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Comparing Three Varieties of *Labisia pumila* (Primulaceae): Phytochemicals and Antioxidation

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Abstract

Labisia pumila is a traditional medicinal herb that is widely used by women to firm the uterus after childbirth. Recent scientific studies have also proven its pharmacological importance. However, the comparison of phytochemicals in the different varieties of the herb is very limited in literature. Therefore, 3 common varieties of *L. pumila* leaves were extracted in a reflux system, and consequently the crude extract was fractionated using reversed phase solid phase extraction (SPE) into individual fractions according to solvent polarity. *L. pumila* var. *alata* (LPA) showed the highest yield of crude extract and total phenolics content which was also in line with high DPPH radical scavenging activity. While, *L. pumila* var. *pumila* with red leaves (LPPR) exhibited higher total flavonoids and triterpenoids than its green leaves counterpart (LPPG). Higher antioxidant capacity was possibly due to the presence of plant co-pigments in LPPR. Glycosylated flavones including orientin were putatively detected in the intermediate polar fraction of LPA based on mass fragments. However, 0 % methanol (polar) and 100 % methanol (less polar) fractions notably contained compounds competing oxygen with nitric oxide radicals from the formation of nitrite ions. Proton NMR spectral analysis proposed N-[1-(2,3-dihydro-1,4-benzodioxin-6-yl)-1-hydroxy-3-(morpholin-4-yl)propan-2-yl]decanamide and 4-[({1,3-bis[(2-ethylpenta-3,4-dien-1-yl)oxy]propan-2-yl}oxy)methyl]hexa-1,2-diene in the 0 % methanol and 100 % methanol fractions, respectively. This study concluded that the plant variety of *alata* was more antioxidative than *pumila*. The detection of orientin and its derivatives are first time reported in LPA. Glycosylated flavones are more likely to be hydrogen atom and/or electron donors contributing to the major antioxidant property of the herb.

Keywords: *Labisia pumila*; orientin, Glycosylated flavones, Solid phase extraction, Antioxidant, Scavenging activity

Introduction

Labisia pumila is a medicinal popular herb from the family *Primulaceae.* It can be widely found in the rainforest of Indochina such as Malaysia, Indonesia, Thailand and Philippines [1]. Often, it is also well known as *Kacip Fatimah*, *Selusoh Fatimah*, *Sangkoh*, *Tadah Matahari* and *Mata Pelanduk Rimba* among the Iban community [2]. Three common varieties of *L. pumila* have been identified in Malaysia, and they are *L. pumila* var. *lanceolate*, *L. pumila* var. *alata* and *L. pumila* var. *pumila* [3]. The varieties differ in term of morphological appearance, especially the plant leaf structure and colour [1,4]. Overall, *L. pumila* var. *alata* has long petioles and slightly broad ovary shaped leaves, *L. pumila* var. *pumila* has winged petioles, whereas *L. pumila* var. *lanceolate* has terete and long petioles. *L. pumila* var. *pumila* has also either green or red coloured leaves depending upon the amount of sunlight exposed to the plants.

The most commonly used variety is *L. pumila* var. *alata* [5]. It is traditionally prepared as herbal decoction by boiling its leaves and roots, or the whole plant in water. The herbal decoction is given to pregnant women to induce childbirth during pre-partum, to contract the birth channel and tone abdominal muscle during post-partum, as well as to recover body strength during confinement period [6]. The other

scientifically proven pharmaceutical properties of *L. pumila* included anticancer [7,8], antioxidant [9], antimicrobial [10], anti-inflammatory [11] and phytoestrogen therapy [12].

Even though *L. pumila* has long been used as ethnomedicine and its remarkable pharmacological significances have also been proven, the main chemical constituents that are contributing to those properties are still remained unknown. There is no conclusive technical data to support the role of individual phytochemical groups in relation with the reported bioactivities. Phytochemicals such as flavanols (catechin and epicatechin), flavonols (kaempferol, quercetin and myricetin), flavones (apigenin, luteolin and velutin), isoflavones (daidzin, puerarin and genistin), flavanone (naringin) and phenolic acids (gallic acid, coumaric acid and ferulic acid) were identified in previous studies [13-15].

Often, natural products with high antioxidant property possess tremendous pharmacological importance. Natural antioxidants are also favourable and being a good alternative choice in counteracting radicals associated with diseases like cancer, diabetic, high blood pressure and arthritis. The antioxidants are likely to be reducing agents, hydrogen donors, and singlet oxygen quenchers, in addition to the metal chelating potentials. The common antioxidant assays for natural product samples are DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), FRAP (ferric reducing antioxidant power) and ORAC (oxygen radical absorbance capacity). Calorimetric methods which examine the mechanism followed by the antioxidative compounds via hydrogen atom transfer (HAT), single electron transfer (SET) or both pathways simultaneously. DPPH and ABTS assays determine antioxidant compounds that follow both HAT and SET mechanisms in inhibiting radicals. FRAP assay analyses compounds that performing SET, whereas ORAC estimates compounds that follow HAT pathway. Another common assay is using Griess reagent which is used to spectrophotometrically measure antioxidant (SET) and anti-inflammatory activities by inhibiting nitric oxide radicals. Plant antioxidants may react as radical scavengers and compete oxygen with NO radicals, and leading to the reduction of nitrite ion production.

In the present study, the antioxidant and phytochemical profiles of the 3 common varieties of *L. pumila* were investigated for comparison. This comparison is very important because many herbal products formulated from *L. pumila* extract including health supplement, cosmetic and food based products are increasingly available in the market. Understanding the underlying antioxidative mechanism in relation with the phytochemicals could explain the pharmacological significance of *L. pumila*. The right selection of the plant variety would enhance product efficacy, particularly products with functional claims. The complexity of *L. pumila* extract could be reduced by performing the sequential extraction techniques, namely reflux extraction and followed by C18 reversed phase solid phase extraction (SPE) to fractionate the crude extract into individual fractions according to solvent polarity. Fractions containing the assigned polarity of compounds would better explain the antioxidant property which is usually the basis to support the pharmacological importance of herbal plants.

Materials and methods

Chemicals and plant materials

Liquid chromatography grade of solvents such as methanol, ethanol, acetonitrile and dimethyl sulfoxide (DMSO), strong acids such as sulfuric acid (98 %), formic acid (≥ 98 %), hydrochloric acid (37 %) and phosphoric acid, and other chemicals such as 2,4,6-Tri(2-pyridyl)-1,3,5-triazine (TPTZ, ≥ 98 %) and sulfanilamide were bought from Merck (Darmstadt, Germany). Folin Ciocalteau reagent (2 N), 2,2-diphenyl-1-picrylhydrazyl (95 %, DPPH), ferric chloride (45 %), vanillin (≥ 97 %), quercetin (≥ 98 %), diosgenin (≥ 93 %) and tetradeuteromethanol were sourced from Sigma-Aldrich (St. Louis, USA). Sodium carbonate and aluminum chloride were purchased from Fisher Scientific (Pittsburgh, PA), Gallic acid (98 %) was sourced from Acros Organics (Pittsburgh, PA). Sodium nitroprusside dihydrate and N-1-napthylethylenediamine dihydrochloride were purchased from Bio Basic (Markham, Ontario, Canada). Chromabond C18ec cartridge (500 mg, 5 mL, Macherey-Nagel, Düren, Germany) was used for fractionation.

The 3 varieties of herbal plants *L. pumila* (Blume) Fern.-Vill. var. *alata* Scheff. (LPA, PID 270817-17), *L. pumila* (Blume) Fern.-Vill. var. *pumila* with green leaves (LPPG, PID 250817-17) and *L. pumila* (Blume) Fern.-Vill. var. *pumila* with red leaves (LPPR, PID 260817-17) were collected from Pusat Pertanian Parit Botak (Batu Pahat, Johor, Malaysia) in September 2017. They were analysed in the Forest Research Institute Malaysia (Kepong, Selangor) for species authentication. The plant photos of the species are present in **Figure 1**.

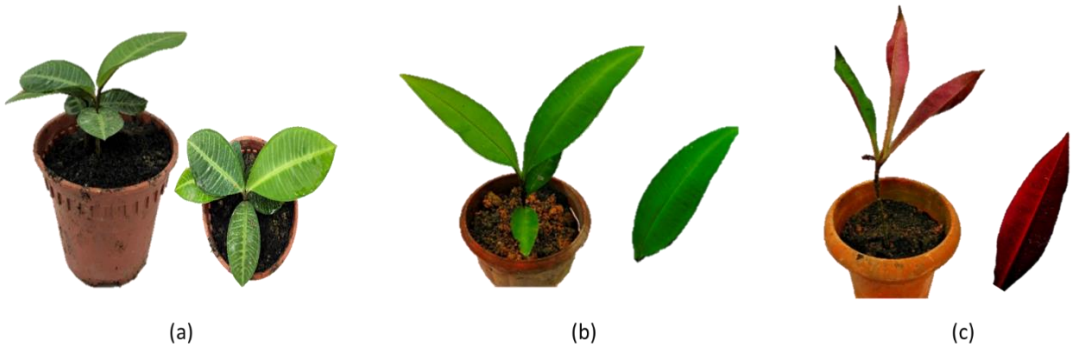


Figure 1 The plant varieties of *Labisia pumila*; (a) *L. pumila* var. *alata*, (b) *L. pumila* var. *pumila* (green leaves) and (c) *L. pumila* var. *pumila* (red leaves).

Reflux extraction

A reflux extraction system was used to extract phytochemicals from the dried and ground plant leaves (2.5 g) in 70 % methanol (125 mL) for 2 h at 80 °C. The supernatant was harvested after centrifugation at 3,000 rpm. The supernatant was then filtered into a beaker and dried in an oven at 50 °C. The weight of the dried crude extract was recorded and kept in a refrigerator (0 °C) until further analyses. All experiments were conducted in triplicates, unless otherwise stated.

Solid phase extraction

Solid phase extraction was used to fractionate the crude extract into fractions using pre-packed cartridges. A C18ec cartridge was pre-conditioned with methanol (5 mL), and then equilibrated with deionized water (5 mL). The crude extract (5 mg/mL) was prepared in 50 % methanol, and 1 mL of the crude extract solution was loaded onto the cartridge. The fractionation was performed by eluting 3 mL of 0 % (MeOH-0), 50 % (MeOH-50) and 100 % methanol (MeOH-100) in sequence into 3 different tubes. The collected fractions were dried in an oven at 50 °C. New cartridge was used for each sample. The dried fractions were weighted and reconstituted in 50 % methanol for Ultra Performance Liquid Chromatography integrated with tandem Mass Spectrometry (UPLC-MS/MS).

Total phenolic content

The total phenolic content of crude extract and individual fractions from each plant variety was estimated using Folin Ciocalteau reagent according to the procedures explained by Chua *et al*. [13] About 1 mL (0.01 g/mL) of sample at different concentrations was mixed with 5 mL of the 10-fold diluted Folin-Ciocalteu reagent in the test tube. The mixture was incubated at 25 °C for 5 min, and then added with 4 mL of (75 g/L) of sodium carbonate. The test tube was shaken gently to mix well the mixture. The absorbance of the mixture was measured after 30 min using an UV-Vis spectrophotometer (UV-1,800, Shimadzu, Japan) at 765 nm. A calibration curve of gallic acid (50 to 450 mg/mL) was constructed. The results were expressed as milligram gallic acid equivalent (GAE) per gram dried extract as shown in Eq. (1).

$$\mathrm{TPC} = \frac{C_s}{m} \mathrm{x}\, V \quad (1)$$

TPC is total phenolic content (mg GAE/g), C_s denotes for sample concentration obtained from the calibration curve (mg/mL), V is the volume of extract (mL) and M is the weight of sample (g).

Total flavonoid content

The total flavonoid content of crude extract and individual fractions from each plant variety was spectrophotometrically estimated according to the method explained by Chua *et al*. [13]. An aliquot of 1 mL (1 mg/mL) sample was mixed with l mL of 2 % methanolic aluminum chloride. The mixture was incubated at 30 °C for 15 min, and followed with the measurement of absorbance at 430 nm using an UV-vis spectrophotometer (UV-1,800, Shimadzu, Japan). The results were expressed as microgram quercetin equivalent (QE) per gram dried extract as shown in Eq. (1).

Total terpenoid content

The total terpenoid content of crude extract and individual fractions from each plant variety was estimated according to the method described by Chua *et al*. [16]. An aliquot of 250 µL (1 mg/mL) of sample was mixed with 250 µL vanillin (8 g/100 mL ethanol) and then added with 2.5 mL of sulfuric acid (72 %). The mixture was heated at 60 °C for 10 min, and then cooled on ice flakes for 5 min. The absorbance of the mixture was read by an UV-vis spectrophotometer (UV-1,800, Shimadzu, Japan) at 544 nm. The results were expressed as microgram diosgenin equivalent (DE) per gram dried extract as shown in Eq. (1).

DPPH ASSAY

DPPH assay was performed to measure the free radical scavenging activity of crude extract and individual fractions from each plant variety according to the method described by Chua *et al.* [13]. A 2 mL sample (100 - 500 µg/mL) in methanol was added to 2 mL DPPH solution (0.1 mM). The mixture was kept aside in a dark place for 30 min and the absorbance of the mixture was measured at 517 nm. The percentage of DPPH• scavenging was estimated using Eq. (2).

$$\text{Radical inhibition (\%)} = \frac{A_o - A_1}{A_o} \times 100 \quad (2)$$

A_0 is the absorbance of control and A_1 is the absorbance of sample.

FRAP assay

The ferric reducing antioxidant power (FRAP) was determined according to the procedures reported by Muthukrishnan and Manogaran [17]. A 100 µL freshly prepared FRAP solution consisted of 25 mL 300 mM acetate buffer at pH 3.6, 2.5 mL 10 mM TPTZ in HCl (40 mL) and 2.5 mL 20 mM ferric chloride solution was mixed with sample (100 µL) at different concentrations in a 96-well microplate. The absorbance of sample was read at 593 nm. Ascorbic acid (0.004 - 2.000 mg/mL) was used as standard chemical. The reducing power was evaluated using Eq. (3).

$$\text{Ferric reducing antioxidant power (\%)} = \frac{A_1 - A_o}{A_S - A_o} \times 100 \quad (3)$$

A_o is the absorbance of control, A_S is the absorbance of ascorbic acid and A_1 is the absorbance of sample.

Griess assay

Griess assay was carried out according to the procedures described by Abdelwahab *et al*. [18] with some modifications. Griess reagent was prepared by mixing 25 mL of 1 % sulfanilamide and 25 mL of 0.1 % naphthylethyenediamine dihydrochloride in 2.5 % phosphoric acid (H_3PO_4). Sample was dissolved in dimethyl sulfoxide (DMSO) and then prepared in different concentrations (0.004 to 2.000 mg/mL). Sodium nitroprusside (10 mM) was prepared in phosphate saline buffer. A 50 µL sample was added into 200 µL sodium nitroprusside and incubated in a 96-well plate for 2 h at 25 °C. Then, 50 µL of the mixture was withdrawn and added into 100 mL Griess reagent for further incubation in a dark place for another 10 min. The absorbance was recorded at 546 nm after incubation. The nitrite scavenging activity can be determined using Eq. (2).

HPLC analysis

High performance liquid chromatography (Dionex Corporation Ultimate 3,000; Sunnyvale, CA) was used to separate compounds using a C18 reversed phase column (XSelect HSS T3 XP, 2.1×100 mm, 2.5 µm) at 254 nm. The mobile phase was consisted of (A) 0.1 % formic acid in water and (B) acetonitrile and flowed at 0.15 mL/min in a gradient program of 10 min, 10 %B; 10 - 13 min, 10 - 90 %B; 13 - 20 min, 90 %B; 20 - 21 min, 90 - 10 %B and 21 - 30 min, 10 %B. Samples (1 mg/mL) were reconstituted in 50 % methanol and filtered prior to injection (5 µL).

MS/MS analysis

An ultra-performance liquid chromatography (Waters Acquity, Milford, MA) was connected to a triple quadrupole-linear ion trap tandem mass spectrometer (Applied Biosystems 4,000 Q TRAP; Life Technologies Corporation, Carlsbad, CA) and an electrospray ion source for compound screening. A C18 reserved phase Acquity column (150×4.6 mm, 1.7 µm) was used to separate compounds before flown into the mass analyser. The mobile phase was consisted of (A) water acidified with 0.1 % formic acid and (B) acetonitrile. The gradient was: 0 - 5 min, 10 %B; 5 - 15 min, 10 - 90 %B; 15 - 25 min, 90 %B; 25 - 26 min,

90 - 10 %B; 26 - 30 min, 10 %B for system equilibration prior to the next run. The flowrate was 0.20 mL/min and the injection volume was 5 µL. All samples were filtered with 0.2-µm nylon filters prior to injection.

The mass spectra were acquired from m/z 100 – 2,000 in both positive and negative modes at the scan rate of 1,000 amu/s. The capillary of the ion source was maintained at 400 °C, whereas its voltage was set at 5.5 kV for positive mode and 4.5 kV for negative mode. Nitrogen was used as nebulising gas at 40 psi and drying solvent at 40 psi. The declustering potential was set at 40 V, and collision exit energy at 10 V. The enhanced mass spectra (EMS) linked with information dependent acquisition to 2 parallel enhanced product ion (EPI) scan was used to screen compounds.

Proton NMR analysis

The selected 2 LPA fractions (LPA-0 % MeOH and LPA-100 % MeOH) were analyzed by a Bruker Avance III 400 spectrometer for proton NMR analysis. It was operated at a frequency of 400.13 MHz equipped with a 5 mm PABBO BB-probe head for spectra acquisition at 300 K. The spectra were recorded by reconstituted samples in deuterated solvent (water and methanol). The acquisition time was 4.0 s. The spectral data were uploaded to the module of structure elucidator for compound searching against the database of proton NMR in ACD/Labs version 2016. The suggested molecular structure was evaluated based on the lowest average deviations of Hierarchical Organization of Spherical Environments (HOSE) code-based algorithm, incremental approach and neural networks.

Results and discussion

Antioxidant property in relation with phytochemicals

The extraction yield of the plant varieties ranged from 10.9 to 13.7 %w/w (**Table 1**). The plant extracts were then analyzed and compared for their TPC, TFC and TTC using calorimetric assays. LPA was found to have the highest TPC (691.76 mg GAE/g), whereas LPPR showed to have the highest TFC (25.11 µg QE/g) and TTC (56.43 µg DE/g). The difference of phytochemical groups in the plant extracts could approximately achieve up to 50 % amongst the varieties. Therefore, it is important to compare the phytochemicals and bioactivity of the plant varieties, especially for herbal product development.

Table 1 Approximate contents of phytochemical groups in different varieties of *Labisia pumila*.

Plant variety	Yield (%w/w)	TPC (mg GAE/g)	TFC (µg QE/g)	TTC (µg DE/g)
LPA	13.7 ± 1.8^{a}	691.76 ± 112.11^{a}	12.76 ± 1.08^{a}	13.98 ± 2.99^{a}
LPPG	10.9 ± 1.0^{a}	399.37 ± 71.92^{b}	15.19 ± 3.71^{a}	27.55 ± 11.09^{b}
LPPR	12.4 ± 1.4^{a}	568.14 ± 109.76^{a}	25.11 ± 12.74^{b}	56.43 ± 14.53^{c}

LPA, *Labisia pumila* var. *alata*; LPPG, *Labisia pumila* var. *pumila* (green leaves) and LPPR, *Labisia pumila* var. *pumila* (red leaves). TPC, total phenolic content; TFC, total flavonoid content and TTC, total terpenoid content. Means with different superscript letters in a column indicate that they are significantly different at $p < 0.05$ using one-way ANOVA and followed by a paired 2 sample T-test.

The antioxidant capacity of the plant varieties was compared after fractionating their crude extracts into fractions at different concentrations of methanol in a reversed phase SPE. Fractionation may or may not improve the antioxidant capacity of fractions depending upon the compounds in those fractions. The plant crude extracts mostly contained compounds with high polarity to intermediate and less polarity because the plant raw materials were refluxed using 70 % aqueous methanol. Therefore, SPE based fractionation was performed using 0 % methanol to collect highly polar compounds, 50 % methanol to collect intermediate polar compounds, and 100 % methanol to harvest relatively less polar compounds from the crude extracts.

All the plant varieties showed to have higher DPPH radical scavenging activity, particularly LPA. However, LPPR was superior in scavenging nitrite radicals. The performance of samples was strongly relied on the phytochemicals which involved in different antioxidative mechanisms as examined using colorimetric DPPH, FRAP and Griess assays in the present study.

SPE was used to fractionate the crude extracts of the plant varieties into individual fractions and then analyzed for their inhibitory actions as a comparison. The comparison was performed at 70 % inhibition

because the samples had high antioxidant activities, and most samples achieved more than 50 % inhibition even with the use of small concentration of samples down to 0.001 mg/mL. Therefore 70 % inhibition was used as the basis for comparison as presented in **Table 2**. The results showed that the MeOH-50 of all plant varieties exhibited higher antioxidant capacity in terms of DPPH radical scavenging and ferric reducing power. The intermediate polar fractions contained antioxidative compounds involving both HAT and SET mechanisms. On the other hand, the MeOH-0 and MeOH-100 fractions contained compounds with the capability to promote SET in quenching nitrite radicals.

Table 2 Comparison of antioxidant activities based on 70 % inhibition.

Plant varieties	Fractions	DPPH	FRAP	Griess
		mg/mg sample	mg/mg sample	mg/mg sample
Standard	Ascorbic acid/ rosmarinic acid*	0.800 ± 0.022^{a}	1.119 ± 0.364^{a}	$1.463 \pm 0.312^{a*}$
LPA	Crude extract	0.673 ± 0.041^{b}	1.470 ± 0.333^{a}	2.079 ± 0.310^{b}
	MeOH-0	0.580 ± 0.092^{b}	1.396 ± 0.198^{a}	0.759 ± 0.076^{c}
	MeOH-50	0.054 ± 0.010^{c}	0.608 ± 0.089^{b}	1.340 ± 0.601^{a}
	MeOH-100	$0.526 \pm 0.033^{b,d}$	1.354 ± 0.174^{a}	0.886 ± 0.042^{c}
LPPG	Crude extract	2.690 ± 0.121^{b}	2.388 ± 0.182^{b}	2.410 ± 0.809^{b}
	MeOH-0	0.882 ± 0.110^{a}	0.599 ± 0.088^{c}	0.780 ± 0.090^{c}
	MeOH-50	0.483 ± 0.090^{c}	0.637 ± 0.081^{c}	1.642 ± 0.089^{a}
	MeOH-100	3.496 ± 0.232^{d}	1.140 ± 0.105^{a}	0.759 ± 0.061^{c}
LPPR	Crude extract	0.769 ± 0.337^{a}	0.936 ± 0.101^{a}	0.993 ± 0.077^{b}
	MeOH-0	0.439 ± 0.099^{b}	1.222 ± 0.117^{a}	1.154 ± 0.080^{a}
	MeOH-50	0.220 ± 0.047^{c}	0.613 ± 0.209^{b}	2.524 ± 0.419^{c}
	MeOH-100	0.851 ± 0.109^{a}	1.139 ± 0.110^{a}	1.057 ± 0.660^{a}

LPA, *Labisia pumila* var. *alata*; LPPG, *Labisia pumila* var. *pumila* (green leaves) and LPPR, *Labisia pumila* var. *pumila* (red leaves). MeOH-0, 0 % methanol fraction; MeOH-50, 50 % methanol fraction and MeOH-100, 100 % methanol fraction. *denotes rosmarinic acid was used as positive control for Griess assay. Means with different superscript letters for each plant variety in a column indicate that they are significantly different at $p < 0.05$ using one-way ANOVA and followed by a paired 2 sample T-test.

Characterization of Labisia pumila variety alata

Since LPA possessed higher antioxidant property, the crude extract and its fractions were subject to a series of analytic tools such as HPLC, MS and NMR characterization. Based on the HPLC chromatograms, SPE based fractionation had concentrated the phytochemicals into individual fractions according to solvent polarity. MeOH-50 appears to have the highest concentrations of compounds detected at 254 nm (**Figure 2**). Mass spectral data showed to detect few glycosylated flavones, especially in the MeOH-50 (**Table 3**). This is the first study to report the presence of orientin and its glycosylated derivatives in *L. pumila*. Recent studies of Lam *et al.* [26], who reported that orientin has been isolated from various medicinal plants from the *Phyllostachys* species (bamboo leaves), *Passiflora* species (passion flowers), *Trollius* species (Golden Queen), and *Jatropha gossypifolia* (Bellyache Bush). They also conducted an intensive review to report remarkable biological properties attributed to orientin. There were also glycosylated flavones such as isovitexin (apigenin-6-C-glucoside), lucenin-2 (luteolin 6,8-di-C-glucoside) and isoschaftoside (apigenin-6,8-C-arabinopyranosylglucoside), in addition to mono- and di-C-glycosylated orientin in LPA. The mass spectra of the identified compounds are presented in Figure 3. The neutral losses of 60, 90 and 120 Da were characteristic fragments resulting from the cross-ring cleavage of C-hexosyl and C-pentosyl moieties [27]. The MeOH-50 fractions mostly contained polyphenols or flavonoids which could donate hydrogen atom to the odd electron of nitrogen atom in DPPH radicals [28] and reduce ferric to ferrous ions. Chua *et al.* [16] published a comprehensive review on the role and

mechanism of C-glycosylated flavones in the antihyperglycemic pathway for antidiabetic property. The other reported biological activities included antioxidant, anxiolytic [29], antispasmodic [30], hepatoprotective [31], anti-inflammation [32] effects. Therefore, the detection of beneficial orientin and other flavones in the present study proved the wide application of this herb as ethnomedicine in the Malay community to induce and facilitate childbirth, as well as a post-partum medicine [33].

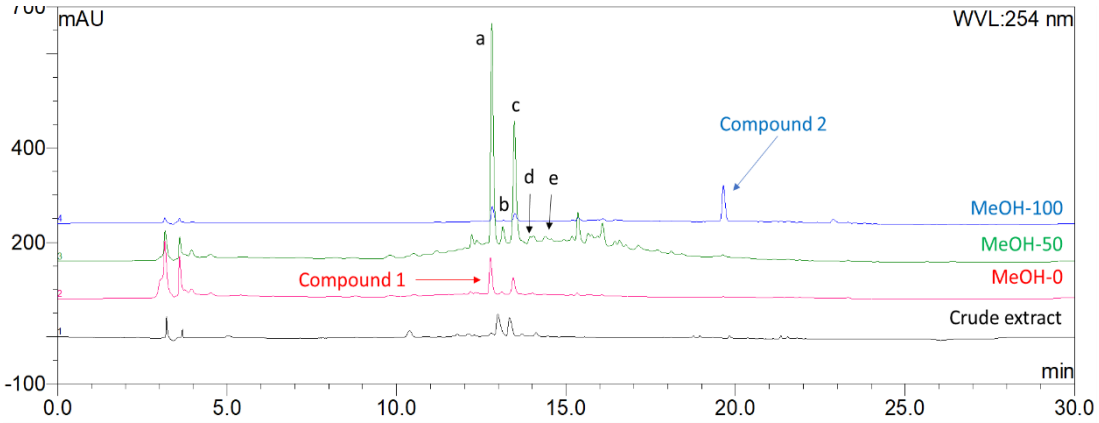
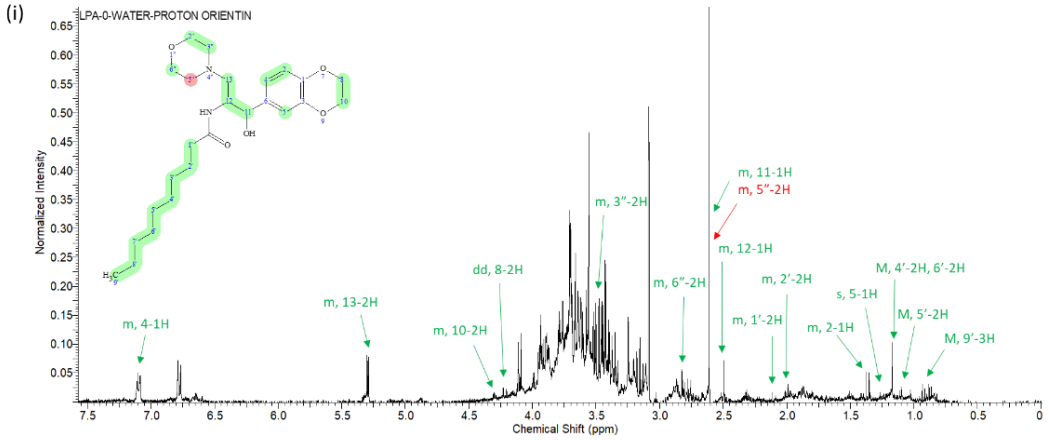


Figure 2 Chromatograms of crude extract and its fractions (MEOH -0, -50 and -100) from *Labisia pumila* var. *alata* using a reversed phase solid phase extraction technique.

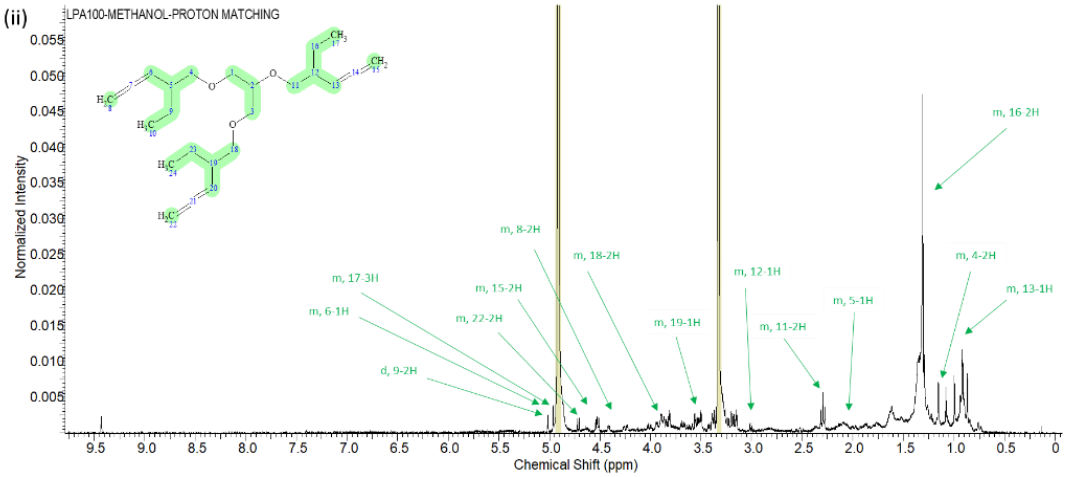
Table 3 Mass fragments of putative compounds in the MeOH-50 fraction of *L. pumila*.

Peak label	Rt (min)	mass per charge (m/z) in negative ion mode	Putative compounds	Reference
a	12.8	563/545/473(-90)/443(-120)/383(-90)/353 (-120)/297	Isoschaftoside	[19]
		579/489(-90)/447(-132)/459 (-120)/429/357/327/309/297	Pentosyl orientin	[20]
b	13.2	593/473(-120)/429/357/327/309/285	Rhamnosyl orientin	[21]
c	13.5	447/357(-90)/327(-30)/297/(-30)/285	Orientin	[22]
d	14.0	465/285(-180)/241/137/165/183	Glycosylated 5-methoxy-7,4'-dihydroxy fla-2-en-3-ol	[23]
		577/457/413/311/293	Isovitexin-2'-O-rhamnoside	[24]
e	14.4	729/609(-120)/447 (-162)/429/359/339/327/309/297/239	O-glycosyl-O-hydroxybenzoyl orientin	[25]
		431/341/323/311(-120)/283/269	Isovitexin	[21,23]

The fractions of MeOH-0 and MeOH-100 were found to be less complex, and therefore, the samples were subject to proton NMR analysis. The NMR spectra were then analyzed by ACD/Labs software for structure elucidation. With the assistance of the software, the samples were detected to have N-[1-(2,3-dihydro-1,4-benzodioxin-6-yl)-1-hydroxy-3-(morpholin-4-yl)propan-2-yl]decanamide (compound 1, 84 % match) in MeOH-0 and 4-[({1,3-bis[(2-ethylpenta-3,4-dien-1-yl)oxy]propan-2-yl}oxy)methyl]hexa-1,2-diene (compound 2, 99 % match) in MeOH-100. The software suggested the best structure based on the lowest average deviations of Hierarchical Organization of Spherical Environments (HOSE) code-based algorithm, incremental approach and neural networks [34]. The small average deviation was 0.004 for compound 1 and 0.056 for compound 2. **Figure 4** shows the compound match based on the detection of proton peaks in the NMR spectra.



^{1}H NMR (400 MHz, DEUTERIUM OXIDE) δ ppm 0.82 - 0.92 (m, 1 H) 1.06 - 1.14 (m, 1 H) 1.14 - 1.22 (m, 2 H) 1.26 (s, 1 H) 1.33 - 1.43 (m, 1 H) 1.51 (s, 1 H) 1.75 - 2.02 (m, 5 H) 2.28 - 2.35 (m, 1 H) 2.49 - 2.53 (m, 1 H) 2.61 - 2.67 (m, 4 H) 2.69 - 2.94 (m, 5 H) 3.08 - 4.00 (m, 114 H) 4.04 - 4.17 (m, 5 H) 4.22 (dd, *J*=10.81, 1.33 Hz, 1 H) 4.26 - 4.32 (m, 1 H) 5.29 - 5.33 (m, 1 H) 6.78 (d, *J*=8.59 Hz, 2 H) 7.08 - 7.12 (m, 2 H)



^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 0.72 - 0.80 (m, 1 H) 0.84 - 1.02 (m, 12 H) 1.05 - 1.09 (m, 2 H) 1.14 - 1.18 (m, 2 H) 1.21 - 1.48 (m, 31 H) 1.50 - 1.66 (m, 7 H) 1.70 - 1.80 (m, 2 H) 1.82 - 1.93 (m, 2 H) 1.97 - 2.21 (m, 4 H) 2.23 - 2.39 (m, 3 H) 2.68 - 2.96 (m, 2 H) 2.97 - 3.05 (m, 1 H) 3.11 - 3.30 (m, 7 H) 3.35 - 3.44 (m, 2 H) 3.48 - 3.57 (m, 3 H) 3.66 - 3.95 (m, 7 H) 3.98 - 4.07 (m, 1 H) 4.12 - 4.27 (m, 2 H) 4.38 - 4.45 (m, 1 H) 4.48 - 4.55 (m, 1 H) 4.60 - 4.68 (m, 1 H) 4.71 (d, *J*=7.53 Hz, 1 H) 4.79 - 4.88 (m, 3 H) 4.94 - 4.97 (m, 1 H)

Figure 4 NMR spectra of (i) N-[1-(2,3-dihydro-1,4-benzodioxin-6-yl)-1-hydroxy-3-(morpholin-4-yl)propan-2-yl]decanamide (compound 1) in 0 % methanol fraction and (ii) 4-[({1,3-bis[(2-ethylpenta-3,4-dien-1-yl)oxy]propan-2-yl}oxy)methyl]hexa-1,2-diene (compound 2) in 100 % methanol fraction. Green describes the difference of chemical shift to be less than 5 ppm, yellow for 5-10 ppm and red for more than 10 ppm of chemical shift.

Conclusions

L. pumila var. *alata* was found to have higher antioxidant property than *L. pumila* var. *pumila*, especially in terms of DPPH radicals scavenging and ferric reducing power. LPA contained higher TPC in which phenolic compounds were more likely to be hydrogen atom and/or electron donors. To compare between the green and red leaves of LPP, LPPR was more antioxidative than LPPG. Possibly, this was due to the presence of plant co-pigments, mostly belonging to flavonoids as proven in the highest TFC. LPPR was also estimated to have higher TTC for plant defense. The high antioxidative variety, LPA extract was fractionated using aqueous methanol and both 0 and 100 % methanol fractions were also subject to proton NMR. Interestingly, compound 1 and compound 2 were detected with more than 90 % match to the database.

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