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Total Phenolic Compounds and Antioxidant Properties in Different Stage of *B.racemosa* and *B.spicata* Leaf

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Abstract. A study was carried out to determine the antioxidant properties and total phenolic compounds from different stages of the leaves of *B.racemosa* and *B.spicata.* The highest total phenolic content (TPC) was shown to be in the methanolic extracts, stage one leaf of *B.racemosa* and *B.spicata*. In the chloroform extracts, the highest TPC were found in stage five leaf of *B.racemosa* and *B.spicata*. The free radical scavenging ability of the plant extract using DPPH assay was evaluated. The highest antioxidant activity were showed from methanolic extract from stage three leaf of *B.racemosa* with IC_{50} at 33.33 µg/ml and in *B.spicata*, was shown in stage four at 42.33 µg/ml as compared to the chloroform extraction that gave below 20% of free radical inhibition. The capacity of extracts to inhibit lipid peroxidation was determined by ferric thiocyanate (FTC) and thiobarbituric (TBA) assay. All extracts were active in antioxidant activity with the value above 88%. This study showed *B.racemosa* and *B.spicata* methanolic extracts contain antioxidant compounds when assayed for free radical scavengers and lipid peroxidation inhibition.

Keywords: *Barringtonia*, lipid peroxidation, free radical scavengers, total phenolic content

1. Introduction

Natural antioxidants like phenolics that are present in plants play important roles in inhibiting free radicals and oxidative chain reactions within tissues and membranes [1, 2]. *Barringtonia racemosa* and *Barringtonia spicata* leaves are widely used to treat ailments besides the young leaf were consumed in the daily diet as vegetables by community in South-East Asia [3, 4]. According to [5], the ethanol and chloroform extract of *B.racemosa* roots showed antibacterial activity. The bark is claimed to be specific for gastric ulcers [6] and toxic to aphis [7]. *B.racemosa* leaf extracts also showed anti-inflammatory [8] and antifungal activity [9].

The biological activity of the leaf sometimes can be differ based on its developmental stage, hence will gave varieties of results [10]. Hence, a study was carried out to determine the total amount of phenolics and to evaluate the antioxidant properties in different stages of the leaves. The young leaf was divided into five stages: stage one means first leaf from the shoot tip, followed by stage two as the second leaf until the fifth leaf. Such study would contribute to further knowledge relating to the screening of antioxidant compounds in these plants species.

2. Material and Methods

2.1. Plant materials

The leaves were obtained from trees planted in the herb garden in the Research Station of Malaysian Agricultural Research and Development Institute (MARDI) Jerangau, in Kuala Terengganu.

2.2. Extraction of extracts

The dried leaves (±5g) were macerated with 500 ml of methanol (Fisher, USA) at room temperature for three days. The solvent was then filtered and evaporated in a rotary evaporator (Buchi, Switzerland) under vacuum at 40°C. The crude extract was stored in a refrigerator at 4°C until used for assays. The above procedure was repeated using chloroform (Fisher, USA) as solvent.

2.3. Total Phenolic Content (TPC) assay

The amounts of total phenolics were determined using Folin-Ciocalteu's reagent (Sigma-Aldrich, USA) according to the method of [11] with some modifications. The extracts (0.5 ml) and gallic acid (Sigma-Aldrich, USA) (as standard in various concentrations) were mixed with 2.5 ml of Folin-Ciocalteu's reagent (diluted 1:10) and 2 ml of 7.5% of sodium carbonate. The mixture was incubated at room temperature for 90 min, and then absorbance was measured at wavelength 765nm with a UV-Vis spectrophotometer (Shimadzu Corporation, Japan). The amount of total phenolic compounds was calculated as mg of gallic acid equivalents (GAE) from the calibration curve and expressed as mg gallic acid equivalent/g dry weight of the plant material. The data were presented as the average of triplicate analyses.

2.4. Antioxidant assays

2.4.1. Free radical scavenging ability onto 1,1-diphenyl-2-picrylhydrazyl (DPPH)

The scavenging activity of extracts against DPPH was evaluated spectrophotometrically by slightly modified method of [12], with adaptation on the micro-plate reader. The reaction mixture consist of 100 µl of 0.3 mM DPPH (Sigma-Aldrich, USA) and 50 µl of leaf extracts and standards were incubate for 30 min. The remaining DPPH was measured their absorbance value by micro-plate reader (Infinite M 200, Tecan, Switzerland) at 517 nm. The data were presented as the average of triplicate analyses. The concentration of each sample reaction for 50% scavenging of DPPH free radicals (IC_{50}) were determined graphically by plotting inhibiton percentage of DPPH free radicals. The inhibition of DPPH free radicals in percentage (I %) was calculated as given below:

$$I\ \% = [(Abs_{\ control}/Abs_{\ sample})/Abs_{\ control}] \text{ X } 100$$

2.4.2. Ferric thiocyanate (FTC) assay

The assay was conducted in a linoleic acid emulsion to determine the amount of peroxide at the initial stage of lipid peroxidation. As described by [13], a mixture of 4 mg of sample was placed in 4 ml of ethanol (Merck, Germany), 4.1 ml of 2.52 % (v/v) of linoleic acid (Sigma-Aldrich, USA) in ethanol, 8 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of water was placed in a vial and placed in an oven at 40°C in the dark. The same reaction medium without any additive was used as a control.

To 0.1 ml of this solution, 9.7 ml of 75% (v/v) of ethanol and 0.1 ml 30% (w/v) of ammonium thiocyanate (Sigma-Aldrich, USA) was added. Exactly three minutes after the addition of 0.1 ml of 0.02 M ferrous chloride (Sigma-Aldrich, USA) in 3.5% (v/v) of hydrochloric acid (Fisher, USA) to the reaction mixture, the absorbance was measured at 500 nm with a UV-Vis spectrophotometer (Shimadzu Corporation, Japan) every 24 hours until the absorbance of the control reached maximum. The inhibition percent of linoleic acid peroxidation was calculated same with DPPH assay. The data were presented as the average of triplicate analyses.

2.4.3. Thiobarbituric acid (TBA) assay

The method according to [13] was used to determine the formation of malonaldehyde, a product from lipid peroxidation. One ml sample solution was prepared and incubated as in the FTC method. A volume of 2 ml of 20% (w/v) of trichloroacetic acid (Sigma-Aldrich, USA) and 2 ml TBA aqueous solution were added to 1 ml of sample. The mixture was then boiled for 10 min. After cooling at room temperature, it was centrifuged at 3,000 rpm for 20 min (Refrigerated Centrifuge, Sigma) and the absorbance of the supernatant was measured at the wavelength of 532 nm. Antioxidant activity was determined based on the absorbance on the final day of FTC assay. The inhibition percentage of linoleic acid peroxidation was calculated same with DPPH assay. The data were presented as the average of triplicate analyses.

3. Results and Discussion

3.1. Total Phenolic Content assay

The highest levels of total phenolic compounds were detected in stage 1 for both species in methanol extract (Table 1). The decline level of total phenolic compounds started from stage 2 to stage 5 indicated the beginning of leaf senescence. These results suggest that leaf of earlier stage were most active in biosynthesis and accumulation of secondary metabolites [14, 15, 16]. Extraction by methanol showed higher content of

phenolics compare to chloroform. The finding in agreement with [17, 18] who found methanol was better due to its ability to inhibit the reaction of polyphenol oxidase that oxidize phenolics, easy to evaporate and higher extraction efficiency [19]. In addition, the colour measurement of Folin-Ciocalteu reagent is non-specific to the phenol only, but other non-phenolics substances such as water, fat, sugars, proteins and other pigments [20].

Table 1. Total phenolic compounds of *B.racemosa* and *B.spicata* leaf. Results are expressed as mg of gallic acid equivalents (GAE) per g of dried weight (mg/g DW).

	B.racemosa		*B.spicata*	
Leaf stages	Methanol	Chloroform	Methanol	Chloroform
Stage 1	$0.34\pm0.01^{c,3}$	$0.013\pm0.01^{a,b,1}$	$0.19\pm0.06^{c,2}$	$0.022\pm0.01^{c,1}$
Stage 2	$0.22\pm0.08^{b,2}$	$0.016\pm0.01^{b,c,1}$	$0.10\pm0.05^{a,b,1}$	$0.020\pm0.01^{b,1}$
Stage 3	$0.11\pm0.01^{a,2}$	$0.009\pm0.01^{a,1}$	$0.15\pm0.01^{b,c,3}$	$0.023\pm0.01^{c,1}$
Stage 4	$0.08\pm0.01^{a,2}$	$0.010\pm0.01^{a,b,1}$	$0.15\pm0.01^{b,c,3}$	$0.022\pm0.01^{c,1}$
Stage 5	$0.08\pm0.00^{a,4}$	$0.018\pm0.01^{c,2}$	$0.07\pm0.00^{a,3}$	$0.013\pm0.01^{a,1}$

Values with different letters in the same column were significantly ($p<0.05$) different between stage of leaf. Values with different numbers in the same row were significantly ($p<0.05$) different between species and solvents. Values represent the mean ± SD according to Duncan's Multiple Range Test.

3.2. Antioxidant assays

3.2.1. Free radical scavenging ability onto 1,1-diphenyl-2-picrylhydrazyl (DPPH)

In methanol extract, stage 5 leaf of *B.racemosa* gave the highest antioxidant activity with 93.68% and in *B.spicata*, it was observed in stage 1 (92.24%). In contrast, chloroform extraction gave only 20% of antioxidant activity (data were not presented). In comparison of the results from Table 1 and Table 2, it showed that most of the sample with high amount of total phenolic compounds did not gave high inhibition of free radical activity. Thus, it can be conclude that the inhibition percentage of *Barringtonia* was not reflected by the amount of total phenolic compounds.

Besides that, methanol extraction gave higher activity as compare to chloroform and showed that more polar solvent gave higher antioxidant activity [21, 22]. According to [23], changed in solvent polarity alters its ability to dissolve a selected group of antioxidant compounds and influences the antioxidant activity estimation. Thus it was indicating that different types of antioxidant compounds could be present in the *B.racemosa* and *B.spicata.*

Table 2. Inhibition percentage (I%) and IC_{50} (µg/ml) of free radical radical scavenging activities from DPPH assay in *B.racemosa* and *B.spicata* leaf extract at the 500 µg/ml.

Samples	*B.racemosa*		*B.spicata*		Quercetin	
	I%	IC_{50}	I%	IC_{50}	I%	IC_{50}
Stage 1	93.14 ± 0.03^{c}	36.00 ± 2.00^{a}	$92.24\pm0.22^{b,c}$	$44.33\pm0.58^{a,b}$	92.84±0.09	25.33±1.15
Stage 2	$92.92\pm0.07^{b,c}$	47.33 ± 0.58^{b}	$92.45\pm0.07^{c,d}$	$47.67\pm0.58^{b,c}$	Ascorbic acid	
Stage 3	$92.69\pm0.15^{a,b}$	33.33 ± 4.16^{a}	92.73 ± 0.06^{e}	50.67 ± 2.31^{c}	93.36±0.13	42.00±2.65
Stage 4	92.39 ± 0.39^{a}	35.00 ± 1.73^{a}	$91.98\pm0.25^{a,b}$	42.33 ± 2.52^{a}	BHT	
Stage 5	93.68 ± 0.29^{d}	35.33 ± 2.31^{a}	91.92 ± 0.11^{a}	60.67 ± 2.31^{d}	92.91±0.74	34.67±0.58

Values represent the mean of triplicate ± SD according to Duncan's Multiple Range Test. Values with different letters in the same column were not significantly ($p<0.05$) different.

3.2.2. Ferric thiocyanate (FTC)

The absorbance values of the control reached the maximum on day six of incubation so the percentage inhibition of lipid peroxidation was calculated on that day (Table 3). The highest percent inhibition was showed in stage 5 (93.83%) for *B.racemosa* methanolic extract and for *B.spicata*, the highest value were from chloroform extract, stage 2 leaf (94.55%). Generally, each extract showed very strong antioxidant activity with the percent inhibition ranged from 88.59% to 94.55 %. High percents of lipid peroxidation inhibition for all the extracts tested indicate that the *Barringtonia* extracts tested contain antioxidant constituent that could inhibit lipid peroxidation.

Table 3. Percentage inhibition of lipid peroxidation as measured by the FTC method.

	B.racemosa		*B.spicata*	
Leaf stage	Methanol	Chloroform	Methanol	Chloroform
Stage 1	93.13±0.37^{b,2}	92.78±0.78^{c,1,2}	93.72±0.41^{a,2}	91.79±0.76^{a,1}
Stage 2	93.34±0.81^{b,2}	89.60±0.75^{a,b,1}	94.39±0.00^{a,2,3}	94.55±0.37^{c,3}
Stage 3	93.40±0.12^{b,2,3}	90.64±0.73^{b,c,1}	94.18±0.45^{a,3}	92.65±0.31^{a,b,2}
Stage 4	88.59±0.71^{a,1}	91.63±0.31^{c,d,2}	93.61±0.75^{a,3}	93.14±0.14^{b,3}
Stage 5	93.83±0.49^{b,2}	88.59±0.49^{a,1}	94.41±0.85^{a,2}	93.27±0.88^{b,2}
Standards	BHT	Quercetin	α-tocopherol	
	94.14±0.26	89.76±0.57	86.66±3.05	

Values with different letters in the same column were not significantly ($p<0.05$) different between stage of leaf. Values with different numbers in the same row were not significantly ($p<0.05$) different between species and solvents. Values represent the mean of triplicate ± SD according to Duncan's Multiple Range Test.

3.2.3. Thiobarbituric acid (TBA) assay

The absorbance was measured on the day seven of FTC assay incubation with low absorbance value corresponding to high inhibition percentage of lipid peroxidation. As shown in Table 4, the inhibition percentage of lipid peroxidation of most samples were very high where most sample showed higher than 90% of inhibition and higher than quercetin and α–tocopherol. The results have indicated that methanolic and chloroform extracts of *B.racemosa* and *B.spicata* could inhibit the linoleic acid peroxidation.

Table 4. Percentage inhibition of lipid peroxidation as measured by the TBA assay

Leaf Stages	*B.racemosa*		*B.spicata*	
	Methanol	Chloroform	Methanol	Chloroform
Stage 1	91.09±1.83^{a,2}	91.46±0.22^{a,2}	86.75±3.27^{a,1}	96.51±0.28^{a,3}
Stage 2	94.42±0.51^{b,2}	93.50±0.17^{b,1}	97.56±0.55^{b,4}	95.34±0.29^{b,3}
Stage 3	96.23±0.22^{c,2}	93.20±0.23^{b,1}	96.71±1.35^{b,2}	95.38±0.34^{b,2}
Stage 4	96.75±0.17^{c,3}	93.39±0.82^{b,1}	96.19±0.26^{b,2,3}	95.75±0.34^{b,2}
Stage 5	96.49±0.23^{c,2}	92.50±1.78^{a,b,1}	96.34±0.22^{b,2}	95.12±0.55^{b,2}
Standards	BHT	Quercetin	α –tocopherol	
	98.00±0.01	87.31±2.72	91.08±0.28	

Values represent the mean of triplicate ± SD according to Duncan's Multiple Range Test. Values with different letters in the same column were not significantly ($p<0.05$) different between stage of leaf. Values with different numbers in the same row were significantly ($p<0.05$) different between species and solvent.

4. Conclusions

Various extracts and fractions could contain different types of antioxidant compounds, which demonstrate varying activity in the three *in vitro* assays used in this study. Based on the previous study, *B. racemosa* contain gallic acid, ferrulic acid, naringin, rutin, luteolin and kaempferol and lycopene as the active compound. This identification indicates that *Barringtonia* species is a rich source of phytonutrient. A more detailed investigation have to be focused on the amount of active compounds, the availability of relevant toxicity data in order to evaluate the plants and its medicinal potential as it is easily accessible source of natural antioxidants supplement. This paper has partially identified the stages of leaves to do the essays but a more detailed study will be required to identify which of the active compounds are more in the leaves, which people eat in their daily consumption.

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6. References

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