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# Determination of *in-vitro* antioxidant activity of leaf extracts of *Rhizophora mucronata* Lam. (Rhizophoraceae)

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**Abstract**
The present study was conducted to evaluate the antioxidant activity of potential medicinal plant *Rhizophora mucronata*. Shade dried leaves of *R. mucronata* was powdered and extracted with various solvents by cold maceration method. Antioxidant activity of *R. mucronata* was determined by using different methods namely DPPH, hydroxyl radical scavenging activity, superoxide radical scavenging activity, ABTS radical scavenging activity and reducing power assays, and its $IC_{50}$ values were found. The extracts exhibited marked dose dependent *in-vitro* antioxidant activity. The results indicated that the methanol extract is the most potent antioxidant. This holds great promise for the use of *R. mucronata* as a source of strong antioxidant compounds.

**Keywords:** Medicinal plant, pharmacological properties, antioxidant activity, DPPH, ABTS

## Introduction
Free radicals in the living cell may occur due to various environmental factors such as ultraviolet radiation, chemical reactions and some metabolic processes. Accumulations of them cause initiate deterioration of biomolecules such as proteins, lipids, carbohydrates and nucleic acids and are implicated in several diseases such as cardiovascular diseases, aging, cancer, atherosclerosis, Parkinson's disease, Alzheimer's disease, inflammatory diseases. The oxidative stress results when the balance between the generation of ROS and antioxidant defense system of the body is disturbed. The free radicals generated in the body are neutralized by the body's natural antioxidant defenses, e.g. glutathione, glutathione peroxidase, catalase, and superoxide dismutase. Synthetic antioxidants have been shown to possess carcinogenic activity, which leads to a need for the replacement of synthetic antioxidant with naturally occurring ones. Therefore, there is a necessity for development of safer antioxidants particularly from natural sources. In recent days, researchers mainly focused on natural oxidants and their health benefits. Plants generate a variety of antioxidant compounds as protection to reactive oxygen species (ROS) and free radicals. Plants are the main source of natural antioxidants in the form of phenolic compounds (phenolic acids, flavonoids and polyphenols). Most of the anti-inflammatory, digestive, antinecrotic, neuroprotective and hepatoprotective drugs derived from natural origin have been reported to have antioxidant/radical scavenging mechanism as part of their activity [1].
*Rhizophora mucronata* Lam. (Rhizophoraceae), a true mangrove, is widely distributed along the delta of Indian Sunderbans (21°32 and 22°40 N and between 88°05 and 80°00 E) [2]. The bark, root, leaves, fruits and flowers of *R. mucronata* have been traditionally used as medicine in the coastal areas of Asian subcontinents for treating health ailments such as diabetes [3, 4], diarrhea [5], hepatitis [6], inflammation [7], and cognitive function [8]. The perspective of its use as anti-diabetic medicine was supported with numerous scientific reports, but more information is still required [9, 10]. Chemical identity of *R. mucronata* has also been carried out, and the presence of secolabdane diterpenoid (rhizophorin A) [11], phomoxanthone [12], lupiol, beta-sitosterol [13], gallic acid, coumarin, quercetin [14] and tannins [15]. The current study, the crude extracts of *R. mucronata* were investigated for their antioxidant properties of super oxide radical scavenging activity, ABTS radical scavenging activity, DPPH scavenging activity, hydroxyl radical & reducing power assays.

## Materials and methods
### Collection of plants
The fresh plant parts of *R. mucronata* (leaves) were collected from Manakudi, Tirunelveli District, Tamil Nadu, India.

The gathered samples were cut into small pieces and shade dried until the fracture is identical and even. The dried plant material was crushed or grinded by using a blender and separated to get uniform particles by using sieve No. 60. The final uniform powder was used for the extraction of active constituents of the plant material.

**Preparation of extract**

100 g of the coarse powder of *R. mucronata* leaves was extracted successively with 250 ml of alcoholic and organic solvents (Peroleum ether, Benzene, Ethyl acetate, Methanol and Ethanol) in a Soxhlet apparatus for 24 hrs. All the extracts were filtered through Whatman No. 1 filter paper independently and all the extracts were deliberated in a rotary evaporator. All the concentrated extracts were subjected for *in vitro* antioxidant activity.

**Antioxidant activity**

**DPPH radical scavenging activity**

The DPPH is a constant free radical and is extensively used to measure the radical scavenging activity of antioxidant component. This process is based on the reduction of DPPH in methanol solution in the company of a hydrogen donating antioxidant due to the arrangement of the non-radical form DPPH-H [16]. The free radical scavenging action of all the extracts was assessed by 1, 1- diphenyl-2-picryl-hydrazyl (DPPH) as per the previously reported process. 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was poured into 3 ml of the solution at dissimilar concentrations (50, 100, 200, 400 and 800 µg/ml). The mixtures were shaken dynamically and allowed to stand at room temperature for 30 minutes. After that the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10s UV: Thermo electron corporation). Ascorbic acid was employed as the reference. The lower absorbance values of reaction mixture indicate higher free radical scavenging action. Using the subsequent formula the ability to scavenge the DPPH radical was found out.

DPPH scavenging activity (% inhibition) = (A0 - A1) / A0 X 100

Where, A0 is the absorbance of the control and A1 is the absorbance of the test samples and reference. All the tests were carried out in triplicates and the outcomes were averaged.

**Hydroxyl radical scavenging activity**

The scavenging ability for hydroxyl radical was estimated according to the modified method of Halliwell *et al.* [17]. Stock answers of $FeCl_3$ (10 mM), ascorbic acid (1 mM), EDTA (1 mM), H2O2 (10 mM) and deoxyribose (10 mM) were put in distilled deionized water. The assay was executed by adding 0.1 ml EDTA, 0.1 ml $H_2O_2$, 0.36 ml of deoxyribose, 0.01 ml of FeCl3, 1.0 ml of the extract of unlike concentration (50, 100, 200, 400 & 800 µg/ml) dissolved in distilled water, 0.1 ml of ascorbic acid, 0.33 ml of phosphate buffer (50 mM, pH 7.9), in sequence. The mixture was then protected at 37 °C for 1 hour. 1.0 ml of the incubated mixture was mergeed with 1.0 ml of 10% TCA and 1.0 ml of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532 nm. The hydroxyl radical scavenging action of the extract is accounted as% inhibition of deoxyribose. The degradation is calculated by using the following equation

Hydroxyl radical scavenging activity = (A0 - A1) / A0 X 100

where, A0 is the absorbance of the control and A1 is the absorbance of the test samples and reference. All the tests were carried out in triplicates and the results were averaged.

**Superoxide radical scavenging activity**

The superoxide anion scavenging activity was computed as explained by Srinivasan *et al.* [18]. The superoxide anion radicals were generated in 3.0 ml of Tris - HCl buffer (16 mM, pH 8.0) containing 0.5 ml of NBT (0.3 mM), 0.5 ml NADH (0.936 mM) solution, 1.0 ml extract of different concentrations (50, 100, 200, 400 & 800 µg/ml) and 0.5 ml Tris - HCl buffer (16 mM, pH 8.0). The reaction was started by adding 0.5 ml PMS solution (0.12 mM) to the mixture, incubated at 25ºC for 5 min and the absorbance was measured at 560nm against a blank sample, ascorbic acid. The percentage inhibition was computed by using the subsequent equation:

Superoxide radical scavenging activity = (A0 - A1) / A0 X 100

where, A0 is the absorbance of the control and A1 is the absorbance of the test samples and reference. All the tests were performed thrice and the results were averaged.

**Antioxidant Activity by Radical Cation (ABTS+)**

ABTS assay was based on the modified method of Huang *et al.* [19]. By reacting 7 mM ABTS solution with 2.45 mM potassium persulphate, ABTS radical cation (ABTS+) was prepared. This mixture is permitted to be in the dark at room temperature for 12-16 hrs previous to use. With ethanol to an absorbance of 0.70 + 0.02 at 734 nm the ABTS+ solution was added. Following this trolox standard to 3.9 ml of diluted ABTS+ solution or addition of 100 µL of sample, absorbance was calculated at 734 nm by Genesys 10S UV-VIS (Thermo scientific) accurately after 6 minutes. Results were stated as trolox equivalent antioxidant capacity (TEAC).

ABTS radical cation activity = (A0 - A1) / A0 X 100

where, A0 is the absorbance of the control and A1 is the absorbance of the test samples and reference. All the tests were executed in triplicates and the end results were averaged.

**Reducing Power**

The reducing power of the extract was found by the method of Kumar and Hemalatha [20]. 1.0 ml of solution holding 50, 100, 200, 400 & 800 µg/ml of extract was mixed with sodium phosphate buffer (5.0 ml, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 ml, 1.0%). The mixture was incubated at 50º C for 20 minutes. Then 5ml of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5ºC) in a refrigerated centrifuge. The upper layer of the solution (5.0 ml) was diluted with 5.0 ml of distilled water and ferric chloride and absorbance read at 700 nm. The experiment was performed thrice and the average of the results was found.

**Results and Discussion**

The properties showing antioxidant quality of *R. mucronata* have been evaluated by measuring their DPPH, ABTS, superoxide radical, hydroxyl radical, reducing ability and ascorbic acid contents using crude extracts aerial parts of this plant. Antioxidant potential of the crude extracts was measured by DPPH radical scavenging activity. DPPH is a protonated radical showing the characteristic absorption maxima at 517 nm which reduces with the scavenging of the

proton radical by normal plant extracts. Hence, DPPH finds applications in the determination of the radical scavenging activity of plant materials [21]. In this study, results showed that all extracts had significant levels of radical scavenging activity in a dose dependent manner (Fig. 1). The DPPH-derived $IC_{50}$ values of the plant extracts are also illustrated in Table 1. Methanol extract showed high antioxidant activity than that of other extracts. The concentration of *R. mucronata* leaves methanol extract needed for 50% inhibition ($IC_{50}$) was 36.17 mg/ml, while ascorbic acid needed 31.04 mg/ml. The result obtained in the study indicates that the extract exhibited good radical scavenging activity but was to a lesser extent compared to standard ascorbic acid.

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage [22]. They were produced in this study by incubating ferric-EDTA with ascorbic acid and $H_2O_2$ at pH 7.4, and reacted with 2-deoxy-2-ribose to generate a malondialdehyde (MDA) like product. This compound forms a pink chromogen upon heating with TBA at low pH (Halliwell *et al*., 1987). When *R. mucronata* extract was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction (Figure 2). The concentration of *R. mucronata* leaves methanol extract needed for 50% inhibition ($IC_{50}$) was found to be 32.56 mg/ml, whereas 30.47 mg/ml (Table 1) needed for ascorbic acid. The result obtained in the study indicates that the methanol extract showed significant OH radical scavenging activity compared to standard Ascorbic acid (Figure 2).

The anion superoxide is one of the most delegate free radicals. In cellular oxidation reactions, superoxide radicals have their initial effects magnified because they produce other kinds of cell-damaging free radicals and oxidizing agents, e.g.,radi hydroxyl radicals [23]. Superoxide radical scavenging capacities of the crude extracts tested varied from 20.86 to 124.16% which represents a variation of standard ascorbic acid. Methanol extract showed the highest antioxidant capacity (146.18%) followed by ethanol (123.92%) and ethyl acetate (111.76%). In this assay, benzene (103.41%) showed the lowest antioxidant potential (Figure 3). The $IC_{50}$ value of methanol extract of *R. mucronata* leaves on superoxide radical was found to be 36.47 mg/ml and 32.14 mg/ml for ascorbic acid, respectively (Table 1).

The relative antioxidant capacity to scavenge the radical ABTS+ has been compared with the standard Trolox. ABTS radical cation was created in the stable form using potassium persulphate. After getting the stable absorbance, the antioxidant plant extract is added to the reaction medium and the antioxidant power was measured by studying decolorization. The different solvent extracts of *R. mucronata* leaves were subjected to be ABTS radical cation scavenging activity and the results are shown in figure 4. The methanol extract of *R. mucronata* leaves exhibited potent ABTS radical cation scavenging activity in concentration dependent manner. At 800µg/ml concentration, the methanol extract of *R. mucronata* leaves possessed 139.65%**.** The quantity of *R. mucronata* methanol extract required to produce 50% inhibition of ABTS radical 34.84 mg/ml whereas 31.47 mg/ml (Table 1) needed for trolox.

Reducing capability of a presumed antioxidant can be assessed by means of its ability to convert Fe3+ to Fe2+. Intensity of Perl's Prussian blue caused by this reduction is measured at 700 nm; a higher absorbance shows higher reducing power. Then, reducing power of the compound can be supplied to its antioxidant potency. The reducing power values of the plant extracts tested in this study are illustrated in Fig. 5. As can be observed from the graph, the methanol extract showed a reducing power nearly equal to that of Ascorbic Acid. The ethanol extract showed a very good reducing power at lower concentrations. However, at higher concentrations, its reducing power was lesser than that of the ethanol extract.

In addition to those pharmaceutical properties of *R. mucronata* reported in the literature, this research showed that leaves of this plant may possess considerable antioxidant activities compared to the rest of the medicinal plants as well as Trolox and ascorbic acid (as positive controls). Thus, further research may be warranted to study active compounds of *R. mucronata* that confer the antioxidant activity. The results presented here might have inferences in the population diet and disease avoidance through diet.

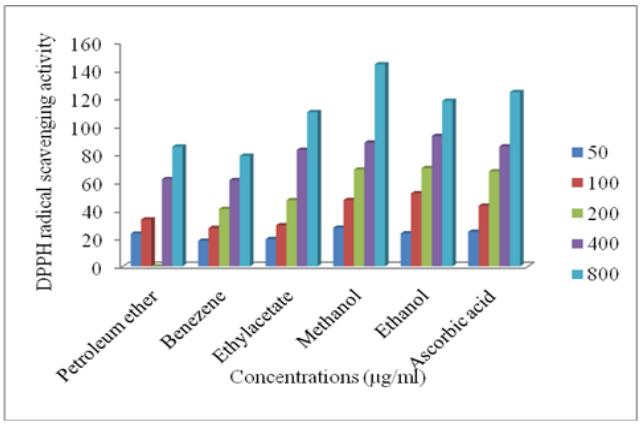


**Fig 1:** Effect of different solvent extract of *R. mucronata* leaf on DPPH assay

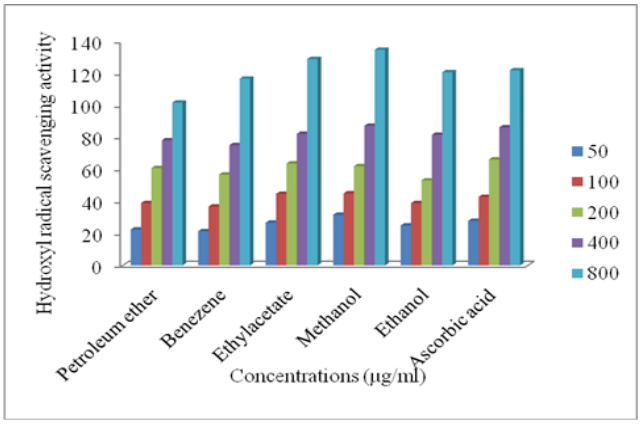


**Fig 2:** Effect of different solvent extract of *R. mucronata* leaf on Hydroxyl assay

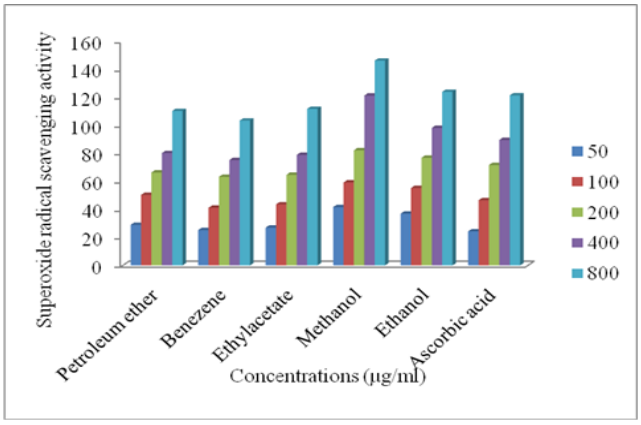


**Fig 3:** Effect of different solvent extract of *R. mucronata* leaf on Superoxide anion assay

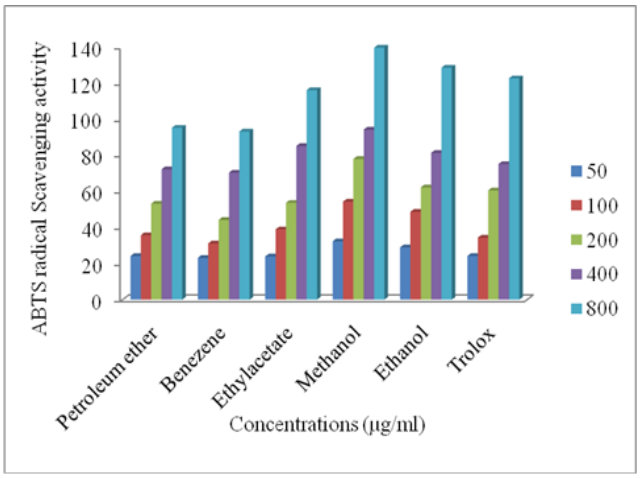


**Fig 4:** Effect of different solvent extract of *R. mucronata* leaf on ABTS assay

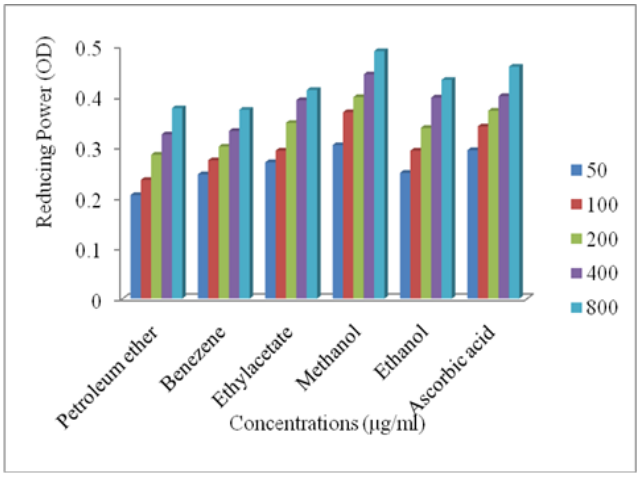


**Fig 5:** Effect of different solvent extract of *R. mucronata* leaf on reducing power assay

**Table 1:** $IC_{50}$ values of different solvent extracts of the leaf extracts of *R. mucronata*

| Solvents | $IC_{50}$ (µg/ml) | | | |
|---|---|---|---|---|
| | DPPH | Hydroxyl | ABTS | Superoxide anion |
| P.ether | 23.56 | 27.12 | 27.45 | 29.27 |
| Benzene | 21.47 | 28.56 | 26.89 | 28.45 |
| Ethyl acetate | 28.55 | 32.94 | 29.13 | 30.47 |
| Methanol | 36.17 | 32.56 | 34.84 | 36.47 |
| Ethanol | 30.89 | 29.84 | 32.46 | 33.12 |
| Ascorbic acid | 31.04 | 30.47 | - | 32.14 |
| Trolox | - | - | 31.47 | - |

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