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28**Contents lists available at** SciVerse **ScienceDirect Industrial Crops and Products journal homepage: www.elsevier.com/locate/indcrop Extraction,**

identification and quantitative HPLC analysis of flavonoids from sarang semut (Myrmecodia pendan) Adam Mekonnen Engidaa, Novy S. Kasima, Yeshitila Asteraye Tsigiea, Suryadi Ismadjib, Lien Huong Huynhc, Yi-Hsu Jua,∗

1**a Department of Chemical Engineering, National Taiwan University of Science and Technology, 43, Sec. 4, Keelung** Road, **Taipei** 106-07, **Taiwan b Department of Chemical Engineering, Widya Mandala Surabaya Catholic University, Kalijudan 37, Surabaya 60114, Indonesia c** Department **of Chemical Engineering,** Can Tho **University,**

Can Tho City, Viet Nam

33**article info Article history: Received 11 December 2011 Received in revised form** 22 April **2012 Accepted** 27 April **2012 Keywords:**

Antioxidant activity Flavonoids HPLC–UV/vis Myrmecodia pendan Phenolics content abstract The objective of this study was to extract and determine total contents of phenolic and flavonoid com- pounds as well as to identify and quantify some flavonoids from sarang semut (Myrmecodia pendan). Water bath extraction at 55 ◦C was employed for extracting flavonoids from sarang semut.

30**The effects of parameters such as extraction time,**

composition of solvent mixture and solvent to sample ratio on extraction were investigated. From (33) factorial design the optimum extracting parameters were deter- mined as follows: extraction time, 4 h; ethanol/water composition, 80%;

15**and solvent to** sample **ratio,** 50 **ml/g. Under** these **optimal conditions,** a **yield of** 13.82% **was**

obtained. The free radical scavenging activity (antioxidant activity) of the extract was evaluated using DPPH radical and it was found that the IC50 occurred at 96.21 ± 9.03 ?g/ml of extract. The total phenol and flavonoid contents were determined using designed methods and found to be 330

3**.61 ± 2.13 mg GAE/g and 63.28 ± 1.75 mg QE/g** of dry extract, **respectively.**

The

31**extract obtained under optimum conditions was analyzed by HPLC and five flavonoid compounds were identified** and quantified; **they are**

kaempferol (13.767 mg/g), luteoline (0.005 mg/g), rutine (0.003 mg/g), quercetin (0.030 mg/g) and apigenin (4.700 mg/g) of dry extract.

5**1. Introduction Flavonoids represent a highly diverse class of secondary plant metabolites with about 9000 structures**

(Martens and Mithofer, 2005). Flavonoids are polyphenolic compounds derived from 2-phenylchromane commonly found in many plants, vegeta- bles, and flowers (Boue et al., 2003; Plochmann et al., 2007; Androutsopoulos et al., 2010; Lisa et al., 2010). They received considerable attention in literature, specifically due to their bio- logical and physiological importance (Zin et al., 2002; Rijk et al., 2006).

29**They have benefits to human health, including** antioxi- dant **activities**

(Tian et al., 2009), metal chelation (Heim et al., 2002; Seyoum et al., 2006) and antiproliferative, anticarcinogenic, antibacterial, anti-inflammatory, antialergic, and antiviral effects (Merken and Beecher, 2000; Ajila et al., 2010; Abrham et al., 2008; Barreca et al., 2011).

39**Flavonoids are able to scavenge free** radi- cals **directly by hydrogen atom donation (Prochazkova et al., 2011).**

5**Basically flavonoids are derivatives of 1,3-** diphenylpropane **-1-one (C6 C3 C6)**

(Erlund, 2004; Barontini et al., 2010; Biesaga, 2011). Derived from the chalcone structure, a flavonoid class containing ∗

17**Corresponding author. Tel.: +886 2 27376612; fax: +886 2 27376644. E-mail address: yhju@mail.ntust.edu.tw (Y.-H. Ju).**

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http://dx.doi.org/10.1016/j. indcrop **.2012.**

04.043 three rings can be formed (Martens and Mithofer, 2005; Mladenka et al., 2010). One of the not-well identified medicinal plants is sarang semut (Myrmecodia pendan) which might be a new potential source of therapeutic agents. Sarang semut lives as epiphyte on cajupu (Melaleuca), cemara gunung (Casuarina), kaha (Castanopsis) and beech (Nothophagus). It is called sarang semut since the inner part of its hypocotyls is used as nest by ants (sarang is nest and semut is ant in Bahasa Indonesian). It belongs to genus Iridomyrmex. Most sarang semut plants are usually colonized by one species of ant. M. pendans is colonized by ant Ochetellus species. Sarang semut is a member of Rubiaceae family with five genera. However, only two of which have association with ants. They are Myrmecodia (45 species) and Hypnophytum (26 species). Hypnophytum formicarum, M. pendans and M. tuberose are believed to have medicinal value (Soeksmanto et al., 2010). There is no report on identification or quantification of bioactive compounds in sarang semut plant, only reports on discussing about its ecology; taxonomy and ethno botany can be found (Soeksmanto et al., 2010). Anti-cancer activity of this plant was tested by using cancer cells derived from human cervix and canine breast, called HeLa cells and MCM-B2, respectively (Soeksmanto et al., 2010). The result of the research showed that the extract of sarang semut plant was capable of inhibiting the growth of HeLa and MCM-B2 cells. A.M. Engida et al. / Industrial Crops and Products 41 (2013) 392–396 393 This effect may be the result of phenolic compounds especially flavonoids contained in the tested extracts. Hence the objective of this research was to extract and deter- mine total phenolic and flavonoid contents using experimental design methods, as well as to identify and determine some indi- vidual flavonoids from sarang semut (M. pendan). 2. Materials and methods 2.1. Chemicals and materials The plant material used in this study was obtained from a tradi- tional medicine plant store in Wamena, Papua, Indonesia. Ethanol, methanol (MeOH, HPLC grade), acetic acid (glacial analytical grade), tert-butyl hydroquinone (TBHQ) and

2**flavonoid standards including catechin, (+)-epicatechin, apigenin, luteolin, myricetin,** quercetin **and** rutine **were purchased from Sigma Aldrich,**

USA. Deionized water was used for HPLC mobile phase. 2.2. Sample preparation Sarang semut was washed with running tap water and then rinsed by distilled water to remove any adsorbed contaminant from sample surface. The cleaned sample was chopped and dried using freeze dryer then placed in an oven at 40 ◦C for 12 h to remove any remaining moisture. The dried material was ground by a blender into powder and passed through a sieve (120 meshes) was collected for extraction. 2.3. Extraction

36**Heat reflux is the most common method for the extraction of bioactive components from natural products**

(Xiao et al., 2008). This extraction method was chosen for the first preliminary study because of its simplicity and manageability. The dried and pow- dered plant material (1 g per batch) was extracted using water bath at 55 ◦C. Three independent variables and three levels; solvent com- position (40%, 60%, 80% of ethanol), extraction time (2, 3, 4 h), and solvent to sample ratio (30, 40, 50 ml/g) with coded values as min- imum (−1), center (0) and maximum (1) were evaluated. All these factors might affect the diffusion of analytes from sample matrix to solvent. Experiments were conducted with full factorial design (33) which refers to three independent variables studied. Three levels were conducted for each independent variable. Altogether, 27 runs were performed

25**under randomized order and the experiments on each sample were carried out in**

triplicate. The mixture was filtered with a Whatman No. 2 filter paper; ethanol was removed using a rotary

32**evaporator at** 68 ◦**C and water was removed by**

freeze drying. The percentage of crude dry extract was determined as follows: Yextract (%) = Mextract × 100 mfeed where Yextract

2**is the extraction yield, Mextract is the crude extract ( ) mass (g) and** mfeed **is the** feed **mass (g)**

2.4. DPPH radical scavenging activity assay Free radical scavenging activity of the plant extract was deter- mined by using DPPH assay according to the procedure described by Basma et al. (2011), with some modifications. 2,2-Diphenyl-1- picrylhydrazyl (DPPH) 0.002% methanolic solution was prepared in a volumetric flask covered with aluminum foil.

38**One milliliter of** the **plant extract in methanol (10–** 150 **?g/** ml) **was mixed with 1 ml** solution **of**

DPPH (0.002%).

10**For blank solution, the** extract was **substituted by methanol.**

Reduction of DPPH by antioxi- dants was monitored at 520 nm using a spectrophotometer (Jasco, UV-550, Japan) in 10 mm quartz cells. Anti-radical

10**activity was based on measurement of the reducing ability of the plant extract toward DPPH radical.** Scavenging effect **was** calculated **as**

(A0 − At) ×

21100/A0, where A0 is the absorbance of the control reac- tion and At is the absorbance in the presence of the extract sample.

The effective concentration of the plant extract at which 50% of DPPH radicals are reduced

22(IC50) was calculated graphically using a calibration curve in the linear range by plotting extract concentra- tion vs. the corresponding scavenging effect.

Data were reported as arithmetic means ± SD for three replications. The performance of the extracts was also compared with that of ascorbic acid.

262.5. Determination of total phenolics content The total phenolics content of the plant extract was determined by Folin–Ciocalteu reagent

(FCR) method according to Michel et al. (2011). For a 2.00 ml total volume, 20 ?l of extract, 20 ?l of aque- ous standard solutions (100–1000 ?g/ml of gallic acid) or 20 ?l deionized water for blank were first mixed with 100 ?l of FCR after adding 1.58 ml deionized water and the contents were kept at room temperature (26.5 ◦C) for 10 min. Later 300 ?l of Na2CO3 aqueous solution (20%) was added between 8 and 15 min and

34incubated at room temperature for 20 min. The absorbance was measured at 765 nm using a spectrophotometer (Jasco, UV- 550, Japan). Total phenolics content

was expressed as ?g of gallic acid equivalent/g of dry extract (?g GAE/g).

272.6. Determination of total flavonoid content Total flavonoid content of the plant extract was determined using aluminum chloride colorimetric method

and using stan- dard solutions (12.5, 25, 50, 80 and 100 ?g/ml of quercetin in 80% methanol). For the analysis, 1 ml extract solution in 80% methanol (1 mg/ml) was mixed with 0.5 ml 95% ethanol (v/v), 0.1 ml alu- minum chloride solution (10% AlCl3), 0.1 ml 1 M potassium acetate and 0.8 ml distilled water to a total volume of 2.5 ml. The

24mixture was well mixed and incubated at room temperature for 30 min. Absorbance was measured at 520 nm vs. reagent blank containing water instead of

sample. Quercetin was used as the standard for the quantification of total flavonoid. Results were expressed as mil- ligrams of quercetin equivalent per gram of dry weight extract (mg QE/g). Total content of flavonoid

was calculated as follows: Total flavonoid content = QE × V/m. Where QE is the quercetin equiva- lence (mg/ml) or concentration of quercetin solution

16established from the calibration curve; V is the volume of extract (ml) and m is the weight (g) of the dry extract

(Basma et al., 2011). Data was reported as arithmetic mean ± SD for three replications. 2.7. Analysis of flavonoids by HPLC HPLC is one of the most powerful tools in analytical chem- istry, with the ability to separate, identify and quantify the target compounds present in any sample that can be dissolved in HPLC compatible liquid (Chiu et al., 2002; Marston, 2007). Sarang semut plant sample was extracted at optimum condition for HPLC analysis using the method of Weisz et al. (2009). A sample (3 g) was heated for 4 h in 150 ml solvent at 55 ◦C. The mixture was filtered with a Whatman No. 2 filter paper, then ethanol was removed using a rotary

32evaporator at 60 ◦C and water was removed by

freeze drying. The dried extract was reconstituted and heated at 80 ◦C for 1 h (to hydrolyze glycosides to aglycons) in a solution of 40 ml 65% aque- ous

6methanol in which 0.5 g/l tert-butylhydroquinone (TBHQ) was dissolved, and 10 ml 6 N HCl was added.

TBHQ was incorporated as antioxidant to avoid flavonoid degradation.

6After cooling, the solu- tion was sonicated for 5 min and

made to a final volume of 100 ml by adding de-ionized water, then filtered through a 0.2 ?m Anotop 394 A.M. Engida et al. / Industrial Crops and Products 41 (2013) 392–396 Table 1 Extraction yield obtained under the experimental condition using complete randomized design (CRD) full factorial. Independent variables Average % yield at each level Range Level 1a Level 2a Level 3a Rb Solvent composition (%) 10.08 ± 2.84 10.67 ± 1.41 11.56 ± 1.89 1.48 ± 3.41 Extraction time (h) 9.76 ± 1.28 11.19 ± 1.42 11.36 ± 1.21 1.60 ± 1.76 Solvent amount (ml/g) 8.66 ± 1.61 11.54 ± 1.31 12.82 ± 0.34 3.16 ± 1.65 a

12Average response for each level on extraction yield

(n = 3). b Range

12(R) value means range between average yields for each level of extraction yield.

syringe filter for HPLC analysis. The HPLC analyses were performed with: Luna 5U-C18 (2) 100A column (250 mm × 4.5 mm, 5 ?m) plus Jasco, quaternary gradient pump (pu-2089) plus Jasco, UV-2077 4? intelligent UV/vis detector.

20The compounds were eluted with a gradient elution of mobile phases A and B.

Solvent A consisted of deionized water and 1% acetic acid and solvent B

25consisted of methanol (HPLC grade) and

1% acetic acid. Acetic acid (1%) was added to reduce peak tailing. Gradient elution

8program was set as follows: 10% B– 17.2% B (18 min),

17.2% B–23% B (12 min), 23% B iso- cratic (10 min), 23%–31.3% B (13 min), 31.3% B–46% B (12 min), 46% B

8–55% B (5 min), 55% B– 100% B (5 min), 100% B isocratic (8 min), 10% B (2 min) and 10% B isocratic (5 min).

The injection volume for all samples was 20 ?l. Flavonoids were monitored at 280 nm and 285 nm at a flow rate of 1 ml/min.

16All determinations were performed in triplicate.

The flavonoids standard stock solutions were initially prepared. Dilutions and injections of these standards were then made until a HPLC chromatogram showed that the flavonoids peak height reached at S/N of approximately 10:1 and 3:1 for LOQ and LOD solutions, respectively.

35Flavonoids were identified by matching the retention time and their spectral characteristics against those of standards

and the contents of flavonoids were determined using calibration curves. Each standard solution (0.1–2.0 ? g/ml quercetin, 0.1–0.5 mg/ml apigenin and rutine, 0.05–0.5 ?g/ml luteoline and 0.01–0.1 mg/ml kaepferol) was dissolved in methanol and subjected to HPLC analy- sis. The calibration curves were constructed by plotting the average peak areas vs. the concentration of each analyte. The chromatogram for the blank solvent was subtracted from sample chromatogram to correct the background error. Finally the quantity of each detected flavonoid was determined using regression equation (Y = ax ± b), where x is concentration and y is the peak area of each flavonoid derived from calibration curve of each respective standard. The linearity was established by the coefficient of determination (R2).

29Slope, intercept, R2 and the other statistical calibration lines were calculated

using Microsoft excel version 7

12.0. 3. Results and discussion 3.1. Optimization of extraction

condition

18**To obtain an efficient extraction of target compounds,**

optimiza- tion of

18**experimental conditions is a critical step in developing an** extraction **method. The** extraction efficiency **of** water bath **extraction**

was examined at different sets of solvent composition, extraction time and solvent to sample ratio under (33) test design. The results shown in Table 1 indicate that there are differences in yield among each set of extraction condition. As can be seen from Table 1, with increasing ethanol composition, extraction time and solvent to sample ratio from 40% to 60%, 2–4 h and 30–50 ml/g, respectively; the yield (%) varies from 10.08 ± 2.84 to 11.556 ± 1.89, 9.76 ± 1.28 to 11.36 ± 1.21 and 8.66 ± 1.61 to 12.82 ± 0.34 corre- spondingly. Statistically the variation was significant with 95% confidence level (p = 0.047). Extraction yield increases with increas- ing level of factors but solvent to sample ratio was found to be the most important determinant of yield with range 3.16 ± 1.65 as shown in Fig. 1. Mean values at the three levels for each param- eter show how extraction yield changes with level of parameter. Based on the obtained mean value of

30**main effects of the** three **most important factors, the** highest **extraction**

yield was obtained at 80% ethanol, 4 h

15**and a solvent to** sample **ratio of** 50 **ml/g. The** maximum **yield**

was obtained at highest levels of all factors. This illustrates the possibility of extracting more antioxidants from the plant on other higher combination of these factors and its potential as antioxidant compound source. But for this preliminary experiment the yield obtained was considered for further analysis. Dry extract obtained under optimum condition was used for the analysis.

9**Data were subjected to** analyses **of variance (ANOVA) and multiple comparison tests were performed using a least significant difference (LSD), suitable for factorial** design, **at** 95% **of confidence level.**

Statistical calculation and analysis were performed using Microsoft excel version 7.0. The yield of crude extract obtained under optimum condition was found to be 13.82%. 3.2. DPPH radical scavenging activity The relatively stable DPPH radical has been

23**widely used to test the ability of** a compound **to act as free radical** scavenger **or hydrogen** donor **and** thus **to evaluate** its **antioxidant activity**

(Li et al., 2008).

13**Phenolic compounds in plants are viewed as powerful in vitro antioxidants** due to **their ability to donate hydrogen or** elec- trons **and to form stable radical intermediates**

(Wang et al., 2007). Plant extract quenched

37**DPPH free** radical **in a dose-** dependent man- ner. **As concentration of the** extract **increased,** its **DPPH quenching activity also increased**

as shown in Fig. 2. The

11**observed antioxi- dant activity of the** extract **may be due to the** neutralization **of free radical character of DPPH; either by transfer of an electron or** hydro- gen **atom.**

The plant extract showed antioxidant activity in the DPPH assay with great antioxidant capacity and the IC50 occurred at 96.21 ± 9.03 ?g/ml of extract. The inhibitory potential of the plant extract was compared with known antioxidant (ascorbic acid) and the result was comparable as shown in Fig. 2. Fig. 1. Variation of extraction yields under complete randomized design (CRD) full factorial in different levels of factors: (a) solvent composition (%), (b) extraction time (h) and (c) solvent to sample ratio (ml/g) at 55 ◦C. A.M. Engida et al. / Industrial Crops and Products 41 (2013) 392–396 395 Table 2 Calibration curves, correlation coefficients (r2),

4**detection limits and quantification limits for HPLC analysis for** compounds extracted, **identified** and quantified **from**

Myrmecodia pendan. Peak no. Compounds Linear equations Squ. LODa (?g/ml) LOQb (?g/ml) correlation coefficients (r2) 8 Kaepferol 7 Luteoline 5 Rutine 10 Quercetin 12 Apigenin y = 1.52x − 1.22 y = 0.39x + 0.04 y = 85.5x − 0.03 y = 0.3x y = 18.8x + 0.14 0.977 0.460 ± 0.033 0.978 1.750 ± 0.096 1 0.720 ± 0.066 1 1.960 ± 0.159 0.995 0.670 ± 0.075 1.533 ± 0.110 5.833 ± 0.320 2.400 ± 0.220 6.532 ± 0.530 2.233 ± 0.250 a LOD (?g/ml) = 3S, where S is the standard deviation of the blank determination (n = 3). b LOQ (?g/ml) = 10 × S. 3.3. Total phenolics and flavonoids contents Total phenol content was reported as gallic acid equiva- lents (GAE) by reference to standard curve (Y = 0.004x + 0.521 and R2 = 0.997). The dry plant extract has a total phenol content of 330.61 ± 2.13 mg GAE/g. These observations suggest that sarang semut plant is rich in phenolics that have potential as value added products. Total flavonoid content was expressed as quercetin equivalents (QE) by reference to standard curve (Y = 0.05x − 0.375 and R2 = 0.991). It was found that the extract has a total flavonoid content of 63.28 ± 1.75 mg QE/g of dry extract. Like any plant prod- ucts, the total phenolic and flavonoids contents and the radical inhibitory effect can be influenced by the plant source and envi- ronment where the plant is collected and cultivated. 3.4. Analysis of flavonoids by HPLC The extract from sarang semut obtained under optimum con- ditions (80% ethanol as solvent, 50 ml solvent per gram sample, extraction time 4 h) was analyzed using HPLC at 280 and 285 nm. Five flavonoid compounds (kaempferol, luteoline, rutine, quercetine and apigenin)

14**were identified from the extract by matching their retention times against those of the standards. Peak assignment was confirmed by injection of**

standards.

However, the results obtained in this work cannot be compared with those reported in literatures. Because firstly this study is the first work on the preliminary identification and quantification of this plant, and secondly the retention times depend on factors such as solvent composition, extract matrix and the gradient elution pro- gram. Fig. 3 shows the HPLC chromatogram of a mixture of eight flavonoid standards for peak comparison with the chromatogram of the crude plant extract (Fig. 4). As shown in Fig. 4, the HPLC chromatogram of the extract obtained under optimum conditions displays twelve peaks which were detected at 280 nm. Peaks 4, 7, 9,

20**Fig. 2. DPPH radical scavenging** capacity **of** plant **extract**

and ascorbic acid. Data represent means ± SD (n = 3). 10 and 12 were identified as rutine, luteoline, kaepferol, quercetin and apigenin, respectively with good resolution. All identified flavonoids were calibrated at 280 nm. Five flavonoids including kaempferol, luteoline, rutine, quercetin and apigenin were quantified. Kaempferol was found to have the highest concentration (13.767 mg/g), followed by apigenin (4.700 mg/g). The other three all existed in relatively smaller con- centrations; quercetin (0.030 mg/g), luteoline (0.005 mg/g), rutine (0.003 mg/g). All results (total phenolic content, total flavonoid, individual flavonoids as well as the radical inhibitory effect) shown in this study may vary depending on the source of sarang semut as well as the environment where it is cultivated.

4**The summary of linear equations, squared correlation** coeffi- cients, **limit of determination (LOD) and limit of quantification (LOQ) of the**

five quantified flavonoids are presented in Table 2. Fig. 3. Chromatogram of eight available flavonoid standards monitored at 280 nm and identified with retention time (min) catechin (17.593), (+)-epicatechin (43.110), rutine (62.433), luteoline (73.178), myrcetin (74.777), kaepferol (76.197), quercetin (78.147) and apigenin (87.160). Fig. 4. HPLC chromatogram of the crude extract obtained under optimum condi- tions: 80% ethanol, 4 h and 50 ml per gram sample. Peak no. 4 (rutine), 7 (luteolin), 9 (kaepferol), 10 (quercetin) and 12 (apigenin) were identified peaks. 396 A.M. Engida et al. / Industrial Crops and Products 41 (2013) 392–396 4. Conclusion This investigation has showed that water bath extraction method at constant temperature (55 ◦C) and optimum con- ditions of solvent composition (80% ethanol), extraction time (4 h) and solvent/g sample (50 ml), resulted maximum yield (13.82%). The extract was evaluated for radical-scavenging activity (1,1-diphenyl-2-picryldrazyl radical). The extract showed radi- cal scavenging activity (IC50 value of 96.21 ± 9.03 ?g/ml) and its quenching activity was comparable to that of ascorbic acid. The total phenol and flavonoid contents of sarang semut (M. pendans) extract were found to be 330

3**.61 ± 2.13 mg GAE/g and 63.28 ± 1.75 mg QE/g** of dry extract, **respectively.**

From HPLC analysis of crude extract, five flavonoids including kaempferol (13.767 mg/g), luteolin (0.005 mg/g), rutine (0.003 mg/g), quercetin (0.030 mg/g) and apigenin (4.700 mg/g) were identified and quan- tified in this plant. Hence the plant can be considered as a potential source for flavonoids particularly and phenolic compounds gener- ally. The findings from this work may add to the overall value of the medicinal potential of plants. Acknowledgment

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