

Full Length Research Paper

# Total phenolic contents, antioxidant and antimicrobial activities of *Bruguiera gymnorhiza*

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In the present work, the antioxidant and antibacterial activities of methanolic, ethanolic and chloroform crude extract of leaves and barks of *Bruguiera gymnorhiza* were investigated. The antioxidant activity of the crude extracts were evaluated using the enzymatic and non enzymatic methods namely superoxide dismutase determination, reducing power assay and 2, 2-diphenyl-1-picrylhydrazil free radical scavenging assays. Folin-ciocalteu reagent method was used to estimate the amount of total phenolic compounds of the extracts. Ethanol extract of barks showed the best result in all antioxidant assays which was positively co-related with the total phenolic contents. There was no significant difference between the IC<sub>50</sub> value of ethanol extract of bark and the ascorbic acid. *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus* were used as the test pathogenic bacteria in this study. Both ethanol and methanol extracts could inhibit the growth of all pathogenic bacteria while chloroform extract of leaves showed no activity against any bacteria. Antimicrobial effect of ethanol extract of barks was higher than all other extracts.

**Key words:** Antioxidant, antimicrobial, *Bruguiera gymnorhiza*, phenolics, mangrove plant.

## INTRODUCTION

In general, mangroves are trees and shrubs, which grow in saline coastal habitats in the tropics and subtropics. The mangrove dwellers get food and wide variety of traditional products and artifacts from mangroves. Extracts and chemicals from mangroves are used mainly in folkloric medicine (e.g. bush medicine), as insecticides and pesticides and these practices continue to this day (Bandaranayake, 2002). Promising antibacterial activity of ethyl acetate extract of *Avicennia marina* mature leaves (Abeyasinghe and Wanigatunge, 2006), methanol extract of *Excoecaria agallocha* leaves and shoots (Chandrasekaran et al., 2009) and antifungal activity of methanol extract of *Excoecaria agallocha* and *Bruguiera gymnorhiza* trunks (Kazuhiko, 2002) are some other

examples of pharmaceutical potential of mangrove plants. *Bruguiera gymnorhiza* is a common mangrove tree which supports as a wall of mangrove forest and is widely distributed in Thailand and southeast Asia, southern and eastern Africa, Australia, Micronesia and Polynesia (Hou, 1970). In China, fruits of *B. gymnorhiza* have been used to treat diarrhea (Bamroongrugs, 1999).

The medicinal use of fruits includes application of the treatment of shingles and eye diseases (Rudjiman, 1992 and Othman, 1998). The bark has been used as an astringent treatment of diarrhea and malaria (Rudjiman, 1992; Othman, 1998). The roots and leaves have been used to treat burn (Othman S, 1998).

Based on review of literature, bioactivity of different varieties/cultivars of a plant might show different values.

Henríquez et al. (2009) reported the different antioxidant activities of five Chile apple cultivars. Different parts of a plant might also show different bioactivity potentials. For example, dissimilar antioxidant values from different parts of *Andrographis paniculata* were reported by Rafat et al. (2010a). For example, Muthu et

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**Abbreviations:** SOD, Superoxide dismutase; DPPH, 2,2-diphenyl-1-picrylhydrazil; DMSO, dimethyl sulfoxide.

al. (2010) showed methanol extract of *Borreria hispida* had higher antibacterial activity than both ethyl acetate and petroleum ether extracts. The references also suggest the application of combination of different assays to indicate a specific bioactivity. As an example, the antioxidant capacity of *Oenanthe javanica* was higher than *Euodia redlevi* using DPPH free radical scavenging assay while the results of superoxide dismutase activity assay showed the higher antioxidant potential of *E. redlevi* compared to *O. javanica* from the same plant extracts (Rafat et al., 2010b).

Although there are a few reports on antioxidant and antimicrobial activities of *B. gymnorhiza*, the present study was designed and carried out based on the reasons mentioned above. In this study, three different ethanol, methanol and chloroform extracts of the Malaysian endogenous *B. gymnorhiza* leaves and barks were examined to evaluate the antioxidant potential and the same crude extracts were also used to indicate the antimicrobial activities of the samples.

## MATERIALS AND METHODS

### Chemicals

Ethanol (95%), methanol, chloroform and dimethyl sulfoxide (DMSO) from system ChemAR® were purchased while butylated hydroxyanisole (BHA) from fluka (Spain) and the tetracycline antibiotic disc (9043) from liofilchem (Italy) were obtained. Folin-ciocalteu reagent (F9252), gallic acid (G7384), SOD determination kit (19160), 2,2-diphenyl-1-picrylhydrazil (D9132), Tween 20 (Cat.: 27,434-8), ascorbic acid (A4544), nutrient broth, Mueller hinton were purchased from Sigma-Aldrich (St. Louis, Mo).

### Plant extracts

Leaves and barks of *B. gymnorhiza* were collected from the Carey Island, Selangor, Malaysia. The collected leaves and bark were washed under tap running water and dried in an incubator at 40°C. Dried leaves and bark were ground to produce fine homogenous powders using an electric blender and the powder was soaked in three selected solvents (95% ethanol, methanol and chloroform) at room temperature in the dark for three days. Each sample was filtered through Whatman® No. 1 filter paper (Whatman International, England) and the filtered solutions were then evaporated to dryness using water evaporator at 40°C. The plant extracts were dissolved in dimethyl sulfoxide (DMSO).

### Total phenolic content evaluation

A protocol based on the Folin-ciocalteu method described by Slinkard and Singleton (1977) was employed to determine the total amount of present phenolic compounds in various extracts of *B. gymnorhiza* leaves and barks. Ethanol, methanol and chloroform extracts (20 µl) at concentration of 5 mg/ml was added to 100 µl of 2N Folin-ciocalteu reagent. Sodium carbonate solution (300 µL) at concentration of 0.2 mg/ml was added after the final volume of mixture was made up to 1600 µl using distilled water. The mixture was then incubated at 37°C for 45 min before the absorbance of solutions were measured at 760 nm using a spectrophotometer.

Lastly, the total phenolic contents were expressed as a gallic acid equivalent (GAE) based on Folin-ciocalteu calibration curve using gallic acid as the standard.

### Antioxidant activity assays

#### Superoxide dismutase (SOD) assay

Superoxide dismutase (SOD) determination assay was carried out using SOD determination kit according to kit's protocol provided by Sigma-Aldrich. Briefly, each plant extract (20 µl) at concentration of 5 mg/ml was added to 200 µl of working solution and was incubated at 37°C for 20 min after adding 20 µl enzyme working solution. Butylated hydroxyanisole (BHA) at concentration of 5mg/ml was used as the positive controls. The mixture of working solution (200 µL) and enzyme working solution (20 µl) containing 20 µl double distilled water (ddH<sub>2</sub>O) named blank1 while blank 2 contained working solution (200 µl) and (20 µl) dilution buffer with each plant extract (20 µl). Blank 3 was prepared same as blank 2 but 20 µl of ddH<sub>2</sub>O was replaced by the plant extract. At last the SOD activity was calculated using the following equation after measurement of the mixtures absorbance at 450 nm in a micro-plate reader.

Inhibition activity (%) =  $\frac{[(\text{blank 1} - \text{blank 3}) - (\text{Sample A} - \text{Sample A's blank 2})]}{(\text{blank 1} - \text{blank 3})} \times 100$

#### Reducing power assay

Reducing power of all extracts was determined based on the method described by Sorenson et al (1986). 0.25, 0.5, 1.0 and 2.0 mg/ml of extracts were tested to determine the reducing power. Each extract dissolved in 1.0 ml of DMSO inside the falcon tube. Then 2.5 ml of 0.2 M phosphate buffer and 2.5 ml of potassium ferricyonide added into the falcon tube. The mixture kept in water bath for 20 min at 50°C. After incubation, 2.5 ml of TCA solution was added to the mixture and then centrifuge the solution at 1000 RPM for 10 min. 2.5 ml supernatant were then transferred into a new tube and 2.5 ml of distilled water and 0.5 ml of ferric chloride was added. The absorbance were taken at 700 nm.

#### Radical scavenging capacity assay

DPPH\* (2, 2-diphenyl-1-picrylhydrazil) was used as the free radical in this study. The assay protocol was obtained from Rafat et al. (2010b). Each plant extract (975 µl) at concentration of 5 mg/ml was added to DPPH\* (25 µl) at concentration of 8 mg/ml. All mixtures were incubated at room temperature in the dark for 30 min before measurement of their DPPH\* scavenging potential. Colour reduction of the mixtures, which is equivalent with scavenging activity of samples, was measured using a spectrophotometer at 520 nm. The potential of scavenging capacity of the examined plant extracts was calculated using the following equation while the solution without plant extract was known as blank.

Radical scavenging capacity (%) =  $\frac{[(\text{Blank} - \text{Sample A}) / \text{Blank}] \times 100$

Ascorbic acid (5 mg/ml) was applied as the positive control in this study.

#### IC<sub>50</sub> value in DPPH assay

The extracts were reset for antioxidant activity at concentration of 0.125, 0.0625, 0.03125 and 0.01625 mg/ml to determine the IC<sub>50</sub>

**Table 1.** Total phenolic contents of ethanol, methanol and chloroform extracts of *Bruguiera gymnorhiza* leaves and barks.

<i>Bruguiera gymnorhiza</i>	Total phenolic contents (mg of GAE/g of dry leaves)
Methanolic extract of leaf	178.73 ± 0.23 <sup>d</sup>
Ethanolic extract of leaf	189.4 ± 0.6 <sup>c</sup>
Chloroform extract of leaf	13.13 ± 0.23 <sup>f</sup>
Methanolic extract of bark	268.47 ± 0.12 <sup>b</sup>
Ethanolic extract of bark	284.93 ± 0.23 <sup>a</sup>
Chloroform extract of bark	23.6 ± 0.35 <sup>e</sup>

Samples presented with different alphabetic letters are significantly different.

value. The assay procedures were similar to the DPPH testing and the IC<sub>50</sub> value for each extract was extrapolated from graphs plotted using the OD value obtained.

#### Antimicrobial activity assay

The antibacterial potentials of leaves and barks of *B. gymnorhiza* ethanol, methanol and chloroform extracts (10 mg/ml) were studied using the paper disc diffusion method of Kil et al. (2009). Two Gram-Negative pathogenic bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and two Gram-Positive pathogenic bacteria (*Staphylococcus aureus* and *Bacillus cereus*) which were identified by Koshy et al. (2009) were obtained from Microbiology Division of Institute of Biological Sciences, University of Malaya. The obtained bacteria were grown in nutrient broth medium to yield a final concentration of 10<sup>7</sup> CFU/ml. Sterilized filter paper discs were soaked in each extract and placed on the Mueller Hinton medium plates after streaking the test bacteria using sterile swab cotton. The diameters of the produced inhibition zones were measured after 24 h incubation of the plates at 37°C. The positive and negative controls in this study were tetracycline (30 µg) and PBS, respectively.

#### Statistical analysis

All experiments were carried out in triplicates. One-way analysis of variance (ANOVA) was used to analyze the data using Microsoft Excel programme. The means were compared by Duncan's multiple range test (DMRT) and p<0.05 was considered to indicate statistical significance.

## RESULTS

#### Total phenolic contents

The standard curve of gallic acid was prepared and the total phenolic content of examined extracts were calculated based on this standard and presented as gallic acid equivalents (GAE) per gram of dry sample. Based on the results obtained in this experiment (Table 1), among all the extracts, ethanol extract of barks of *B. gymnorhiza* showed the highest amount of phenolic compounds. Chloroform extracts of leaves showed the lowest amount of phenolic compounds. There was a significant difference between all three tested extracts.

#### Antioxidant activity assay

##### SOD activity assay

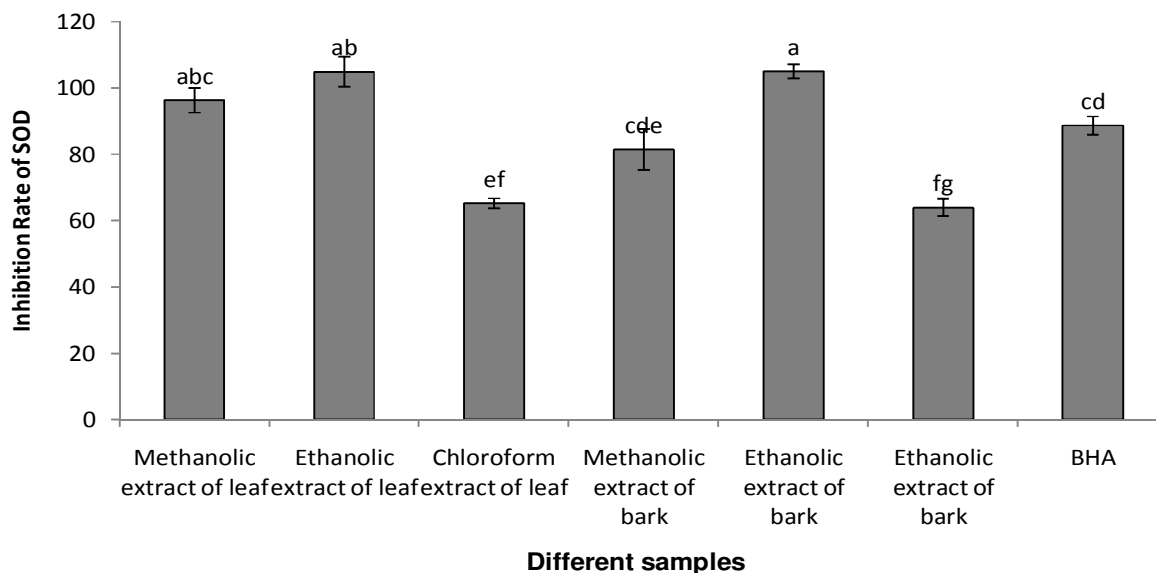
The results of SOD activity assay (Figure 1) showed all samples including BHA (5 mg/ml) with highest antioxidant potential obtained from *B. gymnorhiza* ethanol extract of barks followed by ethanol extract of leaves and methanol extract of leaves. However, the inhibition rate from ethanolic extract of barks is significantly higher than chloroform extract of bark and leaves, methanolic extract of barks and also form positive control (BHA), but not significantly higher than ethanolic and methanolic extract of leaves. The lowest antioxidant capacity was achieved by chloroform extracts as well as it was statistically different from all other extracts in this study.

##### Reducing power assay

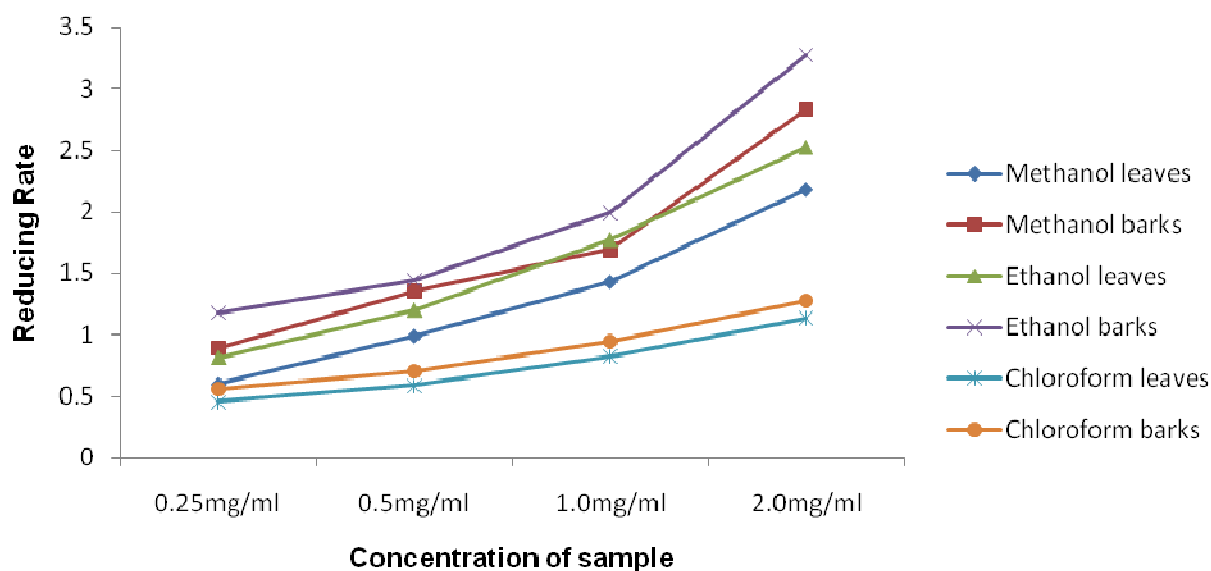
The result of reducing power assay (Figure 2) showed that when the concentrations increased the reducing rates also increased. Among all the extracts, ethanol extract of barks showed the highest reducing rate and chloroform extract showed the lowest reducing rate. The second highest reducing rate found from the methanol extract of barks. As can be observed from graph (Figure 2), barks extracts showed the better result compared with the leaves extract.

#### DPPH free radical scavenging assay

The results of free radical scavenging test (Figure 3) showed none of the extracts were significantly comparable with positive control in terms of scavenging capacity. The best scavenging activity among the examined extracts was achieved from ethanolic extracts of barks which was significantly higher than other extracts except methanolic extracts of barks. However, among all the extracts, chloroform extracts of leaves showed the lowest scavenging capacity.



**Figure 1.** Antioxidant activity of ethanol, methanol and chloroform extracts of *Bruguiera gymnorrhiza* leaves and barks using SOD activity assay presented as inhibition rate. BHA was used as the positive control. Samples presented with different alphabetic letters are significantly different ( $p < 0.05$ ).

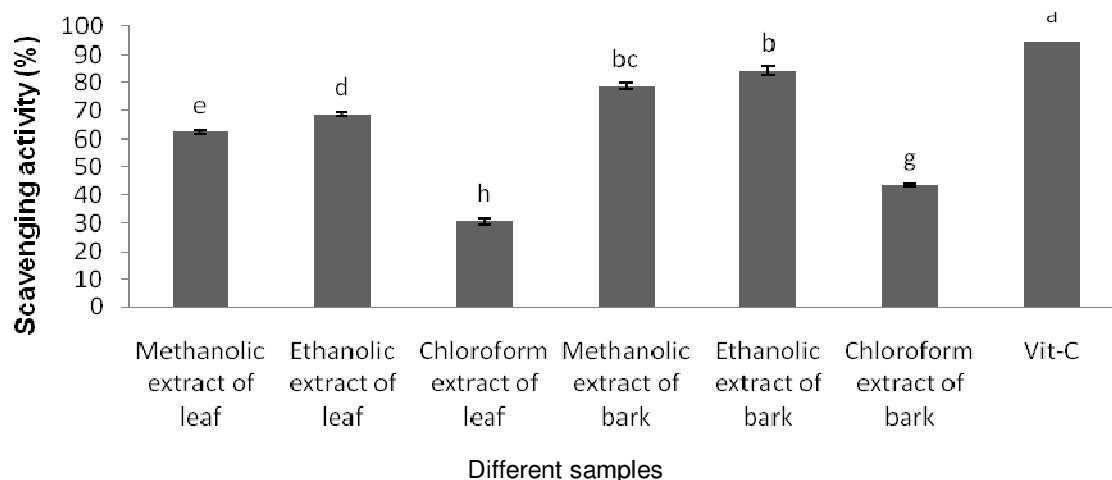


**Figure 2.** Antioxidant activity of ethanol, methanol and chloroform extracts of *Bruguiera gymnorrhiza* leaves and barks using reducing power assay.

### IC<sub>50</sub> value in DPPH assay

The result of IC<sub>50</sub> value of leaves and barks of three different crude extracts has been presented in Table 2. Among all the extracts, ethanol extracts of barks showed the lowest value of IC<sub>50</sub>. However, ascorbic acid showed the better IC<sub>50</sub> value but there is no significant difference

between ascorbic acid and ethanol and methanol extracts of barks and ethanol extracts of leaves. As can be observed from the Table, methanol extract of leaves and chloroform extracts of leaves and barks showed the value of IC<sub>50</sub> is significantly higher than other three extracts. Chloroform extract of leaves showed the highest IC<sub>50</sub> value among all the extracts which means the inhibition



**Figure 3.** Antioxidant activity of ethanol, methanol and chloroform extracts of *Bruguiera gymnorhiza* leaves and barks using DPPH free radical scavenging capacity assay. Ascorbic Acid was used as the positive control. Samples presented with different alphabetic letters are significantly different ( $p < 0.05$ ).

**Table 2.** IC<sub>50</sub> value of in DPPH assay of methanol ethanol and chloroform extracts of *Bruguiera gymnorhiza* leaves and barks.

Sample	Value of IC <sub>50</sub>
Methanolic extract of leaves	0.038 ± 0.003 <sup>de</sup>
Ethanolic extract of leaves	0.029 ± 0.004 <sup>abcd</sup>
Chloroform extract of leaves	0.27 ± 0.017 <sup>g</sup>
Methanolic extract of barks	0.025 ± 0.003 <sup>abc</sup>
Ethanolic extract of barks	0.0197 ± 0.001 <sup>ab</sup>
Chloroform extract of barks	0.193 ± 0.015 <sup>f</sup>
Ascorbic acid	0.018 ± 0.0003 <sup>a</sup>

Samples presented with different alphabetic letters are significantly different ( $p < 0.05$ ).

rate is the lowest.

### Antimicrobial activity assay

The result of antimicrobial activity of methanol, ethanol and chloroform extracts of *B. gymnorhiza* leaves and barks were presented in Table 3. Chloroform extract of leaves showed no inhibition against the growth of any bacteria. Ethanol extracts of leaves and barks showed a higher antimicrobial activity against *E. coli*, *S. aureus*, *P. aeruginosa* and *B. cereus* compared with methanol extracts of leaves and barks. Positive control (30 µg tetracycline) inhibited the growth of all tested bacteria significantly compared to the examined extracts.

### DISCUSSION

The present study confirmed that the extraction solvent in

preparation of samples has an important effect on the antioxidant and antimicrobial activities. It has also confirmed that different explants in different extraction solvent have an important effect on the antioxidant and antimicrobial activities. The results clearly showed that extraction solvents significantly affected the total phenolic content of the prepared extracts. For example, phenolic compounds present in methanol and ethanol extract of *B. gymnorhiza* were approximately more than 10 times the chloroform extract. Cheung et al. (2003) also reported that methanol can extract the highest amount of phenolic compounds compare to petroleum ether, ethyl acetate and water from both their examined samples, *lentinus edodes* and *Volvariella volvacea*. Based on different extraction potential of solvents, combination of different solvents can be suggested. Alberto et al. (2006) applied combination of several solvents such as combination of acetate, methanol and water to extract the phenolic compounds of apple skins.

In this study, the evaluated phenolic content of the

**Table 3.** Antimicrobial activity of methanol, ethanol and chloroform extracts of leaves and barks of *Bruguiera gymnorhiza*.

Bacteria	Inhibition zone						Positive control
	Methanolic extract		Ethanolic extract		Chloroform extract		
	Leaf	Bark	Leaf	Bark	Leaf	Bark	
<i>B. cereus</i>	12.67	15.86	14.56	16.78	-	8.67	17.93
<i>S. aureus</i>	14.34	17.85	15.85	18.95	-	9.77	21.10
<i>E. coli</i>	8.87	9.25	9.91	11.56	-	-	14.30
<i>P. aeruginosa</i>	7.85	8.38	8.9	9.78	-	-	12.16

ethanol extracts of *B. gymnorhiza* barks (284.93 mg/g of dry barks) was higher than other extracts. The amount of phenolic compound found in methanol and ethanol extracts of leaves and barks in this study was higher than the total phenolic contents of *B. gymnorhiza* leaves and barks of aqueous methanol extract (134.16 mg/g of dry leaves and 131.90 mg/g of dry stem bark) reported by Banerjee et al. (2008). This difference might be related to the plants variety difference as several reports showed the total phenolic contents of plants varied with varieties/cultivars of the plants greatly. For example, total phenolic contents in peach influenced by cultivars (Kubota et al., 2000). Ahmed and Beigh (2009) reported that even subspecies of *Brassica oleracea* var. acephala showed variation of phenolic contents.

Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Benavente-Garcia et al., 1997; Manach et al., 2005; Middleton et al., 2000; Puupponen-Pimiä et al., 2001; Samman and Cook 1998). *B. gymnorhiza* can be introduced as a high value source of phenolic compound due to the high amount of this group of compounds. Comparison of amounts of phenolic compounds in different parts of Indian, *B. gymnorhiza* was carried out previously by Banerjee et al. (2008) and it was shown that leaf explants of *R. mucronata* contain the highest amounts of phenolic compounds compare to the stem barks and roots. However, in our study we found that the phenolic compound is higher in barks compared with leaves.

Application of one antioxidant assay alone to show the antioxidant activity of extracts is not recommended (Rafat et al., 2010a). The importance of combination of different methods to evaluate the antioxidant activity of the extracts is confirmed based on the results obtained from this study. Combination of both enzymatic and non-enzymatic methods can result in a more reliable data (Hakiman and Maziah, 2009). All three tested extracts showed the antioxidant capacity based on their phenolic contents. There were positive co-relation with the phenolic contents and the antioxidant activities. Ethanol extract of barks showed the high antioxidant activity

against DPPH scavenging assay, reducing power assay and SOD assay. Phenolic compounds could be a major determination of antioxidant potentials of foods (Parr and Bolwell, 2000), and could therefore be a natural source of antioxidants. However, there are a couple of reports which stated that different solvent extract showed different potentiality in different assay. For example, Sun et al. (2007) showed that methanol extract of *Asparagus officinalis* has a higher antioxidant capacity than acetone extract of the same plant based on the DPPH scavenging method while antioxidant potential of acetone extract is higher than methanol extract in ABTS assay. Methanol extract of *Nelumbo nuficera* Gaertn rhizome obtained the highest antioxidant activity based on DPPH assay while dichloromethane extract showed the strongest antioxidant capacity in  $\beta$ -carotene bleaching experiment (Yang et al., 2007).

From the results we found that the crude extracts were as effective as the positive control (ascorbic acid or Butylated hydroxyanisole) except for the chloroform extracts. In terms of IC<sub>50</sub> there was no significant difference between ascorbic acid and the crude extract of *B. gymnorhiza*. In SOD assay, we have been found that ethanol extracts of leaves and barks were even better than the BHA. The reason might be for the high phenolic contents of their crude extracts *B. gymnorhiza*.

Antimicrobial results of this study showed that both leaves and barks of ethanol and methanol extracts of *B. gymnorhiza* were able to inhibit the growth of both Gram-positive (*S. aureus* and *B. cereus*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*). However, there is no inhibition detected by chloroform extracts of leaves against the growth of pathogenic bacteria. Chloroform extracts of barks showed inhibition against only Gram-positive bacteria (*S. aureus* and *B. cereus*). Ethanol seems to be a better solvent to extract the antimicrobial compounds than methanol and chloroform as the antibacterial activity of ethanol extract was higher than methanol and chloroform extract. Though there was no previous report about antibacterial activity of crude extracts of leaves and barks of *Bruguiera gymnorhiza* but there were some similar reports found about other mangrove plants such as Ravikumar et al. (2010) showed all collar, hypocotyls and bark extracts of *R. mucronata*

had antimicrobial activities against both *S. aureus* and *P. aeruginosa* bacteria. It was in complete agreement with the earlier report by Lim et al. (2006) which showed that tannins extracted from *R. mucronata* barks had antibacterial activity against the growth of both *S. aureus* and *P. aeruginosa*.

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