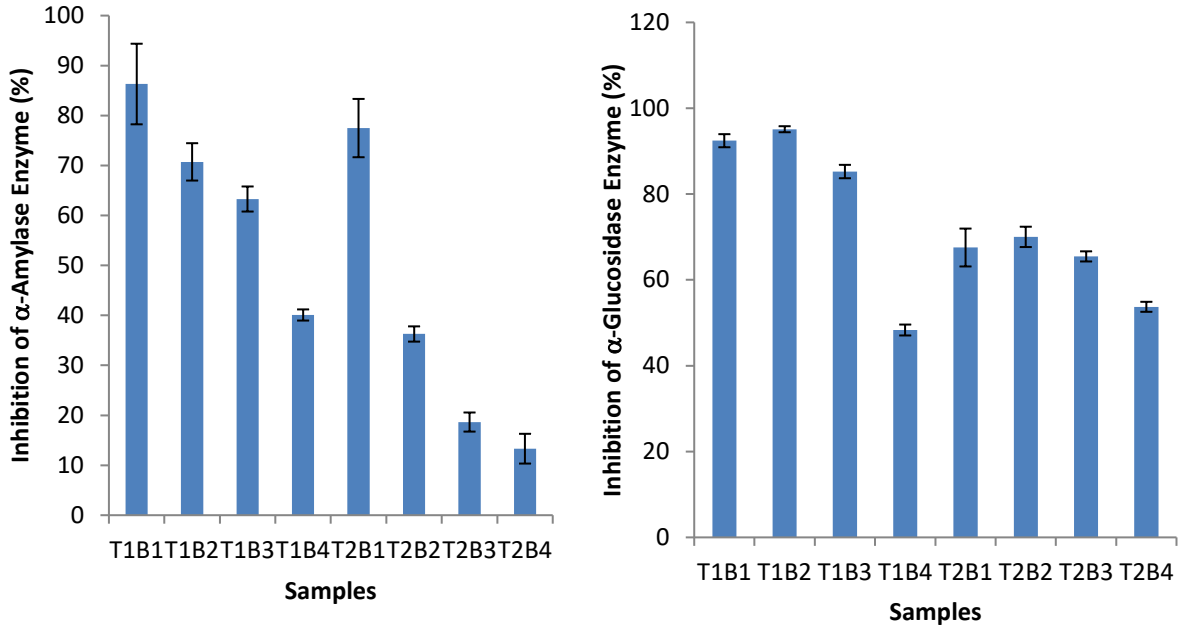

(a) (b)

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage period (a) α-amylase (b) α-glucosidase. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paited t-test at $\alpha \leq 0.05$. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 years; T3B2-steeped at 70 ^{o}C, stored for 5 years; T3B3-steeped at 80 ^{o}C, stored for 5 years; T3B4-steeped at 95 ^{o}C, stored for 5 years.

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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)

	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

834 Significant at the 0.05 level (2-tailed)

835

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Fwd: Comments on PJS Paper Ms 23-158

Paini Sri Widyawati <paini@ukwms.ac.id> Fri, Jun 14, 2024 at 9:31 AM
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

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• [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.

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PJS paper Ms 23-158-Effect of Brewing Temperature and Storage Time on Antioxidant and Antidiabetic Properties of Pluchea Tea (Final).docx
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Effect of Steeping Temperature and Storage Period on the Bioactive Compounds, Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea Indica* Less

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Keywords: antioxidant, antidiabetic, bioactive compound, steeping temperature, *Pluchea indica* Less, storage period

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ABSTRACT

This study was done to determine the effects of steeping temperature and storage period on the bioactive contents, 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 4 steeping temperatures of 60 (T1), 70 (T2), 80 (T3), and 95 (T4) ^{o}C with the storage period of 0 (B1) and 5 (B2) yr resulting in 8 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), total flavonoid (TFC)], antioxidant [(DPPH scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP)] potential and antidiabetic [(α-amylase (AA) and α-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 ^{o}C of the steeping temperature, but decreased until 95 ^{o}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

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acid, kaempferol, myricetin, (+)-catechin, quercetin, 3,4-di-O-caffeoylquinic acid, 3,5- di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid.

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, β-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, β-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 of 2 g of *Pluchea* leaf powder at 95 ^{o}C for 5 min exhibits total phenolic and total flavonoid contents, the ability to scavenge DPPH free radicals, and the capability to reduce ferric ions at 9.3 mg gallic acid equivalent (GAE)/g sample, 22.0 mg gallic acid equivalent (GAE)/g sample, 27.2 mg gallic

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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 ^{o}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 ^{o}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 ^{o}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 ^{o}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.

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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, 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), 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), total tannin content (TTC)], antioxidant [(DPPH free radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP)], and antidiabetic activities [(α-amylase (AA) and α-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 ± 0.09 % dry basis (Widyawati *et al*., 2022). The dried *Pluchea* leaves was pulverized to a 45-mesh size

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powder. The *Pluchea* leaf powder was dried in an oven (Binder, Merck KGaA, Darmstadt, Germany) at 120 ^{o}C for 10 min to reduce microbial organisms. Then, 2 g of the powder were packed into a paper filter infusion bag. Packed samples were stored for 0 (un-stored) and 5 (stored) yr in standing pouch before analysis.

In the research, the one tea bag of *Pluchea* herbal tea that was stored 0 (B1) and 5 (B2) yr, was steeped with 100 mL hot water at various temperatures, including 60 (T1), 70 (T2), 80 (T3), 95 (T4) ^{o}C for 5 min with infusion method obtaining 8 treatment combinations, namely T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, 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, α-amylase, α-glucosidase, pNPG (p-nitrophenyl-α-glucopyranoside), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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 distillated water which was purchased from PT Aqua Industry Surabaya.

METHODOLOGY

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ANALYSIS OF THE BIOACTIVE COMPOUNDS

TOTAL PHENOLIC CONTENT ANALYSIS

Total phenolic content (TPC) of treated *Pluchea* infusion was carried out using the technique by Gao *et al.* (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10 % were mixed in 10 mL volumetric flash and incubated for 5 min. And then 2 mL Na_2CO_3 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 λ 760 nm with gallic acid as the reference standard. The total phenolic content was calculated using the formula: $y=0.00009x+0.008$ with $R^2=0.9941$. The results were expressed as mg gallic acid equivalent (GAE)/g samples.

TOTAL FLAVONOID CONTENT ASSAY

Total flavonoid content (TFC) of the samples was measured based on the reaction between $AlCl_3$ and $NaNO_2$ with the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim *et al*., 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted in a yellow solution. About 30 µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5 % in 10 mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 10 % for 5 min. Then, 2 mL NaOH 1 M and distilled water were added until 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 λ 510 nm with (+)-catechin as the reference standard compound,

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

TOTAL TANNIN CONTENT ANALYSIS

Total tannin content (TTC) of the samples was analyzed by Folin-Ciocalteu method (Chandran and Indira, 2016). Approximately 10 µL *Pluchea* infusion was added with 1 mL Folin-Ciocalteu's phenol reagent 10 % in 10 mL volumetric flash and incubated for 5 min. Then, the mixture was added with 2 mL Na_2CO_3 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 λ 760 nm with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/g samples used the 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 atom to the nitrogen atom in DPPH resulting in the formation of DPPH-H compound exhibiting a yellow-colored solution. About 25 µL *Pluchea* leaf infusion was poured into reaction tube into which was added 3 mL DPPH solution (4 mg/100 mL). After incubation for 15 min in a dark room, the absorbance was measured by a spectrophotometer (Spectrophotometer UV-Vis

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1800, Shimadzu, Japan) at λ. 517 nm. The reference standard compound was gallic acid and the results of analysis were expressed as mg gallic acid equivalents (GAE)/g samples that calculated using formula: y=0.146x+1.7896 with R^2=0.9975.

FERRIC REDUCING POWER ANALYSIS

Ferric-reducing power (FRAP) was determined following the method used by Widyawati *et al*. (2014) method. Approximately 10 µL 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. And then 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 was added 2.5 mL distilled water, 0.5 mL ferric chloride 0.1% w/v, and incubated for 10 min. 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 λ 700 nm. Intensity of the blue color indicated higher reducing capacity. The reducing power expressed as mg gallic acid equivalent (GAE)/g samples was calculated using the formula: y=0.0002x+0,0256 with R^2=0,9906.

ANALYSIS OF THE ANTIDIABETIC PROPERTIES

α-AMYLASE ENZYME INHIBITION CAPACITY ASSAY

In vitro inhibition of α-amylase enzyme (AA) followed the procedure as described by Widyawati *et al*. (2020). Each 500 µL of samples, was mixed with starch 1 % (w/v) and sodium acetate buffer pH 5. Into a 250 µL of the mixture was added an α-amylase solution

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(0.1 g of this enzyme 12.5 unit/mL) then, was dissolved in 50 mL of 0.2 M sodium acetate pH 5. Mixture was shaken into which was and added 2 mL sodium hydroxide 1M. Before the analysis, this mixture was incubated at 37 ^{o}C for 10 min. Then, the capacity of the α-amylase enzyme to hydrolyze the starch to release glucose was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ 540 nm. The inhibition percentage of α-amylase was assessed using the formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 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 test sample with enzyme, Ab is absorbance of test sample without enzyme.

α-GLUCOSIDASE ENZYME INHIBITION CAPACITY ASSAY

The analysis of the α-glycosidase inhibitor activity (GA) was done by Widyawati *et al*. (2020) method with slight modification. About 150 µL samples containing 100 µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100 mL sodium phosphate 0.2 M at pH 7) were reacted with 50 µL α-glycosidase 2 mM (0.0833 unit/mL), and then the mixture was incubated at 37 ^{o}C for 15 min. The reaction was stopped with the addition of 1000 µL sodium carbonate 0.2 M. The amount of these enzymes that didn't react with bioactive compounds of *Pluchea* infusion hydrolyzed p-nitrophenyl-α-D-glucopyranoside (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 λ 405 nm. The inhibition percentage of α-glycosidase was calculated using formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 100 %. Where, ACb is the absorbance

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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, Ab is the absorbance of test sample without enzyme.

ANALYSIS OF PHENOLICS

The phenolic compounds of the samples were analyzed by HPLC based on Kongkiatpaiboona *et al*. (2018) method with modifications. Each *Pluchea* infusion was sonicated for 15 min (Branson 1510) and then the sample was filtered using a filter syringe (Whatmann, 0.2 µm, NYL). About 20 µL of 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 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (2 pieces) (ID 10 mm x 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 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 at 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 reference standard was dissolved in distilled water and prepared similar to the samples before injected in HPLC.

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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 ^{o}C (T1), 70 ^{o}C (T2), 80 ^{o}C (T3), and 95 ^{o}C (T4), and the storage period of 0 yr /un-stored (B1), and 5 yr /stored (B2) resulting in 8 treatment combinations (T1B1, T1B2, T2B1, T2B2, T3B1, T3B2, T4B1, T4B2). 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 $\alpha \leq 0.05$, treatment means of specific phenolic compounds that were identified were expressed as the mean ± SD. The analysis used SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSIONS

BIOACTIVE COMPOUNDS

Phenolic Compounds

The bioactive compounds are active compounds in plants that are essential to protect a 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).

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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 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 un-stored. Further, the highest total phenolic content was observed in samples infused at 95 ^{o}C and stored for 5 yr (at 71.38±4.14 mg GAE/g sample) while the lowest was measured in the un-stored samples and infused at 60 ^{o}C (at 4.39±0.49 mg GAE/g sample). The phenolic content of stored samples that were steeped only at 60 and 95 ^{o}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 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; 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 ^{o}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

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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 temperature and longer storage period.

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 was showed in Table 1. The treatment effects using a 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 un-stored and stored *Pluchea* infusion was significantly different from the rest of the samples between 70 ^{o}C while (+)-catechin concentration of *Pluchea* infusion was only significantly different at 95 ^{o}C. The myricetin content was significantly different at 80 and 95 ^{o}C. The 3,4-di-O-caffeoylquinic acid content showed significant difference at 60, 80, and 95 ^{o}C while 4,5-di-O-caffeoylquinic acid content was only significantly different at 60 ^{o}C.

Results further showed that gallic acids and kaempferol were relatively stable as reflected by the insignificant changes when exposed to the different steeping temperature

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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*-cafffeoylquinic 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 complex blue solution that can detected as total phenolic content.

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 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 $\alpha \leq 0.05$ showed that the total flavonoid content of *Pluchea* infusion was significantly different between the steeped un-stored and steeped stored samples (Figure 1b). The highest total flavonoid content was exhibited by the un-stored samples steeped at 95^{o}C at about 147.42±14.03 mg CE/g sample. Total flavonoid content was significantly lower

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in the stored samples than those of the un-stored 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 the samples infused at 60 ^{o}C at about 4.81±0.58 to 17.42±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 ^{o}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 ^{o}C about 9.22 ± 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 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. 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.

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Antioxidant Activity

Antioxidant activity is capability of compounds to inhibit the oxidation of macromolecules from biological target that involve 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 antioxidant that have antioxidant capability that depends on their redox properties. The structure of phenolic compounds determines the effectivity to donate hydrogen atom 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 reducing agents and singlet oxygen quenchers (Ali *et al*., 2005; Huang *et al*. 2005).

DPPH Free Radical Scavenging Activity

DPPH (2,2-diphenil-1-picrylhydrazyl) is a free radical, that is often used to evaluate antioxidant activity because this method is simple that is suitable to measure the donating hydrogen atoms capability of herbal infusion. This reaction can cause the purple color of DPPH to change to 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 un-stored steeped samples. It can also be observed that the free radical scavenging property was significantly different among the

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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.

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The free radical scavenging property observed in the study was not in consistent with the results of the study by Moraes-de-Souza *et al.* (2008). The research shows that total phenolic content of herbal infusion is low correlated with free radical scavenging activity. However, Dobrinas *et al.* (2021) informed that total phenolic content 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 ferric reducing antioxidant power (FRAP) increased at higher steeping temperature and longer storage period. The lowest FRAP was observed in the un-stored samples which were steeped at 60 ^{o}C at 3.95 ± 0.17 mg gallic acid equivalents (GAE)/g samples, and the highest was exhibited in *Pluchea* infusion which was stored for 5 yr at 95 ^{o}C at 48.63 ±10.83 mg gallic acid equivalents (GAE)/g samples (Figure 2b). FRAP increased significantly as the steeping temperature was increased. FRAP of the samples stored for 5 years was also significantly higher than the un-stored 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 gallat (EGCG), epicatechin gallat (ECG),

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epigallocatechin (EGC), and epicatechin (EC) 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 2-digestive enzymes i.e., α-amylase and α-glucosidase, which are involved 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 α-amylase and α-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 α-amylase enzymes (Figure 3a). The *Pluchea* infusion exhibited a good α-amylase enzyme inhibition activity of more than 50 % and even almost 100 % in un-stored *Pluchea* infusion steeped at 60, 70, and 80 ^{o}C with the highest at 60 ^{o}C, and in stored *Pluchea* leaf infusion which was steeped at 60 ^{o}C. The stored *Pluchea* leaf infusion steeped at 70, 80, and 95 ^{o}C for 5 minutes had lower enzyme inhibition activity of less than 50 % with the lowest at 95 ^{o}C around 13 %. Widyawati *et al.* (2017) found that the ability to inhibit the α-amylase enzyme in un-stored

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Pluchea infusion steeped at 95 ^{o}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 α-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.

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 α-amylase enzyme. Akah *et al*. (2011) reported that phytochemical compounds, such as terpenoids, saponins, flavonoids, glycosides/carbohydrates, and alkaloids are good antidiabetic metabolites or α-amylase enzyme activity inhibitors. Sangeetha and Vedasree (2012) explained that the ability of *Threspesia populnea* extract to inhibit the α-amylase enzyme was determined of their phenolic compound content and protein. Moreover, the presence of α-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 with their protein and polyphenolic content. Aleixandre *et al.* (2022) also stated that phenolic acids have inhibition activity to α-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 α-amylase. The hydroxyl groups can bind by non-covalent interaction (hydrogen bonding, cation-π interactions, salt bridge interactions, ionic interactions, or electrostatic forces) with amino acid residue at the active site in α-amylase

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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 α-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-α-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 α-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 ^{o}C for
497 5 min has an inhibitory effect on the α-glucosidase enzyme of 67.857 %.
498 Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the α-
499 glucosidase enzyme decreased with increasing steeping temperature and storage period.
500 Steeping at 95 ^{o}C of the un-stored *Pluchea* leaf infusion obtained the lowest inhibitory
501 ability, i.e., 48.32 ± 1.27 %, and the highest inhibitory activity was at 70 ^{o}C at 95.11 ±
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 α-glucosidase enzyme tended to be higher
505 than the ability to inhibit the α-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,

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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 α-amylase and α-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 α-glucosidase enzyme activity.

The ability to inhibit the α-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 α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of the two enzymes was different, according to the opinion of McCue *et al*. (2005). The mechanism of the α-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 α-amylase enzyme inhibitor includes blocking carbohydrates, limiting the digestibility and absorption of carbohydrates, and 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 of the α-glucosidase enzyme activity. The ability of bound phenolic compounds to

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inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, that may be occurred in *Pluchea* 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-*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 α-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 un-stored *Pluchea* infusion, and the concentrations of the simple phenolic compounds were increased at higher steeping temperature, but the α-glucosidase inhibition activity of them was reduced. It means that the methyl-esterified quinic acid with the caffeic groups had more potential to inhibit α-glucosidase enzyme than free caffeoylquinic acid.

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 *Pluchea* leaf infusion caused higher antioxidant activity and lower antidiabetic activity.

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CONCLUSION

The Total Phenol (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 un-stored. TPC was highest in the stored and steeped at 95^{o}C and lowest in the un-stored and steeped at 60^{o}C. Un-stored steeped samples exhibited significantly higher flavonoid content than the stored steeped samples. The highest total flavonoid content was exhibited by the un-stored samples steeped at 95^{o}C. The total tannin content of *Pluchea* leaf infusion significantly increased with increasing steeping temperature and storage period. Among the un-stored steeped samples, the tannin content was significantly lowest in the samples steeped at 60^{o}C and highest in the samples steeped at 95^{o}C.

The free radical scavenging property (DPPH) of the stored and steeped *Pluchea* leaf infusion was significantly higher than the un-stored steeped samples. The free radical scavenging property was highest in the stored samples steeped at 80 and 95^{o}C. 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 ferric-reducing antioxidant power (FRAP) significantly increased with increasing steeping temperature and longer storage periods. The lowest FRAP was found in the un-stored samples which were steeped at 60^{o}C and the highest was exhibited in *Pluchea* stored samples which were stored for 5 yr and steeped at 95^{o}C. The FRAP of *Pluchea* leaf infusion was significantly strong and positively correlated with the free radical scavenging property, total phenolic, and total tannin content, but inversely with total flavonoid content. The

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inhibition of the α-amylase activity was generally found to be higher at lower steeping temperatures of the un-stored *Pluchea* leaf infusion than at higher steeping temperatures of the stored sample. The α-amylase enzyme inhibition 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 α-glucosidase enzyme decreased at high steeping temperatures and long storage periods. The highest inhibitory activity was obtained in the un-stored *Pluchea* leaf infusion that was steeped at 70^{o}C while the lowest was obtained in the un-stored sample that was steeped at 95^{o}C. The ability of *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-amylase enzyme. The inhibition of the α-glucosidase enzyme activity 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, 4,5-di-*O*-caffeoylquinic acids.

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STATEMENT ON CONFLICT OF INTEREST

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The authors declare no conflict of interest.

NOTES ON APPENDICES (if any)

The complete appendices section of the study is accessible at

http://philjournsci.dost.gov.ph

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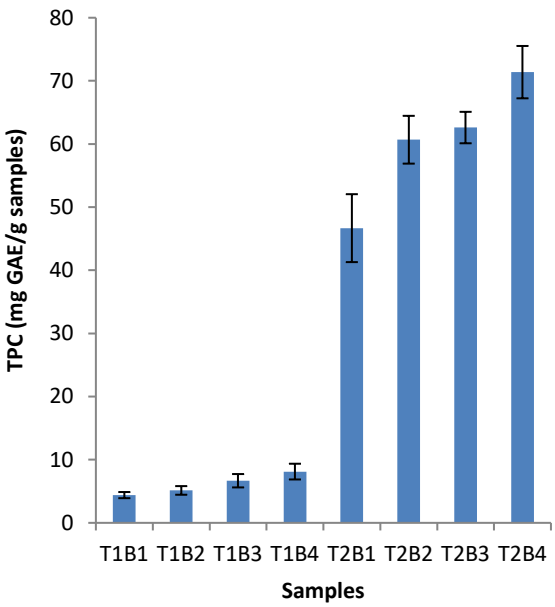

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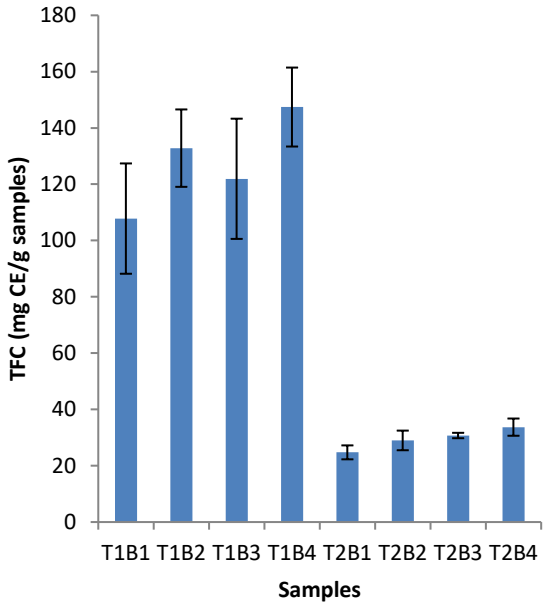

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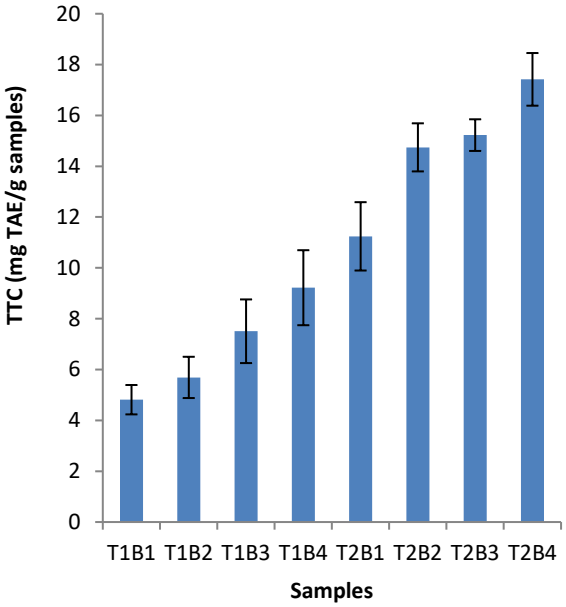

(c)

Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping temperature and storage period (a) Total phenolic content (b) Total flavonoid content (c) Total tannin content. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paited t-test at $\alpha \leq 0.05$. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored;

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T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 yr; T3B2-steeped at 70 ^{o}C, stored for 5 yr; T3B3-steeped at 80 ^{o}C, stored for 5 yr; T3B4-steeped at 95 ^{o}C, stored for 5 yr.

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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

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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 paited t-test at $\alpha \leq 0.05$. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 yr; T3B2-steeped at 70 ^{o}C, stored for 5 yr; T3B3-steeped at 80 ^{o}C, stored for 5 yr; T3B4-steeped at 95 ^{o}C, stored for 5 yr.

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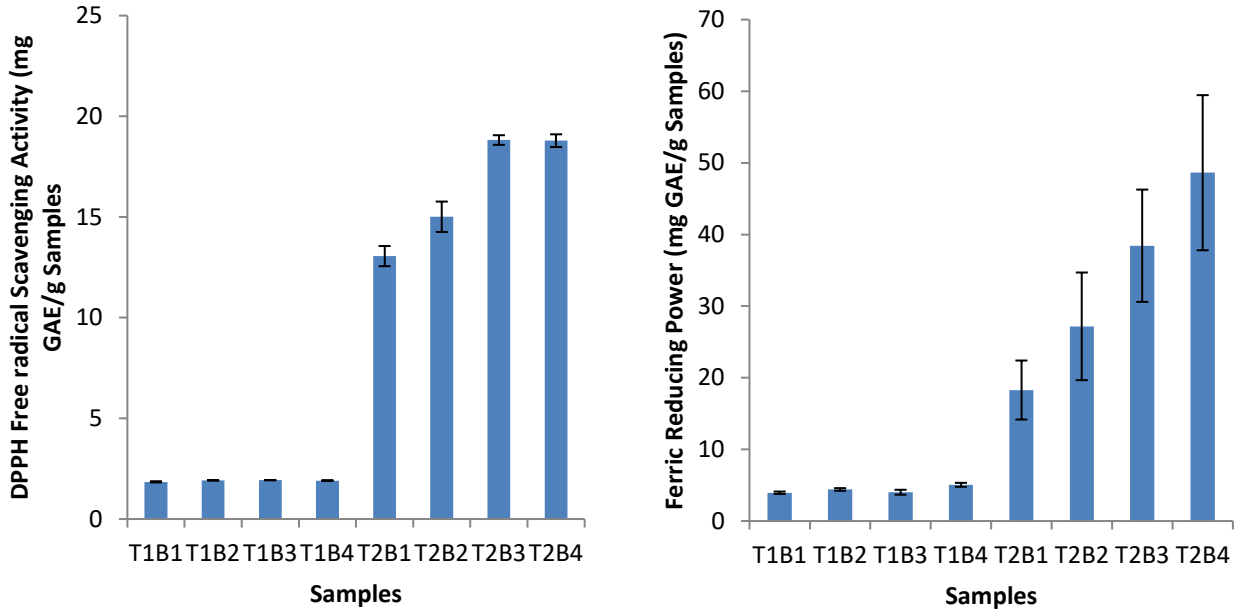

(a) (b)

Figure 2. Antioxidant activity of pluchea tea at different steeping temperature and storage period (a) DPPH (b) FRAP. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paited t-test at $\alpha \leq 0.05$. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 yr; T3B2-steeped at 70 ^{o}C, stored for 5 yr; T3B3-steeped at 80 ^{o}C, stored for 5 yr; T3B4-steeped at 95 ^{o}C, stored for 5 yr.

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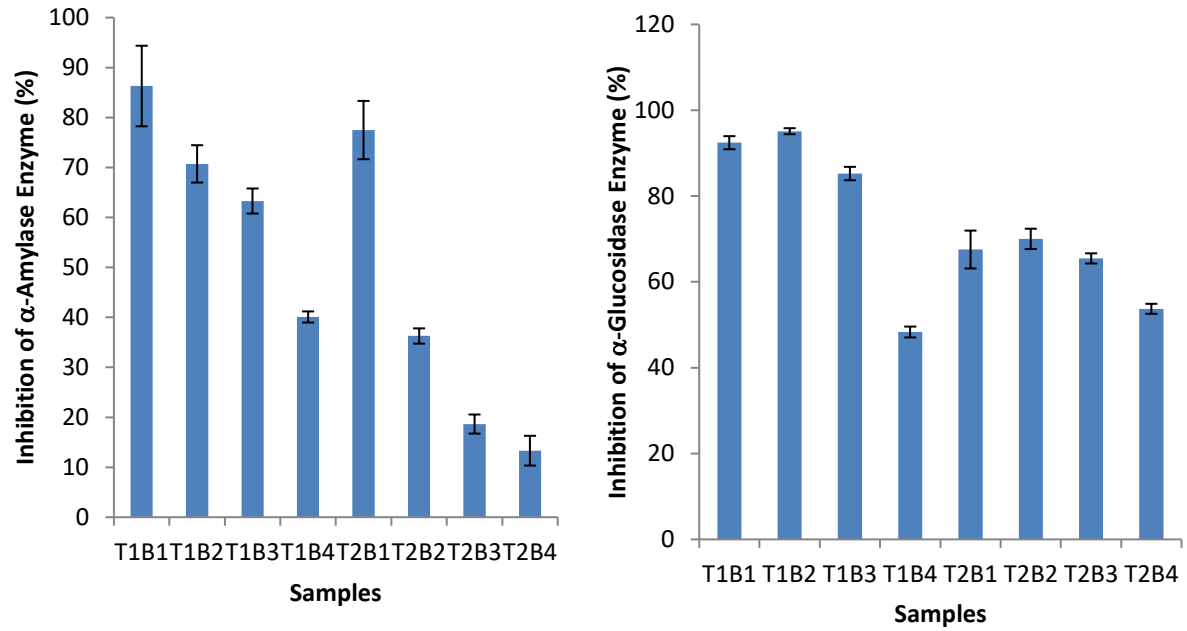

(a) (b)

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperature and storage period (a) α-amylase (b) α-glucosidase. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paited t-test at $\alpha \leq 0.05$. Data were expressed as mean ±standard deviation (n=6). Samples : T1B1-steeped at 60 ^{o}C, un-stored; T1B2-steeped at 70 ^{o}C, un-stored; T1B3-steeped at 80 ^{o}C, un-stored; T1B4-steeped at 95 ^{o}C, un-stored; T2B1-steeped at 60 ^{o}C, stored for 5 yr; T3B2-steeped at 70 ^{o}C, stored for 5 yr; T3B3-steeped at 80 ^{o}C, stored for 5 yr; T3B4-steeped at 95 ^{o}C, stored for 5 yr.

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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)

	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

836 Significant at the 0.05 level (2-tailed)

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Paini Sri Widyawati <paini@ukwms.ac.id> Mon, Jun 17, 2024 at 2:58 PM
To: Philippine Journal of Science <pjs@stii.dost.gov.ph>

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
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Effect of Steeping Temperature and Storage Period on the Bioactive Compounds plus Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea indica* Less

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Keywords: antioxidant, antidiabetic, bioactive compound,
Pluchea indica Less, steeping temperature, storage period

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ABSTRACT

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 four4 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 eight8 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

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(DPPH) and ferric reducing antioxidant power (FRAP)] potential and antidiabetic [~~(~~α-~~α-~~amylase (AA) and α-~~α-~~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.

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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, β-carotene, and vitamin C, whereas bioactive compounds are comprised, *i.e.*

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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, β-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 ~~of~~ 2 g of *Pluchea* leaf powder at 95 °C for 5 min exhibits total phenolic and ~~total~~ flavonoid contents, the ability to scavenge DPPH free radicals, and the capability to reduce ferric ions at 9.3 ~~mg gallic acid equivalent (GAE)/g sample~~, 22.0 ~~mg gallic acid equivalent (GAE)/g sample~~, 27.2 ~~mg gallic acid equivalent (GAE)/g sample~~, and 10.2 mg gallic acid equivalent (~~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) ~~informed~~ stated that the infusion quality of herbal tea extract depends on ~~on several~~a number of factors,

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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~~nd~~ longer exposure periods.

Several studies have mentioned the effect of steeping temperature on the bioactive compound contents and antioxidant activity, ~~such~~ as some white and green teas are effective with hot water at 90 °C for 7 min (Castiglioni *et al.* 2015), on rose~~s~~hip 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), K~~k~~innow-A~~a~~mla 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

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(TFC), and total tannin content (TTC)], antioxidant [~~(~~DPPH free radical scavenging activity (DPPH) and~~,~~ ferric reducing antioxidant power (FRAP)], and antidiabetic activities [~~(~~α-amylase (AA) and α-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 ± 0.09–% dry basis (Widyawati *et al.* 2022). The dried *Pluchea* leaves ~~was~~ 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 ~~were~~ 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, ~~the~~ one tea bag of *Pluchea* herbal tea that was stored for 0 (B1) and 5 (B2) y~~ea~~r~~,~~ 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 ~~8~~ 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.

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Reagents

The reagents used in the analysis include 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, gallic acid, α-amylase, α-glucosidase, ~~pNPG (~~p-nitrophenyl-α-glucopyranoside (pNPG), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic acid, and (+)-catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, Folin–Ciocalteu's p~~P~~henol, 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 distillated water which was purchased from PT Aqua Industry Surabaya.

Analysis of the Bioactive Compounds

Total phenolic content (TPC) analysis. The ~~otal phenolic content (~~TPC~~)~~ of treated *Pluchea* infusion was carried out using the technique by Gao *et al.* (2019). About 10 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10% were mixed in 10 mL volumetric flash and incubated for 5 min. ~~And~~ Then, 2 mL Na_2CO_3 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 λ = 760 nm, with gallic acid as the reference standard. The ~~total phenolic content~~TPC was calculated using the following formula: $y = 0.00009x + 0.008$, with $R^2 = 0.9941$. The results were expressed as mg ~~gallic acid equivalent (~~GAE~~)~~/g samples.

Total flavonoid content (TFC) assay. The ~~otal flavonoid content (~~TFC~~)~~ of the samples was measured based on the reaction between $AlCl_3$ and $NaNO_2$ with the

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aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim *et al.* 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted in a yellow solution. About 30 µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5% in 10 mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 10% for 5 min. Then, 2 mL NaOH 1 M and distilled water were added ~~until~~ 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 λ = 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 ~~otal tannin content (~~TTC~~)~~ of the samples was analyzed ~~by~~ using the Folin-Ciocalteu method (Chandran and Indira 2016). Approximately 10 µL *Pluchea* infusion was added with 1 mL Folin-Ciocalteu's phenol reagent 10% in 10 mL volumetric flash and incubated for 5 min. Then, the mixture was added with 2 mL Na_2CO_3 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 λ = 760 nm, with tannic acid as the reference standard. Calculation of TTC was expressed as mg tannic acid equivalents (TAE)/ g samples ~~used~~ 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

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donate hydrogen atoms to the nitrogen atom in DPPH, resulting in the formation of DPPH-H compound exhibiting a yellow-colored solution. About 25 µL *Pluchea* leaf infusion was poured into the reaction tube, into which ~~was added~~ 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 λ. = 517 nm. The reference standard compound was gallic acid, and the results of the analysis were expressed as mg ~~gallic acid equivalents (~~GAE~~)~~/g samples ~~that~~ calculated using the following formula: y = 0.146x + 1.7896, with R^2 = 0.9975.

Ferric ~~-~~reducing power (FRAP) analysis. ~~Ferric-reducing power (~~FRAP~~)~~ was determined following the method used by Widyawati *et al.* (2014) method. Approximately 10 µL 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. ~~And T~~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, ~~was added~~ 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 p~~P~~otency 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 λ = 700 nm. The i~~I~~ntensity of the blue color indicated a higher reducing capacity. The reducing power, expressed as mg ~~gallic acid equivalent (~~GAE~~)~~/g samples, was calculated using the following formula: y = 0.0002x + 0.~~,~~0256, with R^2 = 0.~~,~~9906.

Analysis of the Antidiabetic Properties

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α-amylase enzyme inhibition (AA) capacity assay. *In vitro* ~~inhibition of α-amylase enzyme (~~AA~~)~~ followed the procedure, as described by Widyawati *et al.* (2020). Each 500 µL of the samples~~,~~ was mixed with starch 1~~-~~% (w/v) and sodium acetate buffer pH 5. Into ~~a~~ 250 µL of the mixture, ~~was added~~ an α~~α~~-amylase solution (0.1 g of this enzyme 12.5 unit/mL) was added and then~~, was~~ dissolved in 50 mL of 0.2 M sodium acetate pH 5. The m~~M~~ixture was shaken, into which ~~was and added~~ 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 α-amylase enzyme to hydrolyze the starch to release glucose was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ = 540 nm. The inhibition percentage of α-amylase was assessed using the formula~~:~~ (ACb − ACa) − (As − Ab) (ACb − ACa) x 100~~-~~% ~~,~~ w~~W~~here~~,~~ 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.

α-glucosidase enzyme inhibition (GA) capacity assay. The analysis of the α-glycosidase inhibitor activity (GA) was done ~~by~~ using the method of Widyawati *et al.* (2020) ~~method~~ with slight modifications. About 150~~-~~µL samples containing 100~~-~~µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100~~-~~mL sodium phosphate 0.2 M at pH 7) were reacted with 50~~-~~µL α-glycosidase 2 mM (0.0833 unit/mL)~~;~~ ~~and~~ then, the mixture was incubated at 37 °C for 15 min. The reaction was stopped with the addition of 1000~~-~~µL sodium carbonate 0.2 M. The amount of these enzymes that did n~~o~~'t react with bioactive compounds of *Pluchea* infusion hydrolyzed ~~p-nitrophenyl-α-D-glucopyranoside (~~pNPG~~)~~ as a substrate to result in p-nitrophenol. The inhibition activity of the *Pluchea*

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infusion was measured by UV-Vvis spectrophotometer (Spectrophotometer UV-Vis-1800, Shimadzu, Japan) at λ = 405 nm. The inhibition percentage of α-glycosidase was calculated using the formula: (ACb − ACa) − (As − Ab) (ACb − ACa) x 100-% —. wWhere, 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 by using high-performance liquid chromatography (HPLC) based on the method of Kongkiatpaiboona *et al.* (2018) method with modifications. Each *Pluchea* infusion was sonicated for 15 min (Branson 1510); and then, the sample was filtered using a filter syringe (Whatmann, 0.2 µm, NYL). About 20 µL 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 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (2 two pieces) (ID 10 mm x 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 minutes; then, 100–% B was maintained for 20 minutes. 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 at of 40 °C. Detection was used at a wavelength of 280 nm. The

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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 four ~~4~~ steeping temperatures, namely 60 °C (T1), 70 °C (T2), 80 °C (T3), and 95 °C (T4), and the storage period of 0 yrear /unstored (B1), and 5 yrear /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 α ≤ 0.05, treatment means of specific phenolic compounds that were identified were expressed as the mean ± SD. The analysis used SPSS 23.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSIONS

Bioactive Compounds

Phenolic compounds. B ~~The b~~ioactive compounds are active compounds in plants that are essential to protect ~~a~~ 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

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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 ~~total phenolic content (~~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 α~~α~~ ≤ 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 ~~total phenolic content~~TPC was observed in samples infused at 95 °C and stored for 5 yr (at 71.38 ± 4.14 mg GAE/g sample), ~~while~~ whereas the lowest was measured in the unstored samples and infused at 60 °C (at 4.39 ± 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, ~~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 the

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antioxidant activity of lychee juice stored at different temperatures of 4 and 45 °C and different long storage (fresh and 72 hours).

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;) and 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 was is showed shown in Table 1. The treatment effects using the a t-test at α ≤ 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, while 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

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significant difference at 60, 80, and 95 °C, ~~while~~ whereas 4,5-di-O-caffeoylquinic acid content was only significantly different at 60 °C.

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*-cafffeoylquinic 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~~ phenol reagent, resulting in a complex blue solution that can detected as ~~total phenolic content~~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 ~~total flavonoid content~~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 α~~α~~ ≤ 0.05 showed that the TFC~~total flavonoid content~~ of *Pluchea* infusion was significantly different between the steeped

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unstored and steeped stored samples (Figure 1b). The highest TFC~~total flavonoid content~~ was exhibited by the unstored samples steeped at 95 °C at about 147.42 ± 14.03 mg CE/g sample. The TFC~~Total flavonoid content~~ 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 ~~total tannin content~~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 ± 0.58 to 17.42 ± 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 ± 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 ± 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 ~~had~~ contributed to the observed increase in the tannin content in the treated samples.

~~Although~~Nonetheless~~,~~ high temperatures and long storage periods can cause the degradation of tannins to catechins. Rusita *et al.* (2019) emphasized that tannins are polar

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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-diphenil-1-picrylhydrazyl)~~ is a free radical~~,~~ that is often used to evaluate antioxidant activity because this method is simple ~~that~~ and is suitable f~~t~~or measuring~~e~~ 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

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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 ~~to~~ 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 ~~which~~ 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 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.

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The free radical scavenging property observed in the study was not ~~in~~ consistent with the results of the study by Moraes ~~–~~de ~~-~~Souza *et al.* (2008). The research shows that the ~~total phenolic content~~TPC of herbal infusion is low~~ly~~ correlated with free radical scavenging activity. However~~,~~ ~~–~~, Dobrinas *et al.* (2021) informed that ~~total phenolic content~~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 ~~ferric reducing antioxidant power (~~FRAP~~)~~ increased at higher steeping temperature~~s~~ and longer storage period~~s~~. The lowest FRAP was observed in the unstored samples~~,~~ which were steeped at 60 °C at 3.95 ± 0.17 mg ~~gallic acid equivalents (~~GAE~~)~~/g samples, and the highest was exhibited in *Pluchea* infusion which was stored for 5 yr at 95 °C at 48.63 ± 10.83 mg ~~gallic acid equivalents (~~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 α~~α~~ ≤ 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 ~~(EGCG)~~, epicatechin gallate ~~(ECG)~~,

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epigallocatechin ~~(EGC)~~, and epicatechin ~~(EC)~~, 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

α~~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 ~~2~~two -digestive enzymes, *i.e.* α-amylase and α-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 α-amylase and α-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 α-amylase enzymes (Figure 3a). The *Pluchea* infusion exhibited a good ~~α-amylase enzyme inhibition activity~~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 α-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 α-

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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.

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 α-amylase enzyme. Akah *et al.* (2011) reported that phytochemical compounds~~,~~ such as terpenoids, saponins, flavonoids, glycosides/carbohydrates, and alkaloids are good antidiabetic metabolites or α-amylase enzyme activity inhibitors. Sangeetha and Vedasree (2012) explained that the ability of *Threspesia populnea* extract to inhibit the α-amylase enzyme was determined ~~of~~ by their phenolic compound content and protein. Moreover, the presence of the α-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 ~~with~~ by their protein and polyphenolic content. Aleixandre *et al.* (2022) also stated that phenolic acids have inhibition activity to α-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 α-amylase. The hydroxyl groups can bind by non-covalent interaction (hydrogen bonding, cation-π~~π~~ interactions, salt bridge interactions, ionic interactions, or electrostatic forces) with amino acid residue at the active site in the α-amylase enzyme. Elevated steeping temperatures and longer storage periods can easily cause the removal of the hydroxyl groups of phenolic compounds ~~that~~, which can reduce

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their ability ~~of~~ to enzyme inhibition. The phenolic acids with a greater number of hydroxyl groups exhibits stronger capability to obstruct the α-amylase enzyme.

α-~~Alpha~~ glucosidase enzyme inhibition activity (GA). α-~~Alpha~~ glucosidase is an important enzyme in carbohydrate digestion, that catalysis the hydrolysis of 1,4-α-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 α-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 α-glucosidase enzyme of 67.857%.

Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the α-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 ± 1.27 ~~-~~%, and the highest inhibitory activity was at 70 °C at 95.11 ± 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 α-glucosidase enzyme tended to be higher than the ability to inhibit the α-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 α-amylase and α-glucosidase enzymes. Dias *et al.* (2021) stated that flavonoid compounds~~,~~ such as rutin,

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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 α-glucosidase enzyme activity.

The ability to inhibit the α-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 α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of the two enzymes was different, according to the opinion of McCue *et al.* (2005). The mechanism of the α-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 α-amylase enzyme inhibitor includes blocking carbohydrates, thereby limiting the digestibility and absorption of carbohydrates, ~~and~~ 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 ~~of~~ the α-glucosidase enzyme activity. The ability of bound phenolic compounds to inhibit α-glucosidase enzymes was higher than free phenolic compounds. The presence of polymerization and degradation reactions, ~~that~~ which may ~~be~~ occur~~red~~ in *Pluchea* infusion during storage, affects the structure and profile of phenolic and non-phenolic

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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 α-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 unstored *Pluchea* infusion, and the concentrations of the simple phenolic compounds were increased at higher steeping temperature, but the ~~α-glucosidase inhibition activity~~GA of them was reduced. It means that the methyl-esterified quinic acid with the caffeic groups had more potential to inhibit α-glucosidase enzyme than free caffeoylquinic acid.

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~~TPC and ~~total tannin content~~TTC. The increase in the simple phenolic concentration of the *Pluchea* leaf infusion caused higher antioxidant activity and lower antidiabetic activity.

CONCLUSION

The ~~Total Phenol (~~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

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phenolic compounds than the samples that were steeped and unstored. TPC was highest in the stored and steeped at 95 °C and lowest in the unstored and steeped at 60 °C. Unstored steeped samples exhibited significantly higher flavonoid content than the stored steeped samples. The highest TFC~~total flavonoid content~~ was exhibited by the unstored samples steeped at 95 °C. The ~~total tannin content~~TTC of *Pluchea* leaf infusion significantly increased with increasing steeping temperature and storage period. Among the unstored 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 ~~free radical scavenging property (~~DPPH~~)~~ of the stored and steeped *Pluchea* leaf infusion was significantly higher than the unstored 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 ~~ferric-reducing antioxidant power (~~FRAP~~)~~ significantly increased with increasing steeping temperature and longer storage periods. The lowest FRAP was found in the unstored samples ~~which~~ that were steeped at 60 °C, and the highest was exhibited in *Pluchea* ~~stored~~ samples ~~which~~ 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, ~~total phenolic~~TPC, and ~~total tannin content~~TTC~~,~~ but inversely with TFC~~total flavonoid content~~. The ~~inhibition of the α-amylase activity~~AA was generally found to be higher at lower steeping temperatures of the unstored *Pluchea* leaf infusion than at higher steeping temperatures of the stored sample. The ~~α-amylase enzyme inhibition~~AA capacity of the *Pluchea* leaf infusion showed a significantly strong and negative

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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 α-glucosidase enzyme decreased at high steeping temperatures and long storage periods. The highest inhibitory activity was obtained in the unstored *Pluchea* leaf infusion that was steeped at 70 °C, ~~while~~ whereas the lowest was obtained in the unstored sample that was steeped at 95 °C. The ability of *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-amylase enzyme. The ~~inhibition of the α-glucosidase enzyme activity~~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.

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STATEMENT ON CONFLICT OF INTEREST

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The authors declare no conflict of interest.

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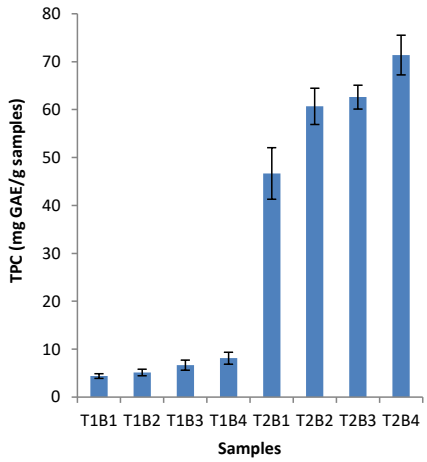

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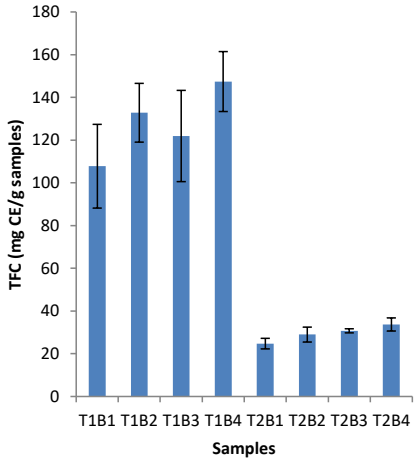

[b]

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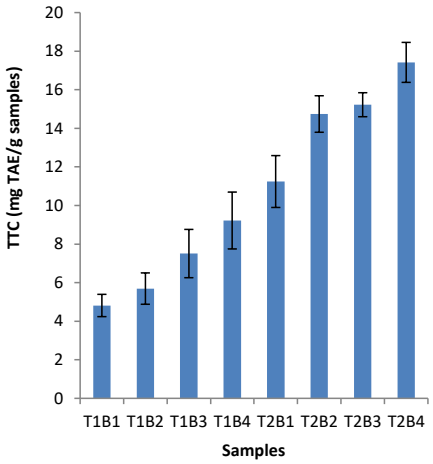

[c]

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 ± 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.

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771 **Table 1.** Simple phenolic compound profile of *Pluchea* Infusion at different steeping temperatures and storage periods.

Phenolic compounds	Steeping temperature (ºC)	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

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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 α ≤ 0.05 continued analysis using a paired t-test at α ≤ 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.

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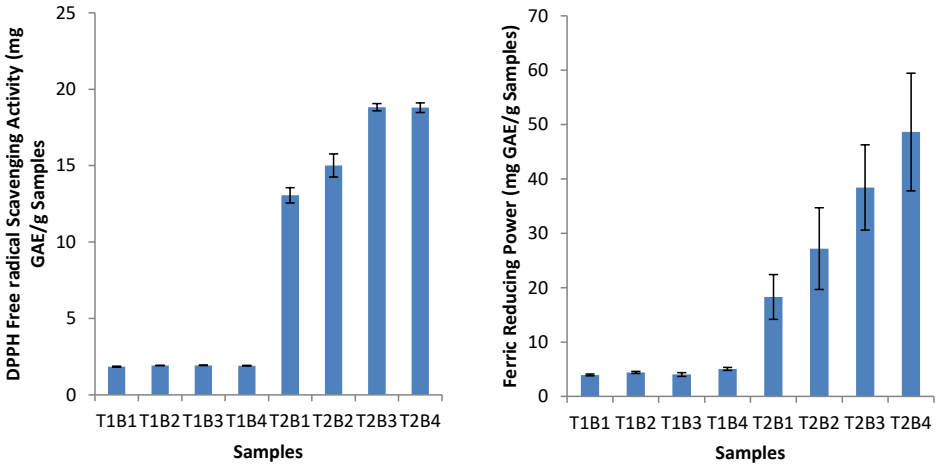

[a] [b]

Figure 2. Antioxidant activity of *Pluchea* tea at different steeping temperatures and storage periods: [a] DPPH; [b] FRAP. Data analysis using ANOVA at α ≤ 0.05 continued analysis using a paired t-test at α ≤ 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.

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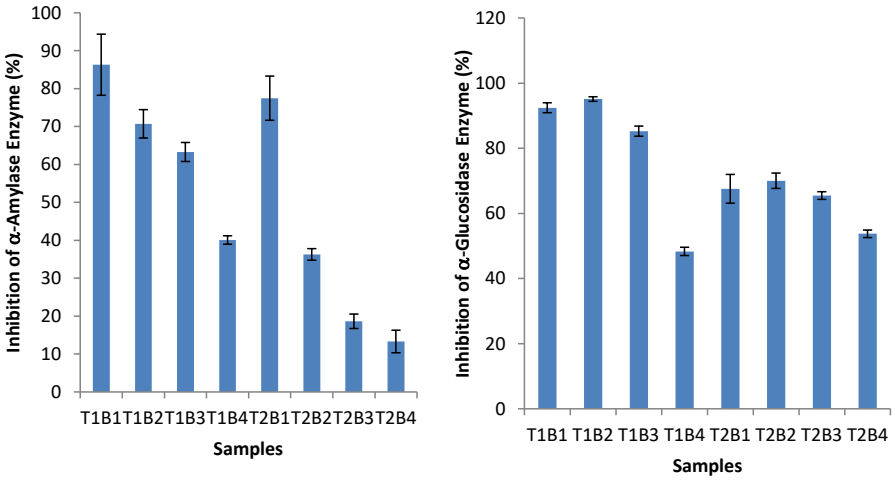

[a] [b]

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperatures and storage periods: [a] α-amylase; [b] α-glucosidase. Data analysis using ANOVA at α ≤ 0.05 continued analysis using a paired t-test at α ≤ 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.

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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	α-Alpha- glucosidase	α-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		
α-Alpha glucosidase	–0.55512	0.349873	–0.71534	–0.5272	–0.55947	1	
α-Alpha amylase	–0.70842	0.429393	–0.8569	–0.69579	–0.80548	0.725161631	1

Significant at the 0.05 level (two-tailed)

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

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
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Effect of Steeping Temperature and Storage Period on the Bioactive Compounds plus Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea indica* Less

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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 [α-amylase (AA) and α-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

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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, β-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, β-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 [α-amylase (AA) and α-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 ± 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, α-amylase, α-glucosidase, p-nitrophenyl-α-glucopyranoside (pNPG), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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 distillated 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 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10% were mixed in 10-mL volumetric flash and incubated for 5 min. Then, 2 mL Na_2CO_3 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 λ = 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 $AlCl_3$ and $NaNO_2$ with the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim *et al*. 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted in a yellow solution. About 30-µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5% in 10-mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 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 λ = 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-µL *Pluchea* infusion was added with 1-mL Folin-Ciocalteu's phenol reagent 10% in 10-mL volumetric flash and incubated for 5 min. Then, the mixture was added with 2-mL Na_2CO_3 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 λ = 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 µL *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 . = 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 µL 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 λ = 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

α-amylase enzyme inhibition (AA) capacity assay. *In vitro* AA followed the procedure, as described by Widyawati *et al.* (2020). Each 500 µL of the samples was mixed with starch 1% (w/v) and sodium acetate buffer pH 5. Into 250 µL of the mixture, an α-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 α-amylase enzyme to hydrolyze the starch to release glucose was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ = 540 nm. The inhibition percentage of α-amylase was assessed using the formula (ACb − ACa) − (As − Ab) (ACb − ACa) x 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.

α-glucosidase enzyme inhibition (GA) capacity assay. The analysis of the α-glycosidase inhibitor activity (GA) was done using the method of Widyawati *et al.* (2020) with slight modifications. About 150-µL samples containing 100-µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100-mL sodium phosphate 0.2 M at pH 7) were reacted with 50-µL α-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-µL 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 λ = 405 nm. The inhibition percentage of α-glycosidase was calculated using the formula (ACb − ACa) − (As − Ab) (ACb − ACa) x 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 Kongkiatpaiboona *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 µm, NYL). About 20 µL 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 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (two pieces) (ID 10 mm x 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 ± 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 ± 4.14 mg GAE/g sample), whereas the lowest was measured in the unstored samples and infused at 60 °C (at 4.39 ± 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.

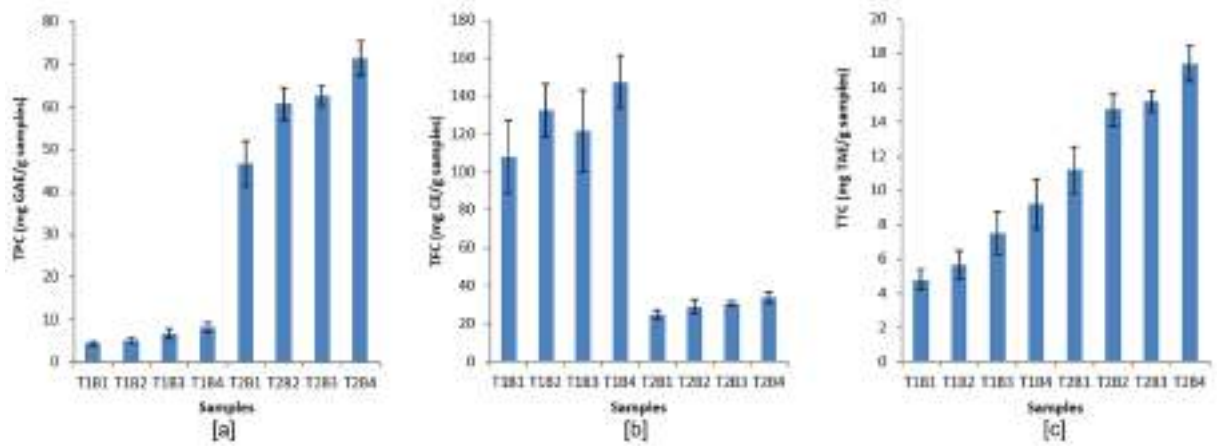

Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping temperatures and storage periods: [a] total phenolic content, [b] total flavonoid content, and [c] total tannin content. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paired t-test at $\alpha \leq 0.05$. Data were expressed as mean ± 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.

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*-cafffeoylquinic 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 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 ± 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 ± 0.58 to 17.42 ± 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 ± 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 ± 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

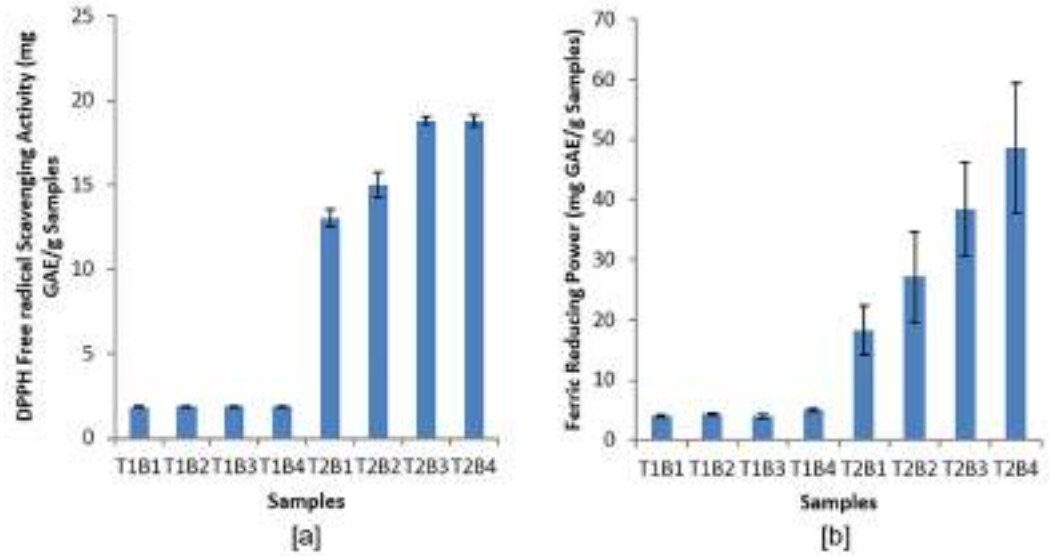

Figure 2. Antioxidant activity of *Pluchea* tea at different steeping temperatures and storage periods: [a] DPPH; [b] FRAP. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paired t-test at $\alpha \leq 0.05$. Data were expressed as mean ± standard deviation (n = 6). Samples : 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.

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	α-glucosidase	α-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		
α-glucosidase	–0.55512	0.349873	–0.71534	–0.5272	–0.55947	1	
α-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 ± 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 ± 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

α-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.* α-amylase and α-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 α-amylase and α-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 α-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 α-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 α-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.

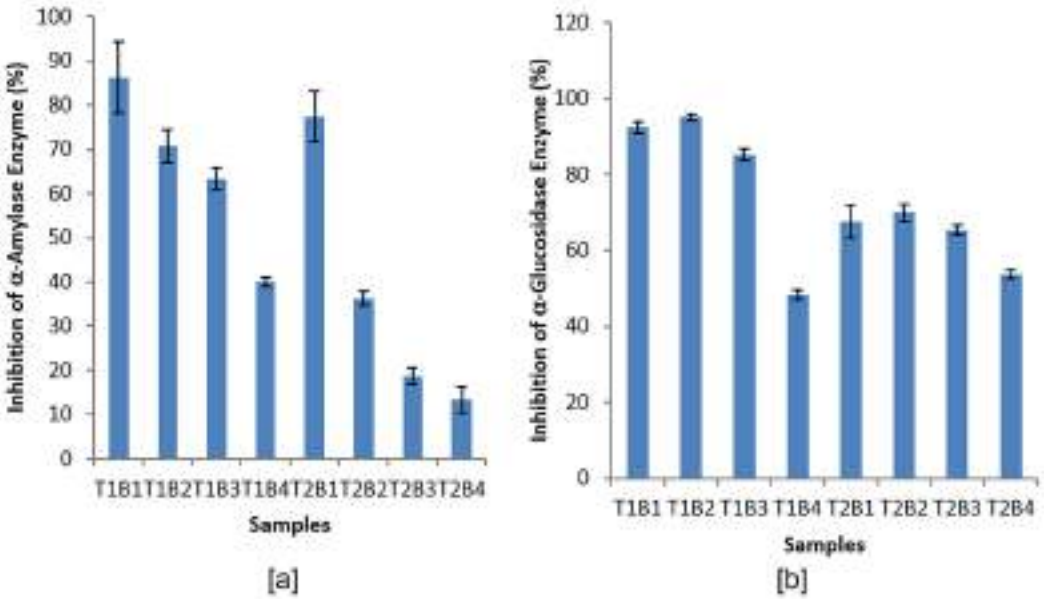

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperatures and storage periods: [a] α-amylase; [b] α-glucosidase. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paired t-test at $\alpha \leq 0.05$. Data were expressed as mean ± standard deviation (n = 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.

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 α-amylase enzyme. Akah *et al.* (2011) reported that phytochemical compounds such as terpenoids, saponins, flavonoids, glycosides/carbohydrates, and alkaloids are good antidiabetic metabolites or α-amylase enzyme activity inhibitors. Sangeetha and Vedasree (2012) explained that the ability of *Threspesia populnea* extract to inhibit the α-amylase enzyme was determined by their phenolic compound content and protein. Moreover, the presence of the α-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 α-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 α-amylase. The hydroxyl groups can bind by non-covalent interaction (hydrogen bonding, cation-π interactions, salt bridge interactions, ionic interactions, or electrostatic forces) with amino acid residue at the active site in the α-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 α-amylase enzyme.

α-glucosidase enzyme inhibition activity (GA). α-glucosidase is an important enzyme in carbohydrate digestion, that catalysis the hydrolysis of 1,4-α-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 α-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 α-glucosidase enzyme of 67.857%.

Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the α-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 ± 1.27%, and the highest inhibitory activity was at 70 °C at 95.11 ± 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 α-glucosidase enzyme tended to be higher than the ability to inhibit the α-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 α-amylase and α-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 α-glucosidase enzyme activity.

The ability to inhibit the α-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 α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of the two enzymes was different, according to the opinion of McCue *et al.* (2005). The mechanism of the α-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 α-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 α-glucosidase enzyme activity. The ability of bound phenolic compounds to inhibit α-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

α-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 unstored *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 α-glucosidase enzyme than free caffeoylquinic acid.

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 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 unstored. TPC was highest in the stored and steeped at 95 °C and lowest in the unstored and steeped at 60 °C. Unstored steeped samples exhibited significantly higher flavonoid content than the stored steeped samples. The highest TFC was exhibited by the unstored samples steeped at 95 °C. The TTC of *Pluchea* leaf infusion significantly increased with increasing steeping temperature and storage period. Among the unstored 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 unstored 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 unstored 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 unstored *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 α-glucosidase enzyme decreased at high steeping temperatures and long storage periods. The highest inhibitory activity was obtained in the unstored *Pluchea* leaf infusion that was steeped at 70 °C, whereas the lowest was obtained in the unstored sample that was steeped at 95 °C. The ability of *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-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.

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STATEMENT ON CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Effect of Steeping Temperature and Storage Period on the Bioactive Compounds plus Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea indica* Less

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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 [α-amylase (AA) and α-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

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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, β-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, β-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 [α-amylase (AA) and α-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 ± 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, α-amylase, α-glucosidase, p-nitrophenyl-α-glucopyranoside (pNPG), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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 distillated 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 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10% were mixed in 10-mL volumetric flash and incubated for 5 min. Then, 2 mL Na_2CO_3 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 λ = 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 $AlCl_3$ and $NaNO_2$ with the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim *et al*. 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted in a yellow solution. About 30-µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5% in 10-mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 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 λ = 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-µL *Pluchea* infusion was added with 1-mL Folin-Ciocalteu's phenol reagent 10% in 10-mL volumetric flash and incubated for 5 min. Then, the mixture was added with 2-mL Na_2CO_3 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 λ = 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 µL *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 . = 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 µL 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 λ = 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

α-amylase enzyme inhibition (AA) capacity assay. *In vitro* AA followed the procedure, as described by Widyawati *et al.* (2020). Each 500 µL of the samples was mixed with starch 1% (w/v) and sodium acetate buffer pH 5. Into 250 µL of the mixture, an α-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 α-amylase enzyme to hydrolyze the starch to release glucose was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ = 540 nm. The inhibition percentage of α-amylase was assessed using the formula (ACb − ACa) − (As − Ab) (ACb − ACa) x 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.

α-glucosidase enzyme inhibition (GA) capacity assay. The analysis of the α-glycosidase inhibitor activity (GA) was done using the method of Widyawati *et al.* (2020) with slight modifications. About 150-µL samples containing 100-µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100-mL sodium phosphate 0.2 M at pH 7) were reacted with 50-µL α-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-µL 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 λ = 405 nm. The inhibition percentage of α-glycosidase was calculated using the formula (ACb − ACa) − (As − Ab) (ACb − ACa) x 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 Kongkiatpaiboona *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 µm, NYL). About 20 µL 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 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (two pieces) (ID 10 mm x 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 ± 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 ± 4.14 mg GAE/g sample), whereas the lowest was measured in the unstored samples and infused at 60 °C (at 4.39 ± 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.

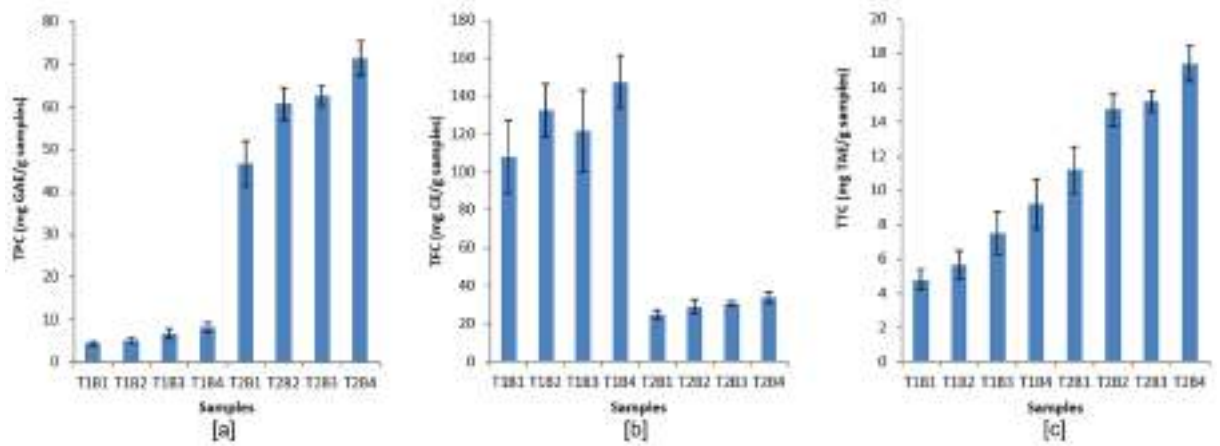

Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping temperatures and storage periods: [a] total phenolic content, [b] total flavonoid content, and [c] total tannin content. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paired t-test at $\alpha \leq 0.05$. Data were expressed as mean ± 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.

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*-cafffeoylquinic 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 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 ± 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 ± 0.58 to 17.42 ± 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 ± 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 ± 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

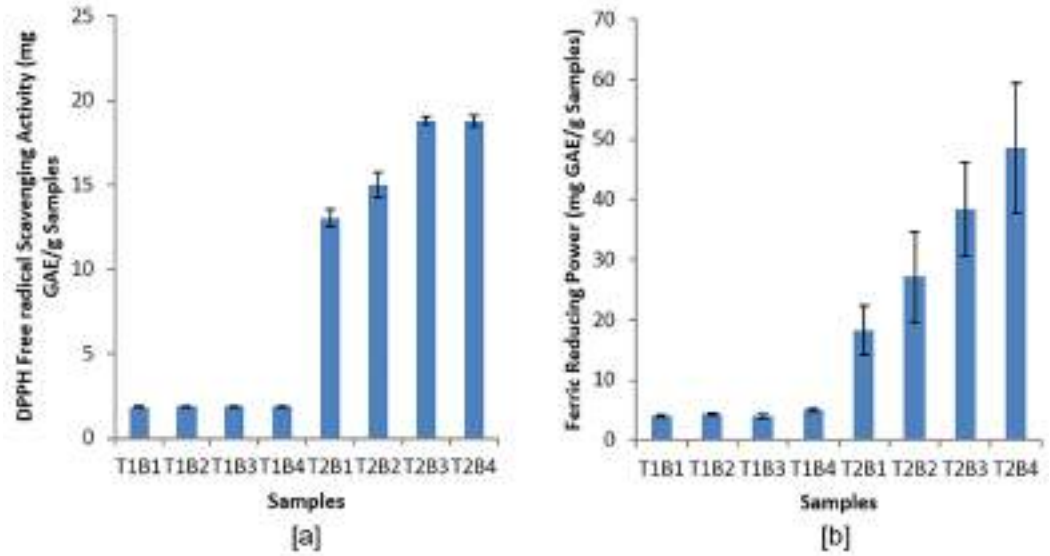

Figure 2. Antioxidant activity of *Pluchea* tea at different steeping temperatures and storage periods: [a] DPPH; [b] FRAP. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paired t-test at $\alpha \leq 0.05$. Data were expressed as mean ± standard deviation (n = 6). Samples : 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.

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	α-glucosidase	α-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		
α-glucosidase	–0.55512	0.349873	–0.71534	–0.5272	–0.55947	1	
α-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 ± 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 ± 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

α-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.* α-amylase and α-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 α-amylase and α-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 α-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 α-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 α-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.

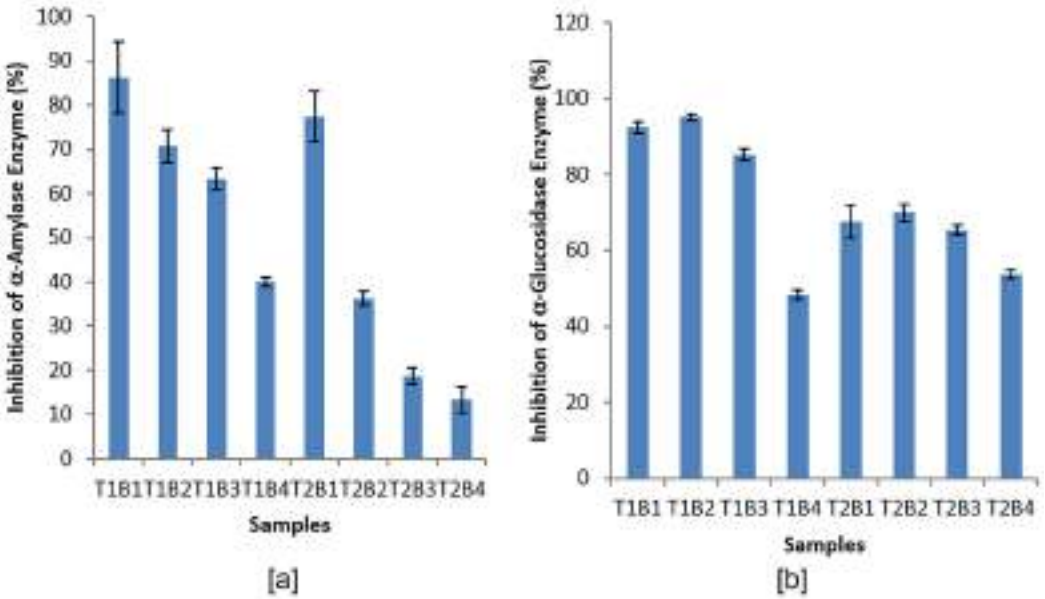

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperatures and storage periods: [a] α-amylase; [b] α-glucosidase. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paired t-test at $\alpha \leq 0.05$. Data were expressed as mean ± standard deviation (n = 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.

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 α-amylase enzyme. Akah *et al.* (2011) reported that phytochemical compounds such as terpenoids, saponins, flavonoids, glycosides/carbohydrates, and alkaloids are good antidiabetic metabolites or α-amylase enzyme activity inhibitors. Sangeetha and Vedasree (2012) explained that the ability of *Threspesia populnea* extract to inhibit the α-amylase enzyme was determined by their phenolic compound content and protein. Moreover, the presence of the α-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 α-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 α-amylase. The hydroxyl groups can bind by non-covalent interaction (hydrogen bonding, cation-π interactions, salt bridge interactions, ionic interactions, or electrostatic forces) with amino acid residue at the active site in the α-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 α-amylase enzyme.

α-glucosidase enzyme inhibition activity (GA). α-glucosidase is an important enzyme in carbohydrate digestion, that catalysis the hydrolysis of 1,4-α-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 α-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 α-glucosidase enzyme of 67.857%.

Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the α-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 ± 1.27%, and the highest inhibitory activity was at 70 °C at 95.11 ± 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 α-glucosidase enzyme tended to be higher than the ability to inhibit the α-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 α-amylase and α-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 α-glucosidase enzyme activity.

The ability to inhibit the α-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 α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of the two enzymes was different, according to the opinion of McCue *et al.* (2005). The mechanism of the α-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 α-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 α-glucosidase enzyme activity. The ability of bound phenolic compounds to inhibit α-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

α-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 unstored *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 α-glucosidase enzyme than free caffeoylquinic acid.

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 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 unstored. TPC was highest in the stored and steeped at 95 °C and lowest in the unstored and steeped at 60 °C. Unstored steeped samples exhibited significantly higher flavonoid content than the stored steeped samples. The highest TFC was exhibited by the unstored samples steeped at 95 °C. The TTC of *Pluchea* leaf infusion significantly increased with increasing steeping temperature and storage period. Among the unstored 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 unstored 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 unstored 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 unstored *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 α-glucosidase enzyme decreased at high steeping temperatures and long storage periods. The highest inhibitory activity was obtained in the unstored *Pluchea* leaf infusion that was steeped at 70 °C, whereas the lowest was obtained in the unstored sample that was steeped at 95 °C. The ability of *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-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.

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STATEMENT ON CONFLICT OF INTEREST

The authors declare no conflict of interest.

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First Draft of PJS Article Ms 23-158

Philippine Journal of Science <pjs@stii.dost.gov.ph> Wed, Jun 19, 2024 at 3:00 PM
To: Paini Sri Widyawati <paini@ukwms.ac.id>

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
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Effect of Steeping Temperature and Storage Period on the Bioactive Compounds plus Antioxidant and Antidiabetic Activities of Infusion from Powdered *Pluchea indica* Less

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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 [α-amylase (AA) and α-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

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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, β-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, β-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 [α-amylase (AA) and α-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 ± 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, α-amylase, α-glucosidase, p-nitrophenyl-α-glucopyranoside (pNPG), (+)-catechin, kaempferol, myricetin, quercetin, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylqiunic 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 distillated 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 µL *Pluchea* infusion and 1 mL Folin-Ciocalteu's phenol reagent 10% were mixed in 10-mL volumetric flash and incubated for 5 min. Then, 2 mL Na_2CO_3 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 λ = 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 $AlCl_3$ and $NaNO_2$ with the aromatic ring of flavonoid compounds, especially flavonol and flavon (Shraim *et al*. 2021). The reaction between $AlCl_3$ and flavonoid compounds resulted in a yellow solution. About 30-µL *Pluchea* infusion was mixed with 0.3 mL $NaNO_2$ 5% in 10-mL volumetric flash and incubated for 5 min. The mixture was added with 0.3 mL $AlCl_3$ 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 λ = 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-µL *Pluchea* infusion was added with 1-mL Folin-Ciocalteu's phenol reagent 10% in 10-mL volumetric flash and incubated for 5 min. Then, the mixture was added with 2-mL Na_2CO_3 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 λ = 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 µL *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 . = 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 µL 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 λ = 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

α-amylase enzyme inhibition (AA) capacity assay. *In vitro* AA followed the procedure, as described by Widyawati *et al.* (2020). Each 500 µL of the samples was mixed with starch 1% (w/v) and sodium acetate buffer pH 5. Into 250 µL of the mixture, an α-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 α-amylase enzyme to hydrolyze the starch to release glucose was measured by UV-Vis spectrophotometer (Spectrophotometer UV-Vis 1800, Shimadzu, Japan) at λ = 540 nm. The inhibition percentage of α-amylase was assessed using the formula (ACb − ACa) − (As − Ab) (ACb − ACa) x 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.

α-glucosidase enzyme inhibition (GA) capacity assay. The analysis of the α-glycosidase inhibitor activity (GA) was done using the method of Widyawati *et al.* (2020) with slight modifications. About 150-µL samples containing 100-µL *Pluchea* infusion and 50 µL pNPG (0.0150 g in 100-mL sodium phosphate 0.2 M at pH 7) were reacted with 50-µL α-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-µL 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 λ = 405 nm. The inhibition percentage of α-glycosidase was calculated using the formula (ACb − ACa) − (As − Ab) (ACb − ACa) x 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 Kongkiatpaiboona *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 µm, NYL). About 20 µL 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 µm × 50 mm x 4.6 mm) with a GVP-ODS Cartridge guard column (two pieces) (ID 10 mm x 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 ± 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 ± 4.14 mg GAE/g sample), whereas the lowest was measured in the unstored samples and infused at 60 °C (at 4.39 ± 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.

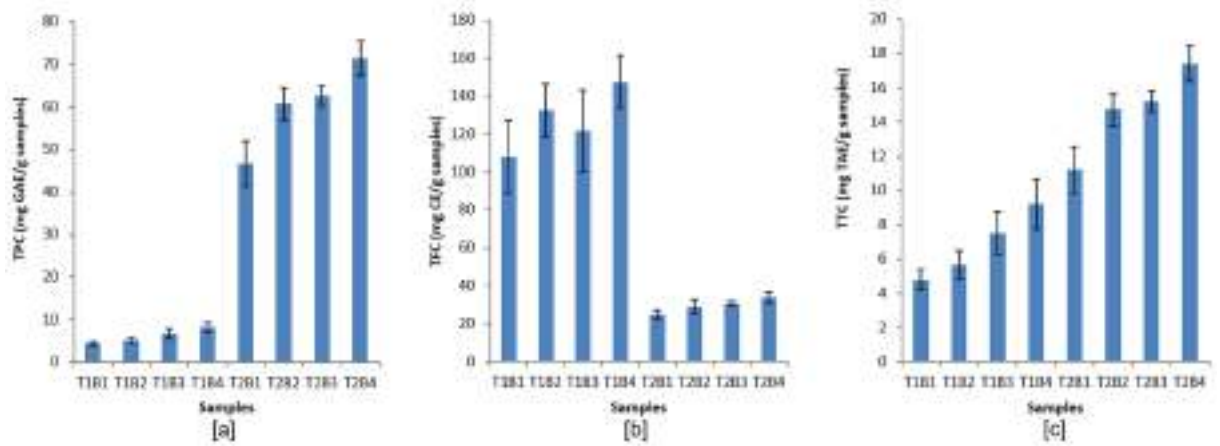

Figure 1. Bioactive compound contents of *Pluchea* infusion at different steeping temperatures and storage periods: [a] total phenolic content, [b] total flavonoid content, and [c] total tannin content. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paired t-test at $\alpha \leq 0.05$. Data were expressed as mean ± 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.

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*-cafffeoylquinic 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 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 ± 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 ± 0.58 to 17.42 ± 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 ± 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 ± 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

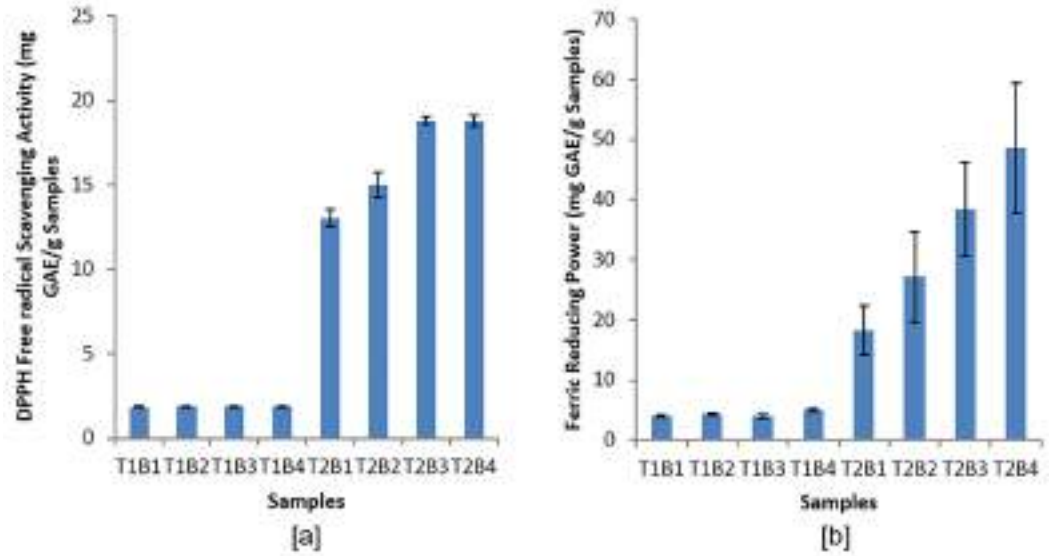

Figure 2. Antioxidant activity of *Pluchea* tea at different steeping temperatures and storage periods: [a] DPPH; [b] FRAP. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paired t-test at $\alpha \leq 0.05$. Data were expressed as mean ± standard deviation (n = 6). Samples : 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.

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	α-glucosidase	α-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		
α-glucosidase	–0.55512	0.349873	–0.71534	–0.5272	–0.55947	1	
α-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 ± 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 ± 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

α-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.* α-amylase and α-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 α-amylase and α-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 α-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 α-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 α-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.

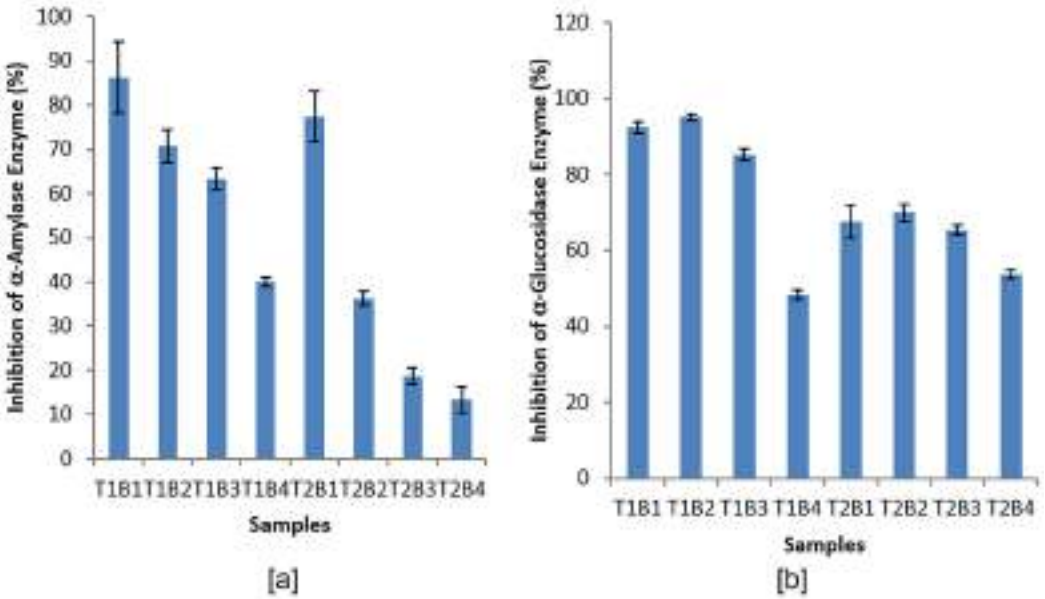

Figure 3. Antidiabetic activity of pluchea tea at different steeping temperatures and storage periods: [a] α-amylase; [b] α-glucosidase. Data analysis using ANOVA at $\alpha \leq 0.05$ continued analysis using a paired t-test at $\alpha \leq 0.05$. Data were expressed as mean ± standard deviation (n = 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.

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 α-amylase enzyme. Akah *et al.* (2011) reported that phytochemical compounds such as terpenoids, saponins, flavonoids, glycosides/carbohydrates, and alkaloids are good antidiabetic metabolites or α-amylase enzyme activity inhibitors. Sangeetha and Vedasree (2012) explained that the ability of *Threspesia populnea* extract to inhibit the α-amylase enzyme was determined by their phenolic compound content and protein. Moreover, the presence of the α-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 α-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 α-amylase. The hydroxyl groups can bind by non-covalent interaction (hydrogen bonding, cation-π interactions, salt bridge interactions, ionic interactions, or electrostatic forces) with amino acid residue at the active site in the α-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 α-amylase enzyme.

α-glucosidase enzyme inhibition activity (GA). α-glucosidase is an important enzyme in carbohydrate digestion, that catalysis the hydrolysis of 1,4-α-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 α-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 α-glucosidase enzyme of 67.857%.

Figure 3b shows that the ability of the *Pluchea* leaf infusion to inhibit the α-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 ± 1.27%, and the highest inhibitory activity was at 70 °C at 95.11 ± 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 α-glucosidase enzyme tended to be higher than the ability to inhibit the α-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 α-amylase and α-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 α-glucosidase enzyme activity.

The ability to inhibit the α-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 α-glucosidase enzyme was greater than the α-amylase enzyme because the mechanism of the two enzymes was different, according to the opinion of McCue *et al.* (2005). The mechanism of the α-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 α-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 α-glucosidase enzyme activity. The ability of bound phenolic compounds to inhibit α-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 α-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 unstored *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 α-glucosidase enzyme than free caffeoylquinic acid.

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 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 unstored. TPC was highest in the stored and steeped at 95 °C and lowest in the unstored and steeped at 60 °C. Unstored steeped samples exhibited significantly higher flavonoid content than the stored steeped samples. The highest TFC was exhibited by the unstored samples steeped at 95 °C. The TTC of *Pluchea* leaf infusion significantly increased with increasing steeping temperature and storage period. Among the unstored 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 unstored 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 unstored 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 unstored *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 α-glucosidase enzyme decreased at high steeping temperatures and long storage periods. The highest inhibitory activity was obtained in the unstored *Pluchea* leaf infusion that was steeped at 70 °C, whereas the lowest was obtained in the unstored sample that was steeped at 95 °C. The ability of *Pluchea* leaf infusion to inhibit the α-glucosidase enzyme tended to be higher than the ability to inhibit the α-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.

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STATEMENT ON CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Paini Sri Widyawati

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Corresponding AUTHOR
(Signature over printed name)

Paini Sri Widyawati

_____
Senior AUTHOR
(Signature over printed name)

Yufita Ratnasari Wilianto

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

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!

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