

## *In Vitro* Antioxidant Activity of Bark Extracts of *Rhizophora mucronata*

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### Abstract

The present study was conducted to evaluate the antioxidant activity of medicinal plant *Rhizophora mucronata* (*R. mucronata*). Shade dried stem bark of *R. mucronata* was powdered and extracted with 95% ethanol and water by cold extraction method. The total phenolic and flavonoid contents of water and ethanol extract of air dried stem bark was estimated by using spectrometric method. Antioxidant activity of *R. mucronata* was determined by using different methods namely DPPH radical scavenging assay, ferric reducing assay, nitric oxide radical scavenging assay, superoxide radical scavenging assay and ABTS radical scavenging assay, and its IC<sub>50</sub> values were found to be 110.85, 193.47, 109.06, 88.69 and 12.56 µg/ml for aqueous extract and 59.63, 242.71, 103.21, 84.95 and 4.21 µg/ml respectively. The extracts exhibited marked dose dependent *in vitro* antioxidant activity. Secondary metabolite isolation and characterization and *in vivo* evaluation of aqueous and ethanolic extracts were under progress.

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## INTRODUCTION

Oxidation is one of the destructive processes, wherein it breaks down the molecules and causes various diseases. During transformation oxygen produces reactive oxygen species (ROS) such as hydroxyl radicals, superoxide, and hydrogen peroxide. They provide uncontrolled reactions. Molecular oxygen is essential for all living organisms, but aerobic species are suffered by injury if they exposed to concentration more than 21%. Free radicals induce oxidative damage to the biomolecules such as protein, lipid, lipoproteins and DNA. Antioxidants act as inhibitors of the oxidation process and are found to inhibit the oxidant chain reactions at a very small concentration and it eliminates the pathological processes. Antioxidants are considered as a possible protection agent for reducing oxidative of human body from the ROS and retard the progress of many chronic diseases as well as lipid peroxidation (Peryor, 1991; Kinsella *et al.*, 1993; Lai *et al.*, 2001).

Medicinal plants contain phenolic compounds and its possessing powerful antioxidant activity. Especially flavonoids play a major class of Phenolic compound present in many medicinal plants and are found to have a potential role in the prevention of various diseases via antioxidant activity. Medicinal plants are a rich source of antioxidants. Antioxidants are important for human health and nutrition. They play a major role in the genesis of various diseases such as cancer, ageing, rheumatoid arthritis, atherosclerosis and inflammation. These

medicinal plants provide rich antioxidants include vitamin C, carotenoids and Phenolic compounds. Some synthetic antioxidants also available but these synthetic antioxidants are suspected to cause a liver damage. Therefore nowadays medicinal plants are used as an antioxidants and it is evaluated by various screening methods (Kokate, 1999; Nikhat *et al.*, 2009; Kekuda *et al.*, 2013). Many medicinal plants become a major candidate for identifying their chemical constituent rich in antioxidant it protects the cellular damage. In the present study different antioxidant assay methods are used to compare the antioxidant activities of different molecules used absorption spectroscopy.

*Rhizophora mucronata* is a small to medium size evergreen tree growing to a height of about 20 to 25 meters (66 to 82 ft) on the banks of rivers. On the fringes of the sea 10 or 15 metres (33 or 49 ft) is a more typical height. The tallest trees are closest to the water and shorter trees are further inland. The tree has a large number of aerial stilt roots buttressing the trunk. The leaves are elliptical and usually about 12 centimetres (4.7 in) long and 6 centimetres (2.4 in) wide. They have elongated tips but these often break off. There are corky warts on the pale undersides of the leaves. The flowers develop in axillary clusters on the twigs. Each has a hard cream-coloured calyx with four sepals and four white, hairy petals. The seeds are viviparous and start to develop whilst still attached to the tree (Gillikin and

Verheyden, 2005). In the present study, aqueous and alcoholic extracts of *R. mucronata* bark were subjected for the antioxidant screening using different *in vitro* methods.

## MATERIALS AND METHODS

### Plant Collection and Extraction

The barks of *R. mucronata* were collected from Pitchavaram mangrove forest. Collected plant materials were shade dried, powdered and used for extraction. The dried powder material of the bark was extracted with ethyl alcohol and water. The solvent was removed under pressure to obtain a total extracts.

### *In vitro* Antioxidant Activity

#### DPPH Assay by Thin Layer Chromatography (TLC)

This preliminary test was performed with a rapid TLC screening method using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). Analytical TLC silica plate (10cm X10cm) was developed under appropriate conditions (CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (61:32:7)) after application of 5  $\mu$ l of each test compound solution (1 mg/ml), dried and sprayed with DPPH solution (0.2%, MeOH). Five minutes later active compounds appeared as yellow spots against a purple background. The purple stable free radical was reduced to the yellow colored diphenylpicryl hydrazine. Ascorbic acid was used as positive control (Takao *et al.*, 1994).

#### Antioxidant Activity by DPPH Staining (Dot Assay)

An aliquot of each sample and standard (Ascorbic acid and Gallic acid) were carefully loaded onto a 10cm X 10cm Silica gel plate (silica gel 60 F254; Merck) and allowed to dry (3 min). Drops of each sample were loaded in order of decreasing concentration (4 to 1  $\mu$ L) along the row. Water sample was used as a control. After 5 min TLC plate was sprayed with 0.2% DPPH in methanol. Discoloration of DPPH indicates scavenging potential of the compound tested (Soler-Rvans *et al.*, 1997).

#### DPPH Radical Scavenging Activity

The free radical scavenging capacity of the extracts of *Rhizophora mucronata* aqueous and alcoholic extract was determined using DPPH. DPPH solution (0.004% w/v) was prepared in 95% methanol. Extracts of *R. mucronata* was mixed with 95% methanol to prepare the stock solution (10 mg/100ml). The concentration of extract solution was 10 mg /100 ml or 100 $\mu$ g/ml. From stock solution 2ml, 4ml, 6ml, 8ml and 10ml of this solution were taken in five test tubes and by serial dilution with same solvent were made the final volume of each test tube up to 10 ml whose concentration was then 20 $\mu$ g- 100 $\mu$ g/ml. Freshly prepared DPPH solution (0.004% w/v) was added in each of these test tubes containing extracts (20-100 $\mu$ g/ml) and after 10 min, the absorbance was taken at 517 nm using a spectrophotometer. Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (10 mg/100ml or 100  $\mu$ g/ml) of extracts. Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was used as blank. % scavenging of the DPPH free radical was measured using the following equation (Blois, 1958).

$$\% \text{ DPPH scavenging activity} = \frac{\text{control} - \text{test}}{\text{control}} \times 100$$

The antioxidant activity of the extract was expressed as IC<sub>50</sub> and compared with standard. The IC<sub>50</sub> value is defined as the concentration (in  $\mu$ g/ml) of extracts that scavenge 50% of DPPH radicals.

### Reducing Power Assay

Reducing power assay (Nikhat *et al.*, 2009) works under the following principle. Substances, which have reduction potential, react with potassium ferricyanide (Fe<sup>3+</sup>) to form Potassium ferrocyanide (Fe<sup>2+</sup>), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. This experiment was carried out as described previously (Yildirim *et al.*, 2001). Briefly, different concentrations of extracts (20-100  $\mu$ g/ml) was mixed with 2.5 ml phosphate buffer (0.2M, pH 6.6) and 2.5 ml potassium ferricyanide [K<sub>3</sub>Fe (CN)<sub>6</sub>] (10g/l), then mixture was incubated at 50 degree C for 20 min. Two and one-half, 2.5 ml of trichloroacetic acid (100g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml FeCl<sub>3</sub> (1g/l) and absorbance measured at 700nm in UV-Visible Spectrophotometer (Systronics UV-Visible Spectrophotometer 119, India). Ascorbic acid was used as standard and phosphate buffer used as blank solution. The absorbance of the final reaction mixture of two parallel experiments was expressed as mean  $\pm$  standard deviation. Increased absorbance of the reaction mixture indicates stronger reducing power.

$$\% \text{ increase in Reducing Power} = \frac{A_{\text{test}}}{A_{\text{blank}}} - 1 \times 100$$

### Nitric Oxide Scavenging Activity

The Nitric oxide scavenging activity of extract of *Rhizophora mucronata* were estimated by Griess reagent method employed by Marcocci *et al.* (1994). Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the SNP in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess reagent. Scavengers of NO compete with oxygen leading to reduced production of NO. Nitric oxide scavenging activity was measured spectrophotometrically. SNP (5 mmol L<sup>-1</sup>) in phosphate buffered saline pH 7.4 was mixed with different concentrations of the extract (20–100  $\mu$ g ml<sup>-1</sup>) prepared in methanol and incubated at 25 °C for 30 min. A control without the test compound, but with an equivalent amount of methanol, was taken. After 30 min, 1.5 ml of the incubated solution was removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% *N*-1-naphthylethylenediamine dihydrochloride). Absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with *N*-1-naphthylethylene diamine dihydrochloride was measured at 546 nm and the percentage scavenging activity was measured with reference to the standard.

$$\% \text{ Nitric oxide scavenging activity}$$

$$= \frac{\text{Control} - \text{test}}{\text{control}} \times 100$$

### Superoxide Radical Scavenging Activity

The Superoxide Radical Scavenging Activity was measured by Nishimiki *et al.* (1972) method. Superoxide anions were generated using PMS / NADH system. The superoxide anions are subsequently made to reduce nitrobluetetrazolium (NBT) which yields a chromogenic product, which is measured at 560 nm. Test solution (20-100  $\mu$ g/ml) in 0.1M phosphate buffer pH 7.4, 625  $\mu$ l of 468  $\mu$ M NADH solution, 625  $\mu$ l of 150  $\mu$ M NBT solution and 625 $\mu$ l of 60  $\mu$ M PMS solution were added to a test tube and incubated at room temperature for 5 min. The

absorbance was read at 560 nm. Linear graph of concentration Vs percentage inhibition was prepared and IC<sub>50</sub> values were calculated.

$$\% \text{ Superoxide radical scavenging activity} = \frac{\text{control} - \text{test}}{\text{control}} \times 100$$

#### ABTS Radical Scavenging Assay

ABTS radical cations were produced by reacting ABTS and APS. The ABTS scavenging capacity of the extract was compared with that of BHT and ascorbic acid and percentage inhibition calculated as For ABTS assay, the procedure followed with some modifications. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of  $0.706 \pm 0.01$  units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (20-100 µg/ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using a spectrophotometer.

$$\% \text{ ABTS radical scavenging activity} = \frac{\text{control} - \text{test}}{\text{control}} \times 100$$

#### Estimation of Total Phenolic Content

Assay used for the determination of total phenolics content employs Folin and Ciocalteu's phenol reagent which response depending on the chemical structure of phenolics (i.e. the higher the number of functional -OH group). Total soluble phenolic compounds in the ethanolic extracts were measured according to the method of Singleton *et al.* (1965) and expressed as gallic acid equivalents. A sample of the ethanolic extract was added to distilled water for a final volume of 2 ml. After, it was mixed with 0.3 ml of a saturated sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) solution and 0.1ml of 1N Folin-Ciocalteu's phenol reagent. The mixture was placed for 1 h at room temperature in the dark. The absorbance was measured at 725 nm against the blank. The total phenolic content was expressed as mg of gallic acid equivalents.

#### Estimation of Total Flavonoid Content

The amount of flavonoids present in the extracts of *Rhizophora mucronata* bark was estimated by Aluminium chloride colorimetric method (Zhishen *et al.*, 1999). A dilute concentration of extract (0.5ml) was mixed with 0.5ml of methanol, 4ml of water, 0.3ml of NaNO<sub>2</sub> (5%) and incubated for 5 min at room temperature. After incubation, 0.3ml of AlCl<sub>3</sub> (10%) was added and again incubated at room temperature for 6 min. Later, 2ml of 1M NaOH and 2.4ml of distilled water were added and the absorbance was measured at 510nm using UV-Visible spectrophotometer. A calibration curve was constructed using different concentrations of Catechin (0-120µg/ml) and the flavonoid content was expressed as µg Catechin equivalents (CE) from the graph.

#### Statistical Analysis

All data were expressed as Mean±Standard Deviation (SD) of the number of experiments (n =3). The IC<sub>50</sub> value was calculated using linear regression analysis of the percent inhibition obtained using different concentrations. The regression equation was obtained and the concentration required to produce 50% effect (IC<sub>50</sub>) was calculated.

## RESULTS AND DISCUSSION

Antioxidant capacity of the *R. mucronata* was eye-detected semi-quantitatively by a rapid DPPH staining TLC method. The results of dot-blot assay showed colored spots where the aqueous and alcoholic extracts of *R. mucronata* bark were dropped. This method was typically based on the inhibition of the accumulation of oxidized products. The generation of free radicals was inhibited by the addition of antioxidants and scavenging of the free radicals shifted the end point. The ascorbic acid and gallic acid were used as a positive control. Initial faint spots appeared, and 1 h later weak spots could be observed in sample row. This yellow spots with strong intensity appeared quickly at the concentration of 4µl of *R. mucronata* per application, and down to the dilution at 1µl of the *R. mucronata* (Figure 1). In the DPPH staining, the alcoholic fraction showed the highest strong dot-blot staining. The yellow area on the plate indicates free radical scavenging (antioxidant) activity. The more intense the yellow colour, the greater the antioxidant activity is as shown in Figure 1 and Figure 2. These results indicate that *R. mucronata* bark extracts have potential antioxidant activity. It was observed between the radical scavenging capacity and polarity of the extracts. The more polarable extracts give the more intense colour and the greater the antioxidant activity.



Fig. 1 - DPPH Dot assay

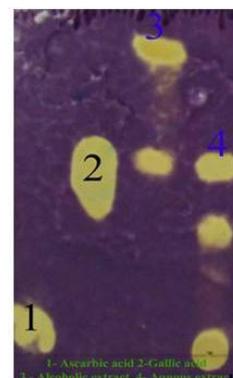


Fig. 2 - DPPH TLC assay

Figure 1 and 2: DPPH assay (Dot assay and TLC assay)

Various methods were employed to assess antioxidant properties of *R. mucronata* bark extracts. DPPH is one of the model systems and widely used to investigate the scavenging activities of several natural compounds such as phenolic and anthocyanins or crude mixtures (extracts) of plants. This assay determines the scavenging of stable radical species of DPPH by antioxidants. The antioxidant activity of phenolic compounds is due to their redox properties, which play an important role in absorption and neutralization of free radicals (Pietta *et al.*, 1998; Shahidi and Wanasundara, 1992; Kekuda *et al.*, 2013). DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. DPPH is one of the compounds that possess a proton free radical and showed a maximum absorption at 517 nm. When DPPH encounter proton radical scavengers, its purple color fades rapidly (Khushad *et al.*, 2003; Buhler and Miranda, 2000; Gooijer *et al.*, 1997). In DPPH radical scavenging assay, *R. mucronata* extracts showed dose dependent scavenging of DPPH radical with the reference ascorbic acid (Figure 2); DPPH radical with the IC<sub>50</sub> value of water extract was 110.85µg/ml and alcoholic extract was 59.63µg/ml while the IC<sub>50</sub> value for the reference ascorbic acid was 12.77µg/ml.

Reducing capacity of extracts was measuring by ferrous ion ( $Fe^{3+}$ ) to Ferric ion ( $Fe^{2+}$ ) conversion. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Yen and Chen, 1995). Aqueous extract (25.06%), alcoholic extract (17.87%) produced lower ferric reducing power when compared to standard which produced 87.5% inhibition at 100 $\mu$ g/ml concentration. The  $IC_{50}$  value of aqueous extract of *R. mucronata* bark is 193.47 $\mu$ g/ml alcoholic extract is 242.71 $\mu$ g/ml, the values were compared with the standard  $IC_{50}$  value of 21.5.

Scavengers of nitric oxide compete with oxygen and lead to reduced production of nitric oxide. In general physiological pH sodium nitroprusside spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions. These nitrite ions are estimated by making use of Griess reagent. Table 1 and 2 illustrates the percentage of inhibition of nitric oxide generation by the extracts of *R. mucronata*. All the extracts and standard chemicals produced 45.29–45.82% of nitric oxide production inhibition at 100  $\mu$ g/ml concentration.

In Superoxide radical scavenging assay, superoxide derived from dissolved oxygen by PMS/NADH coupling reaction and it reduces NBT. Color reduction indicated the

consumption of superoxide anion. Extracts of *R. mucronata* have been showed significant inhibition of superoxide anion generation. In general superoxide anion is generated by neutrophils during host microbe interaction. Extracts of *R. mucronata* showed best superoxide radical scavenging power. About 65% radical inhibition power was exhibited by both the extracts at 100  $\mu$ g/ml concentrations with 88.69 and 84.95  $IC_{50}$  values for aqueous and alcoholic extracts and 32.21 for ascorbic acid.

ABTS (2, 2'-azinobis-3-ethylbenzothiozoline- 6-sulphonic acid) assay is based on the scavenging of light by ABTS radicals (Arnao *et al.*, 2001). An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical, a process which is associated with a change in absorption which can be followed spectrophotometrically. The relatively stable ABTS radical has a green color and is quantified spectrophotometrically at 734nm. Table 1 and 2 illustrate the effect of plant extracts and ascorbic acid on converting  $ABTS^+$  to ABTs, which was measured at 734 nm. Both the extracts produced comparatively similar kinds of results (12.56 $\mu$ g/ml for aqueous extract, 4.21 $\mu$ g/ml for alcoholic extract)  $IC_{50}$  values of extracts.

**Table 1:** Antioxidant activity of aqueous extract of *R. mucronata* bark.

No	Concentration ( $\mu$ g/ml)	DPPH radical scavenging activity	Reducing power assay	Nitric oxide scavenging activity	Superoxide radical scavenging activity	ABTS radical scavenging activity
1	20	18.56 $\pm$ 0.82	2.87 $\pm$ 0.54	11.62 $\pm$ 1.17	9.48 $\pm$ 0.82	66.85 $\pm$ 0.52
2	40	27.87 $\pm$ 1.42	12.20 $\pm$ 0.69	19.20 $\pm$ 0.92	17.45 $\pm$ 0.87	78.30 $\pm$ 1.67
3	60	38.00 $\pm$ 1.50	18.32 $\pm$ 0.90	32.83 $\pm$ 1.03	27.60 $\pm$ 1.32	84.52 $\pm$ 1.16
4	80	41.65 $\pm$ 1.94	19.52 $\pm$ 1.36	36.96 $\pm$ 1.08	39.32 $\pm$ 0.82	88.37 $\pm$ 1.28
5	100	43.38 $\pm$ 1.41	25.06 $\pm$ 0.84	45.29 $\pm$ 1.00	63.42 $\pm$ 1.26	90.82 $\pm$ 0.38
<b><math>IC_{50}</math></b>		<b>110.85</b>	<b>193.47</b>	<b>109.06</b>	<b>88.69</b>	<b>12.56</b>

**Table 2:** Antioxidant activity of Alcoholic extract of *R. mucronata* bark.

No	Concentration ( $\mu$ g/ml)	DPPH radical scavenging activity	Reducing power assay	Nitric oxide scavenging activity	Superoxide radical scavenging activity	ABTs radical scavenging activity
1	20	18.32 $\pm$ 0.85	1.43 $\pm$ 0.25	9.62 $\pm$ 1.03	19.46 $\pm$ 0.77	78.28 $\pm$ 1.16
2	40	41.95 $\pm$ 2.17	5.01 $\pm$ 0.30	26.64 $\pm$ 0.79	24.37 $\pm$ 1.24	80.37 $\pm$ 1.19
3	60	54.08 $\pm$ 1.70	7.47 $\pm$ 1.06	32.37 $\pm$ 0.83	30.19 $\pm$ 0.80	82.42 $\pm$ 1.002
4	80	59.57 $\pm$ 1.07	16.34 $\pm$ 0.80	41.61 $\pm$ 0.86	42.10 $\pm$ 1.70	85.97 $\pm$ 1.75
5	100	70.07 $\pm$ 1.69	17.87 $\pm$ 0.93	45.82 $\pm$ 0.93	65.55 $\pm$ 1.02	93.50 $\pm$ 0.84
<b><math>IC_{50}</math></b>		<b>59.63</b>	<b>242.71</b>	<b>103.21</b>	<b>84.95</b>	<b>4.21</b>

Phenolic compounds are well known powerful chain breaking antioxidant (Shahidi, and Wanasundara, 1992) and these phenols are very important plant constituents because these medicinal plants contain scavenging ability due to their hydroxyl group (Hatano *et al.*, 1989). These compounds stabilize the lipid oxidation and are associated with antioxidant activity. Total phenolic compound of the *R. mucronata* aqueous extract was 80 $\mu$ g/mg and alcoholic extract was 360 $\mu$ g/mg which is equivalent to gallic acid. This is one of the compounds responsible for antioxidant activity. The flavonoid content was estimated by aluminium chloride colorimetric estimation method and the content of total flavonoids was estimated in terms of  $\mu$ g CE/mg of dry extract. The bark alcoholic extract of *R. mucronata* contained higher flavonoid content (38 $\mu$

CE/mg) than aqueous bark extract of *R. mucronata* (10.23 $\mu$ g CE/mg).

## CONCLUSION

The aqueous and alcoholic extracts of bark were shown to exhibit marked *in vitro* antioxidant activity. However, the chemical constituents present in the extract, which are responsible for this activity, need to be investigated, and it is obvious that the constituents like tannins, reducing sugars and proteins present in the extract may be responsible for such activity. The phytochemical tests indicated the presence of alkaloids, glycosides, tannins, and flavonoids in the crude ethanolic extract. These properties may be due to its antioxidant activity. The crude ethanolic extract merits further experiments *in vivo*.

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