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FULL PAPER

Antioxidant activities of *Barringtonia racemosa* leaves

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Abstract

Proximate analysis of *Barringtonia racemosa* (putat) leaves was carried out and total phenolic content (TPC) was measured using total phenolic assay, also known as the Folin Ciocalteu (FC) assay. Antioxidant activities of the methanolic and aqueous extracts were evaluated by ferric reducing antioxidant power (FRAP) assay; the free radical scavenging capacity was determined by 1,1-diphenyl-1-picrylhydrazyl radical scavenging capacity (DPPH) assay. Proximate analysis showed that the leaves contained 70.52% moisture, 0.96% ash, 2.54% protein, 0.26% fat, 1.38% crude fiber, and 25.71% carbohydrates. The TPC amount exhibited by the methanolic extract was 1585.20±25.54 mg GAE/100 g sample, which was higher compared with the aqueous plant extract with 1314.12±66.19 mg GAE/100 g ($p<0.05$). Significant ($p<0.05$) difference in terms of the flavonoid content was observed between the extracts obtained by methanolic and aqueous extraction. Total condensed tannin content showed significant ($p<0.05$) difference between the methanolic and the aqueous extracts, which were 33.11±0.61 mg CE/100 g and 13.38±0.87 mg CE/100 g, respectively. Methanolic extract of *Barringtonia racemosa* leaves showed a higher DPPH scavenging activity than the aqueous extract. FRAP value for the methanolic extract of the sample was higher than the aqueous extract, with a significant difference ($p<0.05$). Results of this study will provide a platform for the future paradigm of these potential natural sources of antioxidants in food and nutraceutical applications.

1. Introduction

Antioxidants are used in foods to prevent food oxidation and prolong shelf life, as well as a dietary supplement to prevent harmful oxidation reactions occurring in the physiological systems of the human body (Venkatesh and Sood, 2011). The natural sources of antioxidants obtained from the human diet include cereals, vegetables, fruits, oilseeds, legumes, cocoa products, beverages, herbs and spices (Pokorny, 2007). Among the types of antioxidants used widely today are enzymes, proteins, vitamins (A, C, E), carotenoids, polyphenols, trace elements and small molecules, such as glutathione (Tabart *et al*., 2007). The antioxidants are important to overcome the overproduction reactive oxygen species (ROS) in our body (Wong *et al*., 2009).

Several studies have been conducted to discover new antioxidant sources with cheaper cost and consistent supply, which will be used in the food industry (Velentao *et al*., 2002). Natural antioxidants are presumed to be safer apart having nutritional and therapeutic values (Li *et al*., 2009).

Barringtonia racemosa (Lecythidaceae family) has long been used in traditional Asian medicine. South-East Asians consume the shoots or young leaves of this plant, either fresh or boiled (Dhiya *et al*., 2007). According to Kong *et al*. (2012), the pounded roots and barks of *Barringtonia racemosa* is believed to reduce itchiness and chicken pox. Besides that, the leaves and barks of *Barringtonia racemosa* can be used as an antidote to snake bites, rat-poisoning and on boils (Nurul *et al*., 2008). By referring to Behbahani *et al*. (2007), the fruits of *Barringtonia racemosa* can be used to treat a cough, asthma, and diarrhea. Due to the various usage of *Barringtonia racemosa* in our folkloric medicine, it is suggested that it has an analgesic effect (Deraniyagala *et al*., 2003).

This study was performed to accomplish the following objectives: analyze and determine the proximate composition, the total phenolic, flavonoid, and condensed tannin contents; and the antioxidant properties of *Barringtonia racemosa* leaves. We used methanol and aqueous as solvents extraction of antioxidant activities

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due to the presence of various chemical groups such as total phenolic, flavonoid and tannins with different chemical characteristics and polarities.

2. Materials and methods

2.1 Plant material and sample preparation

Samples were collected from November 2012 to April 2013 in Kulim, Kedah, Malaysia. *B. racemosa* leaves were identified by Dr. Rahmad Zakaria. Voucher specimen number 11399 was deposited in the herbarium laboratory of the School of Biological Sciences, Universiti Sains Malaysia. Exactly 3 kg of the fresh leaves were separated from the stems and washed under running tap water to remove dirt and particulates.

2.2 Sample extraction

Sample extraction was conducted based on the procedure of Sulaiman *et al.* (2011). Approximately 10 g of fresh samples were washed with distilled water and dried using blotting paper (Whatman 3MM) to remove the remaining water on the leaf surface. The leaves were ground to obtain a fine paste and then soaked in 100 mL of solvent (distilled water or 70% ethanol) for 1 h at room temperature. The obtained extract was filtered using clean muslin cloth and centrifuged (Hittech EBA 20 Centrifuge) at 61,000 × *g* for 15 min. The fresh extracts were stored at 4°C in the dark for further analysis.

2.3 Proximate analysis

Proximate analysis of the samples, which include moisture content, ash, crude protein, fat, and crude fiber, was determined according to the methods set forth by the Association of Analytical Chemist (AOAC), 2000. Percentage of carbohydrate content was determined by the following formula: % Carbohydrate = 100 − (% ash + % fat + % protein + % fiber content)

2.4 Total phenolic content

Folin Ciocalteu (FC) assay was used to find out the total phenolic content (TPC) of the sample based on the procedure of Dhiya *et al.* (2011). Approximately 0.5 mL of the leaf extract containing different concentrations of gallic acid was mixed with 2.5 mL of diluted (1:10) Folin-Ciocalteu reagent and 2 mL of 7.5% NA_2CO_3. The mixture was incubated for 90 min at room temperature. The absorbance of the mixture was measured using UV-vis spectrophotometer at 765 nm. Gallic acid solutions with known concentrations (0, 20, 40, 60, 80, and 100 mg/L) were prepared to create a standard curve.

2.5 Total flavonoid content

Calorimetric assay was used to find out the total flavonoid content of the sample based on the procedure of Nurul *et al.* (2008). Approximately 1 mL of the sample aliquot was added to 4 mL of distilled water (dH_2O). Approximately 0.3 mL of 5% (w/v) sodium nitrite and 0.3 mL of 10% (w/v) aluminium chloride were sequentially added to the mixture after 5 min. Two mL of 1 M sodium hydroxide solution was added on the 6^{th} min; the total volume was increased to 10 mL by adding distilled water. The mixture was then shaken energetically by vortex. The absorbance of spectrophotometer was measured at 510 nm. A standard curve was plotted using catechin solution at various concentrations (0, 20, 40, 60, 80, 100, and 120 mg/L).

2.6 Condensed tannin content

Vanillin assay was used to determine the condensed tannin content of the sample, based on the procedure of Wijekoon *et al.* (2010). About 0.5 mL of the leaf extract of *B. racemosa* was added to 3 mL of vanillin reagent (4% w/v vanillin in methanol) and heterogeneous. About 1.5 mL of concentrated hydrochloric acid was added and the mixture was then vortexed. Afterward, the mixture was covered with aluminium foil for 15 min at 25°C and the absorbance was measured using UV-vis spectrophotometer at 500 nm. A standard curve was plotted using catechin solution at various concentrations (0, 20, 40, 60, 80, 100, and 120 mg/L). The content of condensed tannins in the leave of *B. racemosa* was measured as mg catechin equivalent (CE) per gram (dry weight basis) of the fresh sample.

2.7 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay

Determination of scavenging activity was based on the procedure of Subhasree *et al.* (2009). Methanolic DPPH was freshly prepared by mixing DPPH solution with methanol at a concentration of 100 µmol/L. Approximately 1 mL of the sample extract was mixed with 6 mL of DPPH solution. The control was done by adding 1 mL of methanol to 6 mL of DPPH solution into the test tubes with the lids containing the samples were covered with aluminum foil. Then, the mixture was then shaken energetically and kept without light for 30 min. The absorbance was measured against the blank at 517 nm using UV-vis spectrophotometer (Shimadzu UV-Visible Recording Spectrophotometer Model UV-160A).

2.8 Ferric-reducing antioxidant power (FRAP) assay

FRAP assay was performed according to the procedure of Kong *et al.* (2012). The FRAP reagent was done by adding acetate buffer, 2,4,6-tripyridyl-s-triazine

(TPFZ) solution to 40 mM HCl and 20 mM iron (III) chloride at a volume ratio 10:1:1 and then warmed in the water bath at 37°C prior to analysis. Diluted sample extract (200 µL) was mixed with 3 mL of FRAP reagent. The blank sample was prepared by mixing the FRAP reagent with distilled water. The mixtures were then incubated in the water bath (37°C) for 30 min. Sample absorbance was determined against the blank using UV-Vis spectrophotometer at 595 nm after 30 min. Solutions of $FeSO_4.7H_2O$ with known concentrations (0, 200, 400, 600, and 800 µM) were used to prepare a standard curve. Values obtained were expressed as mmol $Fe^{2\pm}$ per gram (dry weight basis) of fresh sample.

2.9 Statistical analysis

Results obtained were presented as mean values at ± SD. Duncan's test at $p<0.05$, analysis of variance (ANOVA) was done to determine the significant differences for multiple comparisons. SPSS version 20.0 was used to conduct all statistical analysis.

3. Results and Discussion

3.1 Proximate analysis

The moisture content of the fresh sample, which averaged at 70.52±0.25%, was quite high. High moisture content in most fruits and vegetables provides a conducive environment for growth and multiplication of microorganisms (Hassan *et al*., 2007). In addition, the sample is highly perishable, and should thus be stored properly for longer shelf life (Adeleke and Abiodun, 2015). Excessive moisture can lead to a higher tendency for spoilage and rotten products in the agricultural and food industries (Austin *et al*., 2013).

Removal of water and other organic matter present in foods by heating produces an inorganic residue known as ash (Kok, 2012). Ash content is an index of the total mineral element (Aliero and Abdullah, 2009). Ash content of the fresh *Barringtonia racemosa* leaves was 0.96±0.06%, which indicates low mineral content in the plant. Nwachukwu and Ukoha (2007) reported that mineral content is directly proportional to drying. The drier the leaves are, the higher is the ash content. Ash content is directly proportional to drying; hence, the value of ash content in the fresh sample is low because of less surface area and low reaction rate.

Nitrogen content determination in food samples is based on its conversion into simple nitrogen derivatives, which remains to be the official method for determining the overall protein content in many countries, especially for foods where separation of the specific proteins from other components in the sample is not required unless the food contains appreciable amounts of non-protein nitrogen-containing material (Bonomi, 2005). Table 1 showed that the protein content of the fresh matured leaves of *Barringtonia racemosa* ranged from 2.51% to 2.57% and the average protein content was 2.54%. This result is consistent with results of a previous study on protein content in the matured leaves of *Barringtonia racemosa* done by Kok (2012). Ratnayake and Galli (2009) reported that fats and oils consist of a large number of organic compounds including fatty acids, monoacylglycerols, diacylglycerols, triacylglycerols, phospholipids, eicosanoids, resolvins, docosanoids, sterols, sterol esters, carotenoids, vitamins A and E, fatty alcohols, hydrocarbons, and wax esters. The benefits and potential risks of fat consumption should now be considered as key nutrients that affect early growth and development, as well as nutrition-related chronic diseases later in life (Burlingamea *et al*., 2009). The fat content of the fresh sample was 0.26±0.01%.

Crude fiber measures the content of cellulose, hemicellulose, and lignin in foods (Ogunlakin *et al*., 2012). The presence of crude fiber in the intestinal tract aids in the proper peristaltic action of this body part (Kok, 2012). Crude fiber present in fresh *B. racemosa* leaves was in the range of 1.35% to 1.41%, with an average of 1.38%. Carbohydrate content of the leaves was quite low at 25.71±0.41% because of the presence of high amount of water in the leaves. Carbohydrates are ideal sources of energy for the body.

Table 1. Proximate composition of the matured leaves of *Barringtonia racemosa*

Proximate Analysis	(%)
Moisture	70.52 ± 0.25
Ash	0.96 ± 0.06
Protein	2.54 ± 0.03
Fat	0.26 ± 0.01
Crude Fiber	1.38 ± 0.03
Carbohydrate	25.71 ± 0.41

Values are expressed as mean ± standard deviation (n=3).

3.2 Total phenolic content

Phenolic compounds, which are known to have free radical scavenging properties, have shown anti-inflammatory activity and medicinal and physiological functions in animals and plants (Blasa *et al.,* 2010). TFC assay is one of the oldest methods designed to determine the total content of phenolics (total phenols) (Roginsky and Lissi, 2005). This assay involves the transfer of electrons in alkaline medium from phenolic compounds and other reducing species to molybdenum, producing blue complexes that can be detected by spectrophotometer at 750 nm to 765 nm (Magalhaes, 2008).

The amount of TPC in the methanolic extract was 1585.20 ± 25.54 mg GAE/100 g sample, which was higher compared with the TPC obtained from the aqueous plant extract at 1314.12±66.19 mg GAE/100 g ($p<0.05$) (Table 2). A significant difference in the TPC concentration was observed between the two extracts because the phenolic compounds in plants are more soluble in methanol (Roginsky and Lissi, 2005). Thus, methanol is a more efficient solvent for extracting phenolic compounds in fresh matrices of *Barringtonia racemosa* leaves. The acidified organic solvent destroys the plant's cell membranes and simultaneously dissolves the anthocyanins, resulting to a higher amount of TPC.

Table 2. Total antioxidant capacity (DPPH and FRAP) and antioxidant activity of phenolics, flavonoids and condensed tannins of *Barringtonia racemosa* leaves

Analysis	Extraction	
	Methanolic	Aqueous
Total Phenolic Content (mg GAE/100g sample)	1585.20±25.54^{e}	1314.12±66.19^{d}
Total Flavonoid Content (mg CE/100g of sample)	239.35±2.02^{c}	170.96±1.28^{c}
Total Condensed Tannins (mg CE/100g of sample)	33.11±0.61^{a}	13.38±0.87^{a}
DPPH (% inhibition)	85.34±0.84^{b}	79.10±1.17^{b}
FRAP (μM Fe (ll)/g of sample)	291.95±3.34^{d}	109.84±2.56^{b}

Means with different superscript in any column are significantly different at $p<0.05$, same superscript in any column indicates $p>0.05$ with no significant difference, according to Duncan's Multiple-Range Test (n = 3)

The extracting solvents with different polarities influence the solubility of the chemical constituents in the sample and its extraction yield (Sulaiman *et al*., 2011). Alothman *et al.* (2009) reported that the recovery of phenolic compounds is dependent on the solvent used and the polarity of the sample. Moreover, solvent polarity has an important role in increasing phenolic solubility (Naczk and Shahidi, 2006). Higher phenol and flavonoid content in methanolic extracts account for better antioxidant activity results compared with aqueous extracts.

3.3 Total flavonoid content

Flavonoids are one of the most common subcategories of polyphenols, which are found naturally in foods (Venkatesh and Sood, 2011). Flavonoid is one of the bioactive compounds that can scavenge free radicals. Flavonoids have an important role in the defense-system mechanism of plants (Kok, 2012). Environmental conditions, such as air pollution and UV radiation, trigger the production of flavonoids (Dixon and Paiva, 1995). Flavonoids also act as a phytoalexin, which is an antimicrobial compound, in response to the presence of microorganisms (Maimoona *et al*., 2011). Flavonoid content was 239.35±2.02 mg CE/100 g for the methanolic extract and 170.96±1.28 mg CE/100 g for the aqueous extract ($p<0.05$). According to Sulaiman *et al.* (2011), flavonoids have low distribution and composition in the plants compared with the overall phenolics.

3.4 Condensed tannin content

The word 'tannin' refers to a heterogeneous group of polymeric phenolic compounds, and was originally used to describe plant extracts used to tan animal leather (Haslam, 1998). Tannins are polyphenols that occur in vascular plants and exist in two types; the condensed and the hydrolysable tannins (Wei *et al.*, 2010). Condensed tannins, which are also called proanthocyanidins, are the most important studied group of polyphenols abundant in the plant kingdom (Lamy *et al*., 2011).

Using catechin as the standard monomer of condensed tannins in the assay results in red-colored adducts formation (Hagerman, 2002). The total condensed tannin content in the methanolic extract was 33.11±0.61 mg CE/100 g and 13.38±0.87 mg CE/100 g in the aqueous extract ($p<0.05$). According to Wijekoon *et al.* (2011), an abundance of hydroxyl groups and the presence of benzene rings gave the hydrophobic character to tannins. This finding can be the reason why the methanolic extract provided a higher yield of tannins compared with the aqueous extract.

Given that no study has been conducted on *B. racemosa* leaves with condensed tannins previously, the results obtained the current study can be used a reference to other future studies on *B. racemosa* leaves. Several factors can contribute to the different concentrations of condensed tannins in plant tissues, such as plant species, plant part, plant maturity, soil fertility, and growing season (Hagerman, 2002).

3.5 DPPH and FRAP assay

The DPPH inhibition in methanolic extract was 85.34±0.84% and 79.10±1.17% in aqueous extract. The antioxidant effect on DPPH attributed to the hydrogen-donating ability of the solvent according to Adedapo *et al*. (2009). Markowicz *et al*. (2007) reported that, in the presence of hydrogen or any electron donor, the absorption intensity is decreasing, and the radical solution is discolored according to the number of

electrons captured. The methanolic extract exhibited higher antioxidant activity with no significant difference (P>0.05) than the aqueous extract. According to Chan *et al.* (2009), methanol has been recommended for the extraction of phenolic compounds and antioxidants in fresh plant tissues because of its ability to inhibit polyphenol oxidase, which can alter antioxidant activity. High methanol extraction efficiency has been reported in the young leaves of *Camelia sinensis* (Chan *et al.,* 2007).

The reducing ability of the extracts was 291.95±3.34 µM Fe (II)/g in the methanolic extract and 109.84±2.56 µM Fe (II)/g in the aqueous extract. FRAP values for the methanolic extracts were higher than the aqueous extract. The significant difference (P<0.05) observed between these two extracts is probably due to the solvent polarity towards the compounds in the leaves. According to Adedapo *et al.* (2009), increase in antioxidant activity is proportional to the polyphenol content. the active compound in B. racemose leave was lycopene, an antioxidant (Behbahani *et al*., 2007).

4. Conclusion

This study showed that *B. racemosa* (L.) leaves have high antioxidant activity. Methanol was found to be suitable extraction solvent, showed significantly higher yield in DPPH, FRAP, total phenolic, and total flavonoid contents than aqueous extraction. Further studies should be carried out to establish the application and practicability of incorporating *B. racemosa* (L.) powder in any healthy processed products such as fiber powder booster, breakfast cereals, energy/fruit bar, and capsulated daily supplements.

Conflict of Interest

We declare that we have no conflict of interest.

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