International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.11 No.05, pp 201-208, 2018

Antioxidant Activity of Extract of *Rizophora mucronata* leaf

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Abstract : Objective: To evaluate antioxidant activity of *Rhizophora mucronata* **Methods:** antioxidant activity was evaluated by using Ferric reducing activity (FRAP assay), Reducing power activity, Metal chelating activity, Inhibition of peroxides in linoleic acid system, DPPH radical-scavenging activity, Superoxide radical-scavenging activity, Hydrogen peroxide scavenging activity, Nitric oxide radical scavenging activity **Results:** In this present study, different models of antioxidant assays were employed, which could provide a more consistent approach to assess antioxidant and radical scavenging potential of leaves of *R. mucronata*. **Conclusion:** The result obtained in the study led to the conclusion that leaves of the mangrove plant, high level of polyphenolics and show significant antioxidant activity and radical scavenging activity.
Keywords : Antioxidant activity, *Rhizophora mucronata*, Free radical scavenging activity.

1. Introduction

India has a rich culture of medicinal herbs and spices, which includes about more than 2000 species and has a vast geographical area with high potential abilities for Ayurvedic, Unani, Siddha traditional medicines but only very few have been studied chemically and pharmacologically for their potential medicinal value[1, 2] Human beings have used plants for the treatment of diverse ailments for thousands of years [3, 4]. According to the World Health Organization, most populations still rely on traditional medicines for their psychological and physical health requirements [5], since they cannot afford the products of Western pharmaceutical industries [6], together with their side effects and lack of healthcare facilities [7]. Rural areas of many developing countries still rely on traditional medicine for their primary health care needs and have found a place in day-to-day life. These medicines are relatively safer and cheaper than synthetic or modern medicine[8]. People living in rural areas from their personal experience know that these traditional remedies are valuable source of natural products to maintain human health, but they may not understand the science behind these medicines, but knew that some medicinal plants are highly effective only when used at therapeutic doses [9, 10]

International Journal of ChemTech Research, 2018,11(05): 201-208.

DOI= http://dx.doi.org/10.20902/IJCTR.2018.110523

2. Materials and Methods

2.1 Plant Materials and Preparation of Extracts

Rhizophora mucronata a mangrove plant was collected from the inter tidal area of the Neil Island, Port Blair, A& N Islands, India. The collected mangrove leaves were processed on the same day itself. The leaves were washed thoroughly with distilled water and freeze dried. The dried samples were ground to powder and stored in air tight at – 20 °C until further analysis. The powdered leaf material was soaked in the different solvents of varying polarity such as methanol, acetone and ethyl acetate, at room temperature for 24 h with mass to volume ratio of 1:40 (g/ml). The solvents were filtered through Whatman No. 1 filter paper to remove the solid particles. The filtered solvents were evaporated to dryness under vacuum on a rotary evaporator at 40°C. Water extract of R. mucronata was prepared as above by soaking dried powder in distilled water and stirred using a magnetic stirrer at a low speed for 24h.

2.2 Chemicals and Reagents:

All chemicals and reagents used in the experiments were of analytical grade

2.3 In-Vitro Antioxidant Activity of *R.Mucronata*

2.3.1 Ferric reducing activity (FRAP assay)

The FRAP assay was done according to the method described by Benzie and Strain (1999) with some modification. This method is based on reduction of TPTZ-Fe^{3+} complex to TPTZ-Fe^{2+} form in the presence of antioxidants. The stock solutions included acetate buffer (300 mM, pH 3.6), 2, 4, 6-tripyridyl s-triazine (TPTZ) solution (10 mM in 40 mMHCl) and ferric chloride ($FeCl_3.6H_2O$) solution (20 mM). The fresh working FRAP solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution and 2.5 ml $FeCl_3$ solution. Extracts were made up to 2.0 ml with distilled water and 1.0 ml of FRAP solution was added. An intense blue color developed was measured at 593 nm, after an incubation period of 20 min. The absorbance was related to absorbance changes of a ferrous sulphate solution (0 – 100 µM) tested in parallel. All results were based on three separate experiments and antioxidant capacity was expressed as µM $FeSO_4$/ g of dry extract. Quercetin and ButylatedHydroxy Toluene (BHT) were used as positive control

2.3.2. Reducing power activity

The reducing power of extracts was evaluated according to the method described by Yen and Chen (1995) with slight change. Briefly, different amounts of *R. mucronata* extracts (0.05 – 1.0 mg/ml) were incubated with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$] at 50 °C for 20 min. The reaction was terminated by adding 2.5 ml of 10% TCA solution and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (1.0 ml) was mixed with 2.5 ml of distilled water and 1.0 ml of 0.1% ferric chloride ($FeCl_3$) solution and absorbance was measured at 700 nm after incubation at room temperature for 10 min. Quercetin and BHT (10 – 250 µg/ml) were used as positive control. Experiments were performed in triplicate.

2.3.3 Metal chelating activity

The chelating capacity of *R. mucronata* extracts on Fe^{2+} ions was determined according to the method of Dinis et al (1994), wherein Fe^{2+} chelating potential of extracts was monitored by measuring ferrous iron – ferrozine complex at 562 nm. Briefly, extracts (0.05 – 1.0 mg/ml), quercetin, BHT and EDTA (10 – 250 µg/ml) were made up to 4.7 ml with distilled water and then mixture was allowed to react with 0.1 ml of Ferrous chloride (2.0 mM) and 0.2 ml of ferrozine (5.0 mM) for 20 min. Absorbance of mixture was measured at 562 nm against a blank, which contained distilled water, instead of extracts/EDTA/standard antioxidants.

The ability of extracts to chelate ferrous ion was calculated using the following equation

$$\text{Chelating effect } (\%) = \left[\frac{\text{AbControl } 562 - \text{Ab Sample } 562}{\text{Ab Sample } 562}\right] \text{X } 100$$

2.3.4 Inhibition of peroxides in linoleic acid system

The capacity of *R. mucronata* extracts to inhibit formation of peroxides in linoleic acid system was determined according to the thiocyanate method (Osawa and Namiki, 1981). This method is based on the capacity of peroxides to catalyze oxidation of Fe^{2+} to Fe^{3+}. The Fe^{3+} produced is linked to thiocyanate anion, yielding a red complex, which is measured spectrophotometrically at 500 nm. The linoleic acid emulsion was prepared by mixing 0.284 g of linoleic acid and 0.284 g of Tween 20 as an emulsifier and made up to 50 ml with phosphate buffer (0.04 M, pH 7.0). The reaction was set up by mixing *R. mucronata* extracts, quercetin and BHT (250 µg/ml) in 2.5 ml phosphate buffer (0.04 M, pH 7.0) with 2.5 ml of linoleic acid emulsion. It was then incubated in the dark at 37°C. At regular intervals during incubation, degree of oxidation was measured by sequentially adding 4.7 ml of ethanol (75%), 0.1 ml of ammonium thiocyanate (30%) and 0.1 ml of ferrous chloride (0.02 M in 3.5% HCl) to 0.1 ml of sample solution. After incubation at room temperature for 3 min, peroxide level was determined by measuring absorbance at 500 nm. A control was performed with linoleic acid emulsion without extracts or standards. The increase in absorbance is directly proportional to the concentration of peroxides present in the samples

2.3.5 DPPH radical-scavenging activity

DPPH radical-scavenging activity of *R. mucronata* extracts was determined as previously described (Burits and Bucar, 2000). The capacity of extracts to scavenge lipid- soluble 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, which results in bleaching of purple color exhibited by stable DPPH radical, is monitored at an absorbance of 517 nm. Briefly, 1.0 ml of extracts (0.05 – 1.0 mg/ml) and quercetin/BHT (10 – 250 µg/ml) in ethanol were added to 4 ml of 0.004% methanolic solution of DPPH. After incubation for 30 min at room temperature in the dark, absorbance was read against a blank at 517 nm. Tests were carried out in triplicate.

The ability of extracts and quercetin/BHT to scavenge DPPH radical was calculated using the following equation

$$\text{Radical scavenging activity (\%)} = \left[\frac{A_0 - A_1}{A_1}\right] X\ 100$$

Where A_0 was absorbance of negative control (containing all reagents except test compounds) at 517 nm and A_1 was absorbance of the extracts or quercetin/BHT at 517 nm. DPPH scavenging activity of extracts and standard was expressed as IC_{50}, which was interpolated from a graph constructed, using percent inhibition (Y-axis) against concentration (X-axis) of extracts and standards.

2.3.6 Superoxide radical-scavenging activity

The ability of *R. mucronata* extracts, quercetin and BHT to quench generation of superoxide radicals was determined according to the method of Nishikimi et al (1972) with a slight change. Superoxide radicals were generated in PMS-NADH system by oxidation of NADH and analyzed by NBT reduction. 0.1 ml of extracts (0.05 – 1.0 mg/ml) and quercetin/BHT (10 – 250 µg/ml) were mixed with 2.9 ml of phosphate buffer (40 mM, pH 7.4), 1.0 ml of nitrobluetetrazolium (NBT) solution (150 µM in 40 mM phosphate buffer, pH 7.4) and 1.0 ml of NADH (468 µM in 40 mM phosphate buffer, pH 7.4). The reaction was initiated by addition of 1.0 ml of phenazinemethosulphate (60 µM in 40 mM phosphate buffer, pH 7.4) to reaction mixture. After incubation at 25°C for 5 min, absorbance was measured at 560 nm. Negative control was subjected to the same procedures as extracts, except that only solvent was added for negative control. All measurements were made in triplicate.

The ability of extracts and quercetin/BHT to scavenge superoxide radical was calculated using the following equation

$$\text{Superoxide radical scavenging activity (\%)} = \left[\frac{A_0 - A_1}{A_1}\right] X\ 100$$

Where $A_0$was absorbance of negative control at 560 nm and A_1 was absorbance of the extracts or quercetin/BHT at 560 nm. IC_{50} value, which represents concentration of extracts and standards that caused 50% inhibition, was determined by a linear regression analysis.

2.3.7 Hydrogen peroxide scavenging activity

The method of Sinha (1972) originally designed for the estimation of antioxidant enzyme, catalase, was adopted to evaluate hydrogen peroxide scavenging effect of *R. mucronata* extracts. Extracts (0.05 – 1.0 mg/ml) and quercetin/BHT (10 – 250 µg/ml) were incubated with 0.6 ml of H_2O_2 (40 mM in a phosphate buffer, 0.1 M pH 7.4) in the dark for 10 min. A negative control was set up in parallel with entire reagent except extract or standard. After incubation, remaining H_2O_2 was allowed to react with 1.0 ml of dichromate in acetic acid (5% potassium dichromate and glacial acetic acid in the ratio of 1:3) in a boiling water bath for 10 min. Absorbance of green color developed was determined at 620 nm. All experiments were performed in triplicate.

The percentage scavenging of H_2O_2 by *R. mucronata* extracts and standards were calculated using the following equation

$$\text{H2O2 scavenging activity (\%)} = \left[\frac{A_0 - A_1}{A_1}\right] X\ 100$$

Where A_0 was absorbance of negative control and A_1 was absorbance of the extracts or standards. H_2O_2 scavenging activity of extracts and standards was expressed as IC_{50}, which was interpolated from a graph constructed using percent inhibition (Y-axis) against concentration (X-axis) of extracts and standards.

2.3.8 Nitric oxide radical scavenging activity

The method of Rai et al (2006) based on spontaneous generation of nitric oxide (NO•) from sodium nitroprusside (SNP)-buffered solution was used to assess NO• scavenging ability of R. mucronata extracts. Briefly, 0.5 ml of SNP (10 mM) in phosphate buffered-saline was mixed with 0.5 ml of R. mucronata extracts (0.05 – 1.0 mg/ml) and incubated in the dark at room temperature for 2.5 h. A control was set up as above, but sample was replaced with same amount of water. After incubation, 1.0 ml of sulfanilic acid reagent (0.33 % sulfanilic acid in 20 % glacial acetic acid) was added to 0.5 ml of reaction mixture. After 5 min, reaction mixture was incubated further with 1.0 ml 0.1 % naphthylethylenediaminedihydrochloride (NEDD) for 30 min at 25 °C. Absorbance of chromophore formed was read at 540 nm. Results were expressed as a percent of scavenged nitric oxide with respect to negative control. Quercetin and BHT (10 – 250 µg/ml) were used as positive control. All analyses were done in triplicate.

The percentage scavenging of NO• by *R. mucronata* extracts and standards were calculated using the following equation

$$\text{NO} \bullet \text{ scavenging activity (\%)} = \left[\frac{A_0 - A_1}{A_1}\right] X\ 100$$

Where A_0 was absorbance of negative control and A_1 was absorbance of extracts or standards. NO• scavenging activity of extracts and standard was expressed as IC_{50}, which was interpolated from a graph constructed using percent inhibition (Y-axis) against concentration (X-axis) of the extracts and standards.

3. Results

3.1 Antioxidant Properties Of *R. Mucronata*

Several mechanisms have been proposed to be involved in antioxidant activity such as hydrogen donation, termination of free radical mediated chain reaction, prevention of hydrogen abstraction, chelation of catalytic ions and elimination of peroxides (Gordon, 1990). Antioxidant activity is system- dependent and characteristic of a particular system can influence outcome of analysis. Hence, a single assay would not be representative of antioxidant potential of plant extracts. In this present study, different models of antioxidant assays were employed, which could provide a more consistent approach to assess antioxidant and radical scavenging potential of leaves of *R. mucronata*.

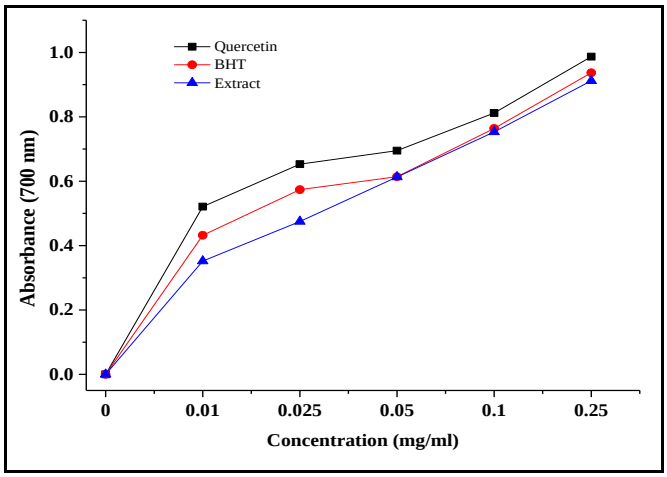

Figure 1: Ferric reducing ability of *R. mucronata* along with standard

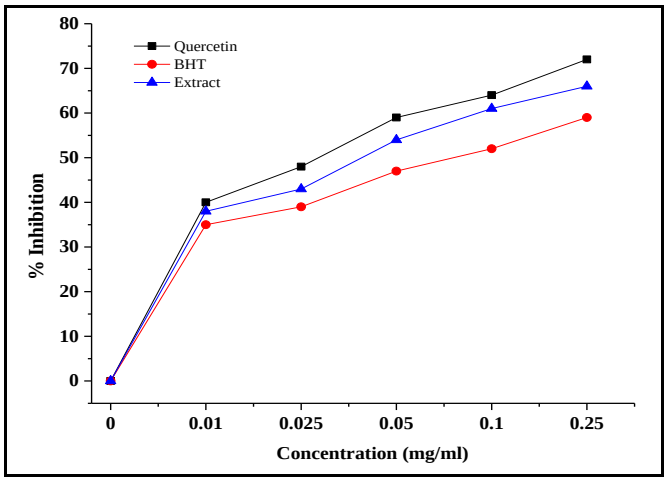

Figure 2: Metal chelating ability of *R. mucronata* along with standard

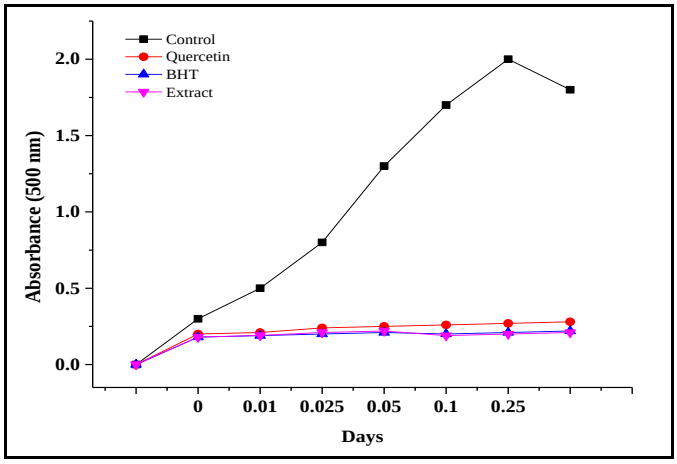

Figure 3: Inhibition of linoleic acid peroxidation by *R. mucronata* extract

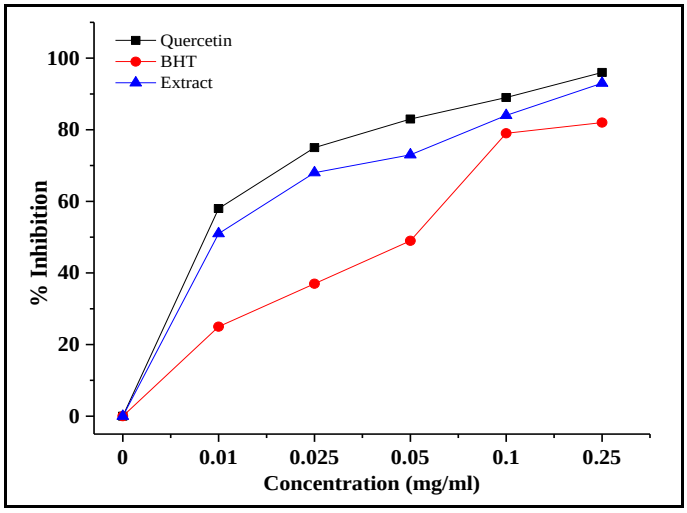

Figure 4: DPPH radical scavenging activity of R. *mucronata* extract

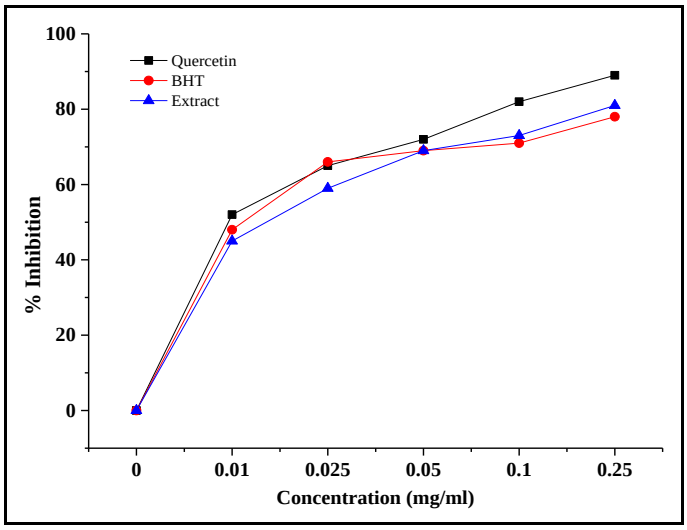

Figure 5: Superoxide radical scavenging activity of *R. mucronata* extract

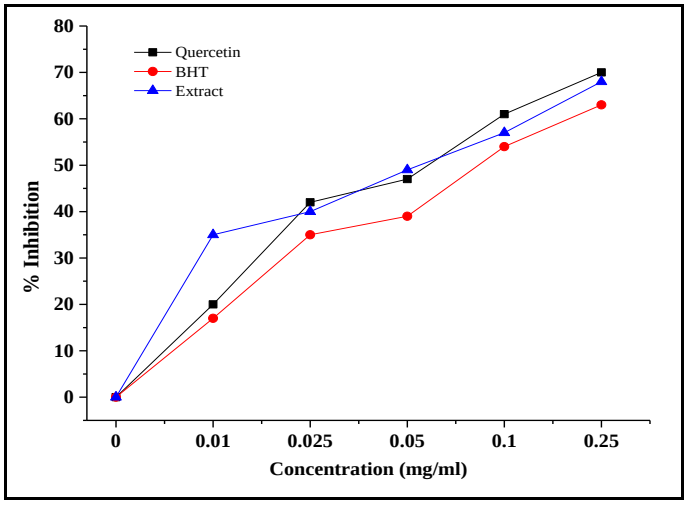

Figure 6: Hydrogen peroxide scavenging activity of *R. mucronata* extract

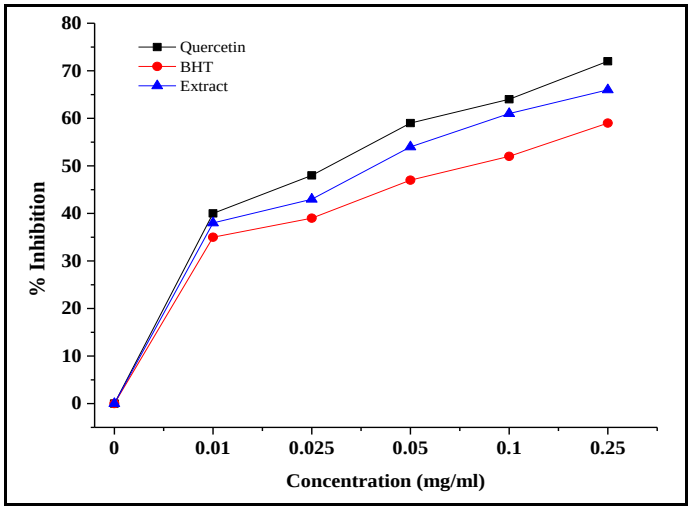

Figure 7: Nitric oxide scavenging activity of *R. mucronata* extract

Findings from this study suggest that *R. mucronata* extracts are able to neutralize superoxide radicals, H2O2 and NO radicals by acting as chain-breaking antioxidants in a dose-dependent manner. Even though, superoxide radicals, H_2O_2 and NO radicals are weak oxidizing agents, they could generate potentially reactive oxygen and nitrogen species such as singlet oxygen, hydroxyl radicals and peroxynitrite. These reactive species are believed to act as inducers of cellular injury through initiation of lipid peroxidation, oxidation of proteins and induction of DNA strand breaks (Halliwell, 1991). Several studies have reported relationship between polyphenolics structure and antioxidant activity, demonstrating that polyphenolics possessing hydroxyl groups on their phenyl rings effectively contribute to chain-breaking antioxidant activity by stabilizing radical form in electron delocation (Rice-Evans, 1995). Among polyphenolics detected in *R. mucronata*, many have hydroxyl groups in their structure, which would make it possible to inhibit free radical-induced chain reactions and thus, could contribute significantly to antioxidant and radical scavenging activity of *R. mucronata*. Furthermore, antioxidant and radical scavenging activity are outcome of combination of diverse polyphenolics having synergistic and/or additive effects.

4. Conclusion

The result obtained in the study led to the conclusion that leaves of the mangrove plant showed significant antioxidant activity and radical scavenging activity based on this study

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