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phytochemical screening and antioxidant activity assessment of the leaf stem and root of (*Labisia paucifolia)*

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Abstract

Researches have focused on medicinal plants in order to extract natural and low cost antioxidants that can help to protect the human body against oxidative stress and replace the synthetic additives that might be carcinogenic. In the present study, the crude extracts of leaves, stems and roots of *Labisia paucifolia* were investigated for their phytochemical constituents and antioxidant activities. The chemical compositions of bioactive compounds were determined using HPLC and GC-MS and the antioxidant activities measured by DPPH and FRAP methods. The results indicated that leaves methanolic extract had highest total phenolic and flavonoid contents with respective values of 2.51 mg gallic acid equivalent g DW^{-1} and 1.29 mg rutin equivalent g DW^{-1}, compared to roots and stems. Meanwhile, the HPLC analysis showed the presence of gallic acid and kaempferol as the major phenolic and flavonoid compounds in all parts of *L. paucifolia.* Other metabolites were detected by GC-MS screening including 9,12,15-Octadecatrienoic acid, methyl ester (22.72%), 4H-Pyran-4-0ne,2,3-dihydro-35-dihydroxy-6-methyl (7.93%) and acetic acid (9.02%) as the main compounds in the leaf, stem and root extracts of *L. paucifolia.* The extracts possess antioxidant activity because the free radical scavenging and ferric reducing power activities were higher in leaf extract at a concentration of 500 µg mL^{-1} with values of 53% and 51%, respectively, as compared to the stem and root, but the activities were around 32-53% lower than those of antioxidant standards such as BHT and α-tocopherol. The present result revealed that *L. paucifolia* extracts contain variable patterns of flavonoids, phenolics and various bioactive volatile compounds and it could be applied as a natural antioxidant source for industrial purposes.

Keywords: *Labisia paucifolia*; antioxidant activities; phytochemicals constituents; HPLC analysis; GC-MS screening.
Abbreviations: HPLC- High performance liquid chromatography; GC-MS- Gas chromatography–mass spectrometry ; DPPH- 1,1-diphenyl-2-picryl hydrazyl; FRAP - Ferric Reducing Antioxidant Power.

Introduction

Plant secondary metabolites have been studied over the last 50 years (Romero et al., 2009). These secondary metabolites play a major role in the adaptation of plants to their environment, but also represent an important source of active phytochemicals or bioactive compounds. Flavonoids are the most diverse group of secondary metabolite belonging to a subfamily of polyphenols which involve in plant growth, reproduction and seed germination. They also involve in protection against pathogens (Prabuseenivasan et al., 2006) and predators (Treutter, 2005). Flavonoids are of interest due to their biological effects. They are able to scavenge free-radical, modulate enzymatic activity, inhibit cell proliferation; modify allergens, carcinogens and viruses (Sun et al., 2004; Harborne and Williams 2000; Cherng et al., 2007; Ramos 2007). Phenolic compounds as another bioactive compounds characterized by hydroxylated aromatic rings with varying substitution patterns and functional derivatives (Ghasemzadeh et al., 2010a; Pereira et al., 2009) and have been reported to have antioxidants, antibacterial, antiviral, anti-cancer and anti-inflammatory properties (Mattila and Hellstrom, 2007; Sacchetti et al., 2005). Research for natural antioxidants agents have become increasingly important due to health implications since the synthetic antioxidant such as BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisole), and TBHQ (*tert*-butyl hydroquinone) could be toxic and could cause carcinogenic effects (Ghasemzadeh and Jaafar 2011; Zainol et al., 2003; Mohdaly et al., 2010). For this reason, there is an increasing interest in identifying natural antioxidant sources from medicinal plants to replace synthetic antioxidants and eliminate these health concerns. Myrsinaceae is a widespread family consisting of 30 genera and about 1000 species of tropical plants, of which about 40 species are medicinal in the Asia-Pacific region, particularly for the treatment of inflammation (Shah et al., 2011). Myrsinaceae are considered their potential medical compounds, quinones and saponins, with a variety of pharmacological activities such as antioxidant, anti-microbial and anti-cancer activities. *Labisia paucifolia* is one of the genuses of this family, a small under shrubs, up to 45 cm high with creeping stems and mainly found in the shady forest with humus-rich soil (Shah et al., 2011). Due to the lack of scientific validation on medicinal potentials of this plant, this research was conducted to analyze the phenolics and flavonoids compounds. The chemical compositions of bioactive compounds of leaves, roots and stems were also investigated and their antioxidant activities determined.

Results and discussion

Total phenolic and flavonoids contents

The leaf part contained higher phenolic and flavonoid contents compared to the root and stem (Table 1). This result is in agreement with Karimi et al. (2011a) in three varieties of *Labisia pumila* Benth. (var. of *pumila*, *alata* and *lanceolata*). Earlier study conducted by Ghasemzade et al. (2010b) using different parts of two varieties of *Zingiber officinale* indicated that the total flavonoid and phenolic contents in the leaves were also more than in the rhizomes, followed by the stems.

Analyses of phenolics and flavonoids compounds

Reversed-phase (RP) chromatography was used to determine of phenolic and flavonoid compounds presented in *L. paucifolia* extract. Gallic acid was the major phenolic compounds present in the leaf, stem and root of *L. paucifolia*, while kaempferol was detected as the major flavonoid compound (Tables 2 and 3; Figures 1 and 2). Overall, the results indicated that the variations of phenolic and flavonoid compounds in the leaf were much more as compare with root and stem. As shown in the Table 3 the myricetin and naringin were only observed in the leaf with values of 25.1± 0.1 and 56.7± 0.3 μg g DW^{-1}. These results are in agreement with Karimi et al. (2011b) in three varieties of *L. pumila* Benth. (Myrsinaceae family). They reported in that all three varieties (*L. pumila* var. *alata*, *pumila* and *lanceolata*), the leaf part contained higher levels of phenolic and flavonoid (kaempferol, naringin and myricetin) compounds compared to the root and stem. Karimi et al. (2011b) only recorded quercitin and rutin in var. *pumila* and *lanceolata*.

Analysis of volatiles in leaves, stems and roots of Labisia paucifolia

The GC-MS analysis of methanolic crude extracts resulted in identification of more than 65 compounds in the leaf, stem and root of *L. paucifolia*, but a few of them were predominant (Table 4) such as 9,12,15-Octadecatrienoic acid, methyl ester, 4H-Pyran-4-0ne,2,3-dihydro-35-dihydroxy-6-methyl and acetic acid in the leaf, stem and root , respectively (Table 4). Leaves exhibited more volatile compounds (36 compounds) compare than stems (21 compounds) and roots (24 compounds). Kumar et al. (2010) investigated 9,12,15-Octadecatrienoic acid, methyl ester is a nature compound of linolenic acid and showed anti-inflammatory activity. Several important biological activities of 4H-Pyran-4-0ne,2,3-dihydro-35-dihydroxy-6-methyl were reported including anti-mutagenic activity against arylamine (Berhow et al., 2000; Xie et al., 2010), anti-alpha-glucosidase activity in patients with diabetes mellitus, (Quan et al., 2003) reactive oxygen scavenging activity (Takara et al., 2007) and also anti-tumour activity (Ban et al., 2007). On the other hand, acetic acid is a well-known antimicrobial agent used in food industry as vinegar (Haesebrouck et al., 2009). Haesebrouck et al. (2009) observed that acetic acid solution (0.5 %) exerted bactericidal effect against *Staphylococcus pseudintermedius, Proteus vulgaris, Pseudomonas aeruginosa and Acinetobacter baumannii*. The presence of these phytochemicals makes *Labisia paucifolia* a potential source of bioactive compounds.

Antioxidant assay of leaves, stems and roots of Labisia paucifolia extracts

As observed in total phenolics and flavonoids contents (Table 1), the leaf part of *L. paucifolia* also possessed higher DPPH (1,1-diphenyl- 2-picrylhydrazyl radicals) free scavenging activities compared to the stem and root (Table 5). The results also demonstrated the IC_{50} (concentration required to inhibit 50% of DPPH radicals) of α-tocopherol, BHT and leaf extract to be 89.77, 60.39 and 489.92 μg mL^{-1}, respectively. FRAP assay similar to the DPPH results indicated that the reductive potential of leaf extracts and standards increased with increasing samples concentration (Table 6). Leaf part compared to stem and root showed higher antioxidant activities, however, they were lower than those of the standards. As comparison, the reductive potential of leaf, stem and root extracts and standards at concentration of 500 μg mL^{-1}were as follow: vitamin C > BHT > α-tocopherol > leaf > stem> root (Table 6).

Materials and methods

Plant material

Seedlings of *Labisia paucifolia* were, respectively, collected from places of origin at Hulu Langat, Selangor and raised under glasshouse for 18 months before used in the study. The GPS location details were 3°0'35.27"N latitude and 101°42'19.38"E longitude. Healthy and uniform seedlings in term of leaf numbers were selected from the three varieties. The leaves, stems and roots of *Labisia paucifolia* were cleaned, separated, and freeze dried for further analysis.

Preparation of extracts

Samples were extracted using methanol as a solvent based on Crozier et al. (1997). Two grams of freeze-dried leaf, stem and root were weighed and placed into a 100 ml conical flask, and added with 40 ml of 80% (v/v) methanol. It was followed by an addition of 10 ml of 6 M HCl. The mixture was refluxed for 2 hours at 90°C and filtered by using Whatman No. 1 filter paper (Whatman, England) followed by evaporation of the filtrate using a vacuumed rotary evaporator (Buchii, Switzerland). The crude extracts were re-dissolved in methanol for total phenolics, total flavonoids, antioxidant activity and RP-HPLC analyses.

Total phenolics determination

Total phenolics content was determined by using Folin–Ciocalteu reagent according to Ismail et al. (2010) and total phenolic results were expressed as mg gallic acid equivalents/g dry matter of the plant material.

Total flavonoids determination

Total flavonoid was determined based on aluminium chloride colorimetric assay described by Ismail et al. (2010). Total flavonoid compound of extracts were expressed as mg rutin equivalent/g dry matter of the plant material.

Table 1. Total phenolics and flavonoids content of the leaves, stems and roots of *Labisia paucifolia*.

Extract	Phenolic content[1]	Flavonoid content[2]
Leaf	2.51[a]	1.29[a]
Stem	0.95[c]	0.43[c]
Root	1.27[b]	0.49[b]

[1] mg gallic acid equivalent g DW^{-1}; [2] mg rutin equivalent g DW^{-1}; Means not sharing a common letter were significantly different at $p \leq 0.05$.

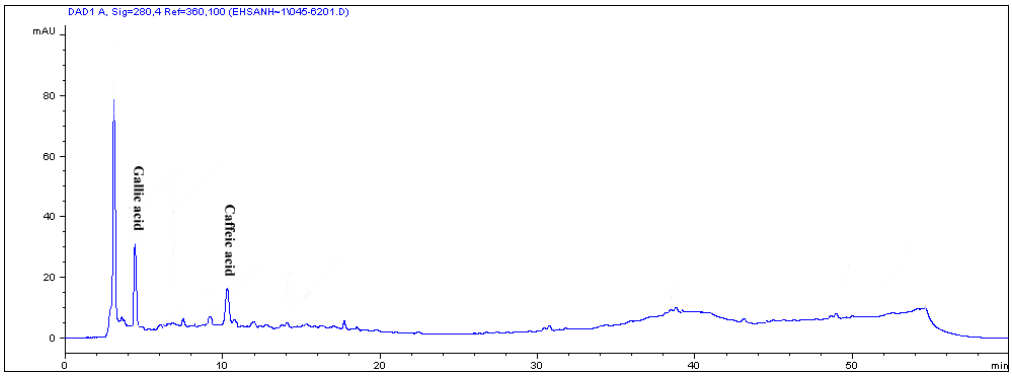

Fig 1. The RP-HPLC chromatogram of phenolic compounds in the leaves of Labisia paucifolia. Identification of compounds: gallic acid and caffeic acid

Table 2. Concentration of different phenolic compounds from methanolic extract of leaf, stem and root of *Labisia paucifolia*.

Extract	Phenolic contents (µg g^{-1} dry sample)		
	Gallic acid	Pyrogallol	Caffeic acid
Leaf	252.3± 0.0[a]	ND	17.5± 0.2[b]
Stem	36.1± 0.0[c]	ND	ND
Root	42.8± 0.1[b]	ND	25.2± 0.0[a]

ND: not detected. All analyses were mean of triplicate measurements ± standard deviation. Means not sharing a common letter were significantly different at $p \leq 0.05$.

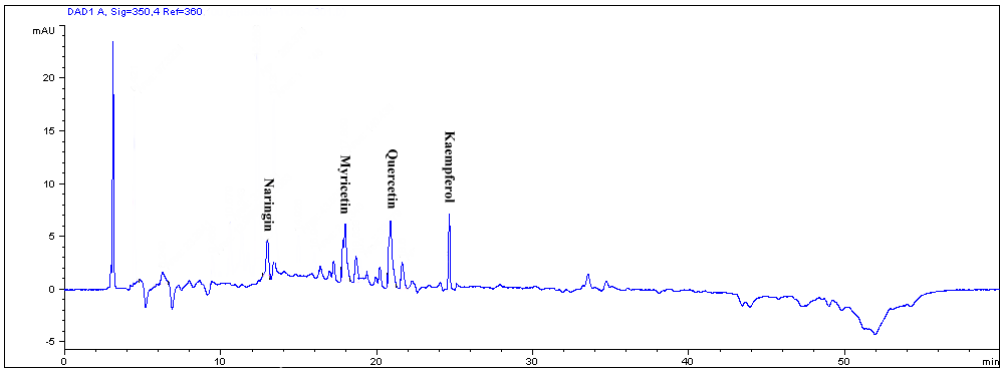

Fig 2. The RP-HPLC chromatogram of flavonoid compounds in the leaves of *Labisia paucifolia*. Identification of compounds: naringin, myricetin, quercetin and kaempferol.

Determination of phenolic and flavonoid compounds by HPLC

The phenolic and flavonoid compounds of the leaf of three varieties of *Labisia pumila* Benth were quantitatively measured by reversed-phase HPLC based on the method described by Crozier et al. (1997) with some modification. The phenolic and flavonoid compounds were identified based on their retention times and quantified according to respective standard calibration curves. Phenolic standards were gallic acid, caffeic acid and pyrogallol. Flavonoid standards were quercetin, rutin, myricetin, kaempferol and naringin. An aliquot of sample extracts was loaded on the HPLC equipped with an analytical column Intersil ODS-3 (5µm 4.6×150 mm, Gl Science Inc). Solvents comprising deionized water (solvent A) and acetonitrile (solvent B) were used. The pH of water was adjusted to 2.5 with trifluoroacetic acid. The phenolic compounds were detected at 280 nm while flavonoid compounds at 350 nm. The column was equilibrated by 85% solvent A and 15% solvent B. Then the ratio of solvent B was increased to 85% in 50 min followed by reducing solvent B to 15% in 55 min. This ratio was maintained to 60 min for the next analysis with flow rate at 0.6 mL/min.

Antioxidant Activity

DPPH radical-scavenging activity

Free radical scavenging activity of extracts were determined with 1,1-diphenyl-2-picryl-hydrazil (DPPH) as free radicals

according to Ismail et al. (2010). The absorbance was measured at 515 nm by using a spectrophotometer. Butylated

Table 3. Concentration of different flavonoid compounds from methanolic extract of leaf, stem and root of *Labisia paucifolia*.

Extract	Flavonoid contents (μg g^{-1} dry sample)				
	Kaempferol	Myricetin	Naringin	Quercetin	Rutin
Leaf	112.8± 0.2[a]	25.1± 0.01	56.7± 0.3	42.3± 0.2[a]	ND
Stem	18.4± 0.1[b]	ND	ND	ND	11.5± 0.3
Root	13.8± 0.1[c]	ND	ND	23.5± 0.0[b]	ND

ND: not detected. All analyses were mean of triplicate measurements ± standard deviation. Means not sharing a common letter were significantly different at $p \leqslant 0.05$.

Table 4. Chemical composition of methanolic extraction in the leaf, stem and root of *L. paucifolia*.

Extract	Number	Composition (%)	Main Compounds
Leaf	1	22.7	9,12,15-Octadecatrienoic acid, methyl ester
	2	20.7	11,14-Eicosadienoic acid, methyl ester
	3	18.8	n- Hexadecanoic acid
	4	11.1	9- Hexadecenoic acid
Stem	1	7.9	4H-Pyran-4-0ne,2,3-dihydro-35-dihydroxy-6-methyl
	2	6.0	13-Methyl-11-pentadecen-1-acetate
	3	5.9	Hexadecanoic acid
Root	1	9.0	Acetic acid
	2	8.9	Hexadecenoic acid
	3	7.5	Heptadecanoic acid

Table 5. DPPH scavenging activities of different parts of *L. paucifolia* at concentration of 500 μg/mL. BHT and α-tocopherol were used as positive controls.

Inhibition (%)		
Part	Leaf	51.3
	Stem	31.2
	Root	42.2
Control	BHT	99.2
	α-tocopherol	99.7

Table 6. Total antioxidant (FRAP) activities of different parts of *L. paucifolia* at concentration of 500 μg/mL. BHT and α-tocopherol were used as positive controls.

Total antioxidant (FRAP) activities (%)		
Part	Leaf	52.8
	Stem	32.1
	Root	35.3
Control	BHT	99.2
	α-tocopherol	96.2
	Vitamin C	99.6

hydroxytoluene (BHT) and α-tocopherol (Fisher Scientifics, USA) were used as standard antioxidants.

Ferric-Reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) of the extracts was determined as described by Yen and Chen (1995). Ascorbic acid (Fisher Scientifics, USA), BHT and α-tocopherol were used as standard antioxidants.

Gas chromatography-mass spectrometry method (GC-MS)

The GC-MS analysis of methanolic crude extract of the leaf, stem and root of *Labisia paucifolia* were quantitatively performed by GC-MS (Shimadzu QP2010PLUS system, Japan) equiped with a type capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) based on the method described by Hossain and Rahman (2011) with some modification. Split less injection was performed with a purge time of 1.0 min. The carrier gas was helium at a flow rate of 1 mL min^{-1}. The column temperature was maintained at 50 °C for 3 minutes, then programmed at 5 °C min^{-1} to 80 °C and then at 10 °C min^{-1} to 340 °C. The inlet temperature was 250 °C, the detector temperature was 340 °C and the solvent delay was 4 min. The identification of the peaks was based on computer matching of the mass spectra with the National Institute of Standards and Technology (NlST 08 and NIST 08s) library and by direct comparison with published data.

Statistical Analysis

The antioxidant activities, total phenolics and flavonoids contents and their profiling were analyzed using analysis of variance (ANOVA) with Statistical Analysis System (SAS) Version 9 (SAS Institute, Cary, NC). Significant differences among tissues (roots, leaf, stem) using means from triplicate analyses ($p < 0.05$) were determined by Duncan's multiple range test.

Conclusions

The antioxidant properties in the herbal plant *Labisia paucifolia* could be attributed to flavonoid, phenolic and bioactive volatile compounds present in the respective crude extracts. Gallic acid and kaempferol were the major phenolic and flavonoid compound in the all parts of *L. paucifolia*

while predominant bioactive volatile differed depending on the tissue analyzed. The leaf is the main source of anti-oxidants, which have shown to possess radical scavenging activities and reducing potential compared to root and stem. These findings are an important first step towards the development of value added products from this plant.

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