Comparative Evaluation of Total Phenolics and Free Radical Scavenging Activity of Aqueous Extracts of *Labisia pumila* var. *alata* from Malaysia and Indonesia

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***Abstract*— Functional food and beverage companies are touting the presence of antioxidants in their products in response to consumer interest in the potential health benefits of antioxidants in the diet. *Labisia pumila* (LPva) or "Kacip Fatimah" is a popular supplement in the Malaysian functional food and beverage market. Today, information on its bioactive compounds remains scarce. A comparative evaluation of total phenolics and free radical scavenging activity of the aqueous extract of LPva were studied using Folin – Ciocalteu assay, aluminum chloride assay, ferric ion reducing power assay and DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay. The results of total phenolics showed that LP from Tilu Mountain-Bogor (LPvaT) contained the highest average of total phenolics. The ratio of total flavonoids/total phenolics (TFC/TPC) of LP from Halimun-Salak Mountain, Bogor (LPvaB) with is 0.27 and contained the highest average of total flavonoids. Antioxidant activity by FRAP assay shows that LPvaT contained the highest average of ability to reduce the ferric ion-TPTZ complex. DPPH assay showed that EC_{50} of LPvaT (78.79 µg/mL) was lowest. Based on these values, LPvaT is found to have the highest antioxidant activity.**

Keywords- Free radical scavenging activity, total phenolics, aqueous extract, Labisia pumila var. alata

I. INTRODUCTION

The advent of technology and education has led to increased awareness in the importance of health thereby, changing public opinion and demand on food. The change demands that food products are not only nutritious but are also safe. Functional foods as defined by Goldberg [1] are foods which have passed processing, having one or more compounds based on research, have physiological functions useful for health, and having sensory characteristics and good acceptability (color, taste, texture and consistency).

The search for newer natural antioxidants, especially of plant origin, has ever since increased. Several plant extracts and different classes of phytochemicals have been shown to have free radical scavenging activity. *Labisia pumila* var alata (Malay: "Kacip Fatimah") is used in traditional medicine preparation in the form of decoction to prepare the birth channel, delay fertility, treat flatulence, dysentery, dysmenorrheal, gonorrhea, and also as a general tonic. Previous studies have reported on LPva as possessing antibacterial effects [2], oestrgenicity in vitro, receptor binding inhibition in vitro, and anti-oedema in vivo [3], prevented the changes in bone biochemical markers in rats [4], as an anti-photoaging cosmetic ingredient [5]. Singh *et. al.,* [6] reported that a dose of 50 mg/kg of an aqueous extract of *LPva* corresponded to no-adverse-effect-level (NOAEL), where as higher doses were associated with some toxicity concerns. Plant phenolic compounds can reduce the deleterious effects of reactive oxygen species (ROS) on a number of biological and pathological processes [7]. Sawa *et. al.* [7] also suggests that scavenging of ROS by plant phenolics may be the basis of purported human health benefits of plants. However, the previous studies on antioxidant properties of aqueous extract of LPva vary between geographical regions are sporadic and lacking.

In this study, we examined the total phenolics, total flavonoids, and free radical scavenging activity of LPva from different geographic in order to gauge the potential of the plant as a functional food ingredient. In our study a relationship between total phenolics content and free radical scavenging activity also demonstrated.

The rest of this paper is organized as follow. Section 2 describes material and methods of this study which consist of preparation of freeze-dried extract, determination of total phenolics content, total flavonoids content, ferric ion reducing power, and DPPH antiradical scavenging effect. Section 3 describes results and discussion of comparison total phenolics, total flavonoids, and antioxidant activity of LPva. Finally the conclusion of this study is described section 4.

II. MATERIAL AND METHODS

In this section, material and methods of this study are discussed.

A. Materials

Chemicals

Gallic acid, catechin, Folin-Ciocalteu's phenol reagent, TPTZ and 1,1–diphenyl 2-picrylhydrazyl (DPPH), were purchased from Sigma Chem. Co. Methanol, $FeCl_3$, Na_2CO_3, $AlCl_3$, $NaNO_2$, NaOH, and ultra pure water were obtained from the chemical store room of the Faculty of Chemical and Natural Resources, Universiti Malaysia Pahang. Reagents were of analytical grade.

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Plant material

Fresh LPva were collected from Raub Pahang, Malaysia (LPvaR); Salak-Halimun Mountain, Bogor, Indonesia (LPvaB); Tilu Mountain, Bogor, Indonesia (LPvaT); Cibeundey Village, Aceh, Indonesia (LPvaA) and Pekandangan Village, Lampung, Indonesia (LPvaL). LPvaC was a commercial product ingredient obtained from a manufacturer in Kuala Lumpur, Malaysia.

B. Methods

Preparation of freeze-dried extract of LP

LP leaves were rinsed to remove debris, dried at 40°C for 3 days and ground into small pieces. After soaking in ultrapure water (1:6) overnight, it was extracted for 6 hours (twice) by Soxhlet. The filtrates were consolidated and lyophilized in a freeze dryer. The lyophilized extract was stored in a dessicator until further use.

For analysis, 0.06 g of freeze dried material was weighed into a centrifuge tube, to which was added 10 mL of ultra pure water. The sample was shaken for 15 minutes and then centrifuged. The supernatant was then transferred into a 10 ml volumetric flask, diluted to the mark with ultra pure water and mixed well by shaking for 15 minutes.

Total phenolics content

The total phenolics content of LP was determined using a modified Folin-Ciocalteu assay [8]. To 0.1 mL extract or ultra pure water blank or gallic acid standard solution (0-200 µg/ml) was add 2.8 mL ultra pure water and 2 mL of 2% sodium carbonate and left standing for 4 minutes. Then, 100 µL Folin-Ciocalteu was added the solutions again left standing for 30 minutes. Measurement was conducted on a spectrophotometer UV-VIS HITACHI 1800 at λ= 760 nm against the blank. Total phenolics were derived from expression **(1)** and expressed as mg gallic acid equivalent (GAE) in g of dry weight of lyophilized plant extract (R^2: 0.993). Samples were analyzed in triplicate.

$$\text{Absorbance} = 0.008 \text{ gallic acid } (\mu g) + 0.083 \qquad (1)$$

Total flavonoids content

Total flavonoids content was measured by aluminum chloride colometric assay with modification. Total flavonoid content was measured using aluminum chloride colorimetric assay [9]. To the extract solution (1 mL) or standard solution of catechin (0-200 µg/mL) was added to separate 10 ml volumetric flasks containing 4 ml of ultra pure water. To the flask was added 0.3 ml of 5% $NaNO_2$ solution and left to stand for five minutes. A solution of 10% $AlCl_3$ (0.3 mL) was added and left to stand for 6 minutes. A solution of 1 M NaOH (2 mL) was added and the total volume diluted to 10 ml with ultra pure water. The solutions were mixed well. Measurement was conducted on a spectrophotometer at λ= 510 nm against a blank. Total flavonoid content were derived from expression **(2)** and expressed as mg catechin equivalents (CE)/g dry weight of lyophilized plant extract (R^2: 0.999). Samples were analyzed in triplicates.

$$\text{Absorbance} = 0.003\text{catechin } (\mu g) + 0.096 \qquad (2)$$

Ferric Reducing Antioxidant Potential (FRAP) assay

The ability to reduce ferric ions was measured using a modified version of the method described by [10]. An aliquot (150 µL) of an extract was added to 850 µL of FRAP reagent (10 part of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 TPTZ solution and 1 part of 20 mM $FeCl_3.6H_20$ solution), add ultra pure water into mark of volumetric flask 10 mL. The reaction mixture was incubated in water bath 37 °C. The increase in absorbance at λ= 597 nm was measured at 30 minutes. Antioxidant activity was expressed as mg Gallic acid equivalent in g dry weight plant material. Ultrapure H_2O was use as a blank. Samples were analysis in triplicate.

Scavenging activity (DPPH) assay

The free radical scavenging activity of the extracts, based on activity of the stable DPPH free radical, was determined by a method described by Kumaran and Kuranakaran [11]. To the extract solution of varying concentrations (1 mL) or blank or standard solution of ascorbic acid (0-100 µg/ml) was added 3 mL of 0.004% DPPH methanolic solution and left standing in the dark for 30 minutes. Measurement was conducted on a spectrophotometer at λ = 517 nm against a blank. The data was derived using expression (**3**) and reported as concentration of antioxidant required for 50% scavenging of DPPH radicals in the specified time period (EC_{50}). Samples were analyzed in triplicates.

$$\% \text{ Inhibition} = [(A_c - A_s)/Ac] \times 100 \qquad (3)$$

Where: A_c = absorbance control or blank,
A_s = absorbance with sample or standard

Statistical analysis

Data were first tested for normality, and then subjected to analysis of variance (ANOVA). Significant differences between mean values were determined using Duncan's Multiple Range test (P=0.05) following one-way ANOVA. Statistical analyses were carried out using the correlation and regression program in *Microsoft Excel 2007*.

III. RESULTS AND DISCUSSION

A. Total phenolics and total flavonoids content

Phenolics are very important constituent of plants. Their free radical scavenging ability is attributed to hydroxyl groups. Total phenolics were measured using an established method employing the Folin-Ciocalteu reagent. The principle of this method is the reduction ability of the phenol functional group. An oxidation and reduction reaction of the phenolic ion takes place in basic conditions. Reduction of the phosphortungstat-phosphormolybdenum complex (Folin-Ciocalteu reagent) by phenolic ions changes the reagent to

dark blue [12]. The color becomes darker, absorbing at progressively higher wavelengths as reduction ability increases with increasing phenolic compounds as is the case with LPva. The results of total phenolics and total flavonoids content of LPva are displayed in Table 1.

TABLE 1. CONTENT TOTAL PHENOLICS AND TOTAL FLAVONOIDS OF LPVA WATER- SOLUBLE EXTRACT

Sample	Total phenolics (mg GAE/g)*	Total flavonoids (mg CE/g)*	ratio TF/TP
LPvaR	86.875 ± 0.00	19.68 ± 0.03	0.23
LPvaB	117.5 ± 0.00	32.28 ± 0.06	0.27
LPvaC	94.58 ± 0.00	14.83 ± 0.06	0.16
LPvaT	140.49 ± 0.12	11.43 ± 0.03	0.08
LPvaA	113.96 ± 0.00	9.48 ± 0.03	0.08
LPvaL	127.36 ± 0.12	9.19 ± 0.03	0.07

*GAE: Gallic acid equivalent.
CE: Catechin equivalent. Values expressed are means ± SD

Table 1 showed that total phenolics content range from 86.88 to 140.49 mg GAE/g of dry material and a significance of $0.000 < \alpha$. This indicates significant differences in total phenolics content of the different LPva extracts. Duncan's multiple ranges test showed that LPvaT (140.49 mg GAE/g dry weight of lyophilized plant extract) contained the highest average of total phenolics followed by LPvaL (127.36 mg GAE/g dry weight of lyophilized plant extract), LPvaB (117.50 mg GAE/g dry weight of lyophilized plant extract), LPvaA (113.96 mg GAE/g dry weight of lyophilized plant extract), LPvaC (94.58 mg GAE/g dry weight of lyophilized plant extract) and LPvaR (86.88 mg GAE/g dry weight of lyophilized plant extract).

Flavonoids are a natural subset of phenolics with antioxidant properties. Plant extracts have reported by [13] which were rich in flavonoids. Table 1 showed total flavonoids of LPva range between 9.17 to 32.28 mg CE/g of lyophilized plant extract (also Table 1). ANOVA analysis showed a significance of $0.000 < \alpha$. It can be conclude that there were significant differences in total flavonoids content of LPva from different geographic origins. Duncan's multiple ranges test showed that LPvaB (32.28 mg CE/g dry weight of lyophilized plant extract) contained the highest average of total flavonoids with a ratio of total flavonoids to total phenolics of 0.27. This is followed by LPvaR (19.69 mg CE/g dry weight of lyophilized plant extract), LPvaC (14.83 mg CE/g dry weight of lyophilized plant extract), LPvaT (11.43 mg CE/g dry weight of lyophilized plant extract), LPvaA (9.48 mg CE/g dry weight of lyophilized plant extract) and LPvaL (9.17 mg CE/g dry weight of lyophilized plant extract). Total phenolics content of LPva in 60% crude methanolic extract and 40% fraction SPE were 950.4 µg GAE/mg dry weight plant and 266 µg GAE/mg dry weight plant [14]. Chua *et. al.*, [14] also reported that total flavonoids content of LPva in 60% methanolic extract and 40% fraction SPE were 46.6 µg Rutin Equivalent (RE)/mg dry weight plant and 76.8 µg RE/mg dry weight plant.

B. *Ferric Reducing Antioxidant Potential (FRAP) assay*

The results of antioxidant activity of LPva to reduce the ferric ion-TPTZ are shown in Fig. 1.

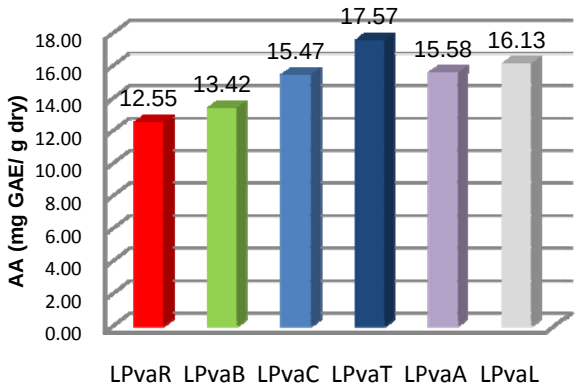

Figure 1. An antioxidant activity of *LPva* leaves on their abilities to reduce the ferric ion-TPTZ complex (n=3)

Fig. 1 showed that antioxidant activities of LPva ranged from 12.55 to 17.57 mg GAE/g weight of lyophilized plant extract. ANOVA analysis showed a significance of $0.000 < \alpha$. It can be conclude that there were significant differences in antioxidant activity content of LPva from different geographic origins. Duncan's multiple ranges test showed that LPvaT (17.57 mg GAE/g dry weight of lyophilized plant extract) contained the highest average of antioxidant activity. This is followed by LPvaL (16.13 mg GAE/g dry weight of lyophilized plant extract), LPvaA (15.58 mg GAE/g dry weight of lyophilized plant extract), LPvaC (15.47 mg GAE/g dry weight of lyophilized plant extract), LPvaB (13.42 mg GAE/g dry weight of lyophilized plant extract) and LPvaR (12.55 mg GAE/g dry weight of lyophilized plant extract).

C. *DPPH free radical scavenging activity*

Lyophilized plant extracts of LPva showed a concentration-dependent free radical scavenging activity by inhibiting the DPPH radical and values are expressed as EC_{50}. The DPPH method is based on the reduction of methanolic DPPH solution in the presence of hydrogen donating antioxidant through formation of the non-radical form, DPPH-H. The scavenging effect increased with increasing concentration of the lyophilized plant extracts. As the extracts reduce the stable DPPH radical, the solution changes color from purple to yellow, arising from diphenylpicrylhydradzine. The lyophilized plant extracts possess hydrogen donating capabilities and acts as free radical scavengers. The results of antioxidant activity of LPva to reduce and decolorized DPPH are shown in Fig. 2.

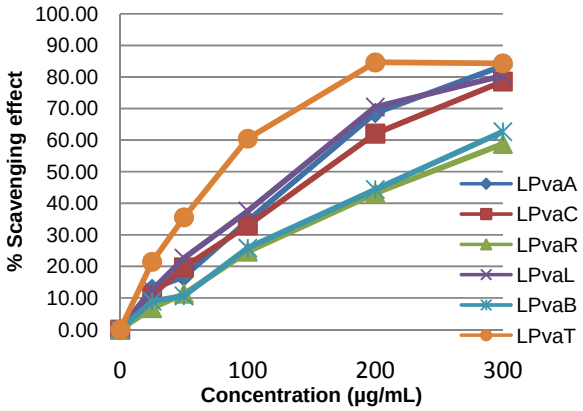

Figure 2. An antioxidant activity of *LPva leaves* on their abilities to reduce and decolorized DPPH (n=3)

Fig. 2 shows that EC_{50} of LPvaR was 244.32 µg/mL followed by LPvaB (231.65 µg/mL), LPvaC (157.40 µg/mL), LPvaA (145.11 µg/mL), LPvaL (137.54 µg/mL) and LPvaT (78.79 µg/mL). The LPvaT lyophilized plant extract showed the highest DPPH radical scavenging activity followed by LPvaL, LPvaA, LPvaC, LPvaB and LPvaR lyophilized plant extracts. Chua *et. al.,* [14] reported that EC_{50} of LPva in 60% methanolic extract and 40% SPE were 1532.5µg/mL and 1092.10 µg/mL.

The free radical scavenging activities of a plant extracts can also be related to its phenolic content. Phenolic compounds have been reported to possess antioxidant activity that allows them to scavenge both active oxygen species and electrophiles, inhibit nitrosation, chelate metal ions, and have potential for auto-oxidation and the capability to modulate certain cellular enzyme activities [15]. The correlation between total phenolics content (TPC) (X) versus antioxidant activity FRAP assay (Y) had a correlation coefficient of R^2= 0.541 (Y=0.067X + 7.457) (Fig. 3).

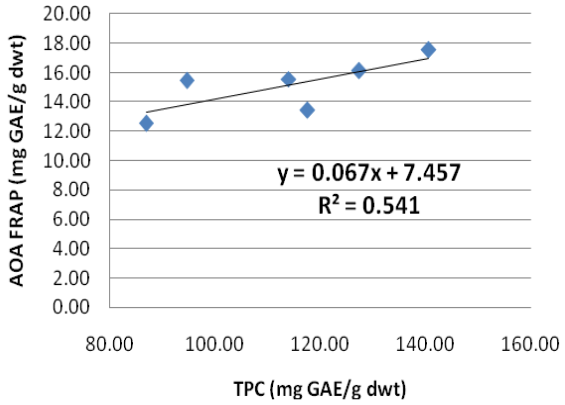

Figure 3. Linear correlation of free radical scavenging activity (Y) Versus the total phenolics content (X) of LPva

This result suggests that 54% of antioxidant capacity of LP arise from contribution of phenolics compounds and 46% from other secondary metabolites, such carotenoids and vitamins, among others. It was clear that antioxidant activity increased proportionally to the total phenolics content and a positive linear relationship exist between antioxidant activities (FRAP values) and total phenolic compounds. The correlation was suggest by [16] between the total phenolics content and free radical scavenging capability of edible seaweed extracts.

IV. CONCLUSION

In this study, we have successfully comparison of total phenolics and free radical scavenging activity of aqueous extract of LPva from Malaysia and Indonesia. Based on the results, LPvaT (140.49 mg GAE/g lyophilized plant material) contained the highest average of total phenolics. In terms of total flavonoids content LPvaR (32.28 ± 0.03 mg CE/g lyophilized plant material) with a ratio of total flavonoids/total phenolics (TFC/TPC) of 0.27 contained the highest average of total flavonoids. Antioxidant activity by FRAP assay showed that LPvaT (17.57. mg GAE/g lyophilized plant material) contained the highest average ability to reduce the ferric ion-TPTZ complex. *DPPH assay* showed that EC_{50} of LPvaT (78.79 µg/mL) had the lowest concentration of EC_{50}. It can be concluded that LPvaT possessed the best antioxidant capability compared to the rest of the LPva samples investigated.

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