International Journal of
Environmental Health and Technology

GREENS TRUST

Antıoxıdant and Free Radıcal Scavengıng Actıvıty of Crude Extract of *Rhizophora Mucronata* Leaves

Received date:11th August-2017, Accepted date: 20th October- 2017

ISSN: 2581-4516 ***(online)***

www.greenstrust.com/ijeht/

Asbin Mary X[1], Syed Ali1[2*], Anuradha V[2], Yogananth N[1]

Abstract: Rhizophora mucronata belongs to family Rhizophoraceae and commonly known as the loop root mangrove, red mangrove and Asiatic mangrove. They have been known to posses secondary metabolites that are useful against cancer and they are used in folklore medicine. Four different extracts of Rhizophora mucronata were evaluated for antioxidant and free radical scavenging activity. In the present study, the free radical scavenging potential of four extracts of Rhizophora mucronata was assessed for scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, hydrogen peroxide radical, 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)(ABTS) radical, Deoxy ribose, β-carotene, Lipid per oxidization, superoxide radical, superoxide dismutase, ferric reducing antioxidant. Total antioxidant capacity and phenolic, flavonoid contents were determined spectrophotometrically. It was found that methanol and ethanol extract shows noticeable effect in DPPH scavenging assay, ABTS scavenging assay, Deoxy ribose assay, Hydrogen per oxide scavenging assay, superoxide radical scavenging assay. Methanol and ethanol extract shows significant activity in total phenolic content and total flavonoids content. Diethyl ether and ethyl acetate extracts shows least activity in all the measures. The finding suggest that the four different extracts of Rhizophora mucronata leaves showed different level of antioxidant activity and it is potential source of natural antioxidants.

Keywords: Rhizophora mucronata; DPPH scavenging assay; ABTS scavenging assay; Total phenolic; Flavonoids.

1. Introduction

Cancer is a notorious that now becomes the major cause of human mortality in the world. Most of the incidence and mortality happen in Asia, with lungs and bronchus, breast and ovarian cancers in women to be the most fatal cancers. Photochemical containing antioxidant properties showed capacity to inhibit carcinogenesis. Several studies discovered these antioxidant compounds are related to other bioactivities. Mangroves are salt-tolerant plants of tropical and subtropical intertidal regions of the world. In spite of the commercial properties, mangroves are used in folklore medicine. Mangroves have been known to posses secondary metabolites that are useful against cancer. Antioxidants are of great importance in terms of reducing oxidative stress that is thought to cause damage to biological molecules [1]. Several studies have described the antioxidant properties of medicinal plants rich in phenolic compounds [2-3]. Free radicals, the partially reduced metabolites of oxygen and nitrogen, are highly toxic and reactive. Free radicals are linked with the majority of diseases like aging, atherosclerosis, cancer, diabetes, liver cirrhosis, cardiovascular disorders, etc. [4-5]. Antioxidants protect the body from damaging oxidation reactions by reacting with free radicals and other reactive oxygen species within the body and hindering the process of oxidation. So diseases linked with free radicals can be prevented by antioxidant therapy which gained an immense importance. Traditionally used natural antioxidants from tea, wine, fruits, vegetables, spices, and medicine (e.g. rosemary and sage) are already exploited commercially either as antioxidant additives or a nutritional supplements [6]. Also many other plant species have been investigated in search of novel antioxidants [7-10]. But generally there is still a demand to find more information concerning the antioxidant potential of plant species. Medicinal plants possess secondary metabolites like alkaloids, glycosides, steroids and flavonoids which are the important sources of drugs [11]. Plant phenolics are commonly found in both edible and non-edible plants, and have been reported to have multiple biological effects, including antioxidant activity. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In addition, they have a metal chelation potential [12]. The phenolic compounds are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food [13]. The importance of natural phenolic compounds from plants materials is also raising interest among scientists, food manufacturers, and consumers due to functional food with specific health effects [14]. Recently, there are numerous methods that have been developed to evaluate antioxidant activities of compounds and of complex mixtures such as plant extract [15]. Oxidative stress is an imbalance between production of reactive oxygen species and antioxidant defences [16]. The redox stress triggers the activation of immune cells which release proinflammatory cytokines, reactive oxygen and nitrogen species causing damage to

[1]PG & Research Deparment of Biotechnology, Mohammad Sathak college of arts and science,Sholinganallur, Chennai-600119, Tamilnadu, India.
[2]PG & Research Department of Biochemistry, Mohammad Sathak college of arts and science,Sholinganallur, Chennai-600119, Tamilnadu, India
** Corresponding Email: syedmicro555@gmail.com*

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biological molecules and inducing imbalances in physiological and pathological pathways [17]. Epidemiological and *in vivo* studies have provided evidence that dietary intake of antioxidant and anti-inflammatory compounds is a key strategy for health promotion by lowering oxidative stress and inflammation [18]. Triterpenes and diterpenoids were isolated from this plant and phenolic compounds were found in the pyroligneous acid of *R*. apiculata [19-21]. It was reported that the bark extract of *R. apiculata* showed antioxidant activities [22-24]. Antioxidants are necessary to supplement the natural antioxidant defences of the body to cure these diseases. However, the synthetic antioxidants might be unsafe; therefore, more attention is being paid to searching for natural antioxidants from plants to prevent oxidative damage [25]. Therefore, the objective of present study was to determine the amount of total phenolic compounds and to determine the antioxidant activity of crude extract of *Rhizophora mucronata* through different free radical scavenging assay.

2. Materials and Methods

2.1. Sample collection and preparation

Rhizophora mucronata leaves were collected from Parangipettai, Chennai. The morphology of the leaves shape, colour were authenticated by Dr. Syed Ali, Head of the Department, Mohammad Sathak College, India. The collected mangrove was brought to laboratory, washed thoroughly under running tap water in order to remove dirt and other contaminants from the leaves and then washed with distilled water. Then the sample was air dried in shade, powdered and stored for further use.

2.2. Sample extraction

The powder was carefully sieved through muslin cloth. The powder was used to prepare hot extracts of *Rhizophora mucronata* using Soxhlet extraction method with ethanol, methanol, diethyl ether, ethyl acetate as solvents. The extracts were evaporated on the rotary evaporator to remove the solvents completely and reduce the volume. The resultant dark mass was then stored in airtight bottles and kept at 4ºC.

2.3. Quantitative determination of phytoconstituents determination of total Phenolic content

Phenolic contents of crude extracts were estimated by the method [26]. 100 µl of aliquot sample was mixed with 2.0 ml of 2% Na_2CO_3 and allowed to stand for 2 min at room temperature. After incubation, 100 µl of 50% Folin Ciocalteau's phenol reagent was added and the reaction mixture was mixed thoroughly and allowed to stand for 30 min at room temperature in the dark. Absorbance of all the sample solutions was measured at 720 nm using spectrophotometer. All the experiment was conducted in three replicates.

2.3.1. Total flavonoid content

The total flavonoid content was determined according to the method [27]. 250 µl of 5% NaNO2 solution was added to 0.5 ml of the stock sample along with 150 µl of 10% AlCl3.H2O solution. After 5 min, 0.5 ml of 1M NaOH solution was added and then the total volume was made up of 2.5 ml with ionized distilled water and the absorbance was read 510 nm.

2.3.2. Total antioxidant activity

Total antioxidant activity was measured by the method [28]. Total Antioxidant Capacity (TAC) reagent consist of 7.45 ml of sulphuric acid (0.6 mM solution), 0.9942 g of sodium sulphate (28 mM solution) and 1.2359 g of ammonium molybdate (4 mM solution) were mixed together in 250 ml distilled water. 300 µl of extract was dissolved in 3 ml of TAC reagent. Blank was maintained with distilled water replacing the TAC reagent. Absorbance of all sample mixtures was measured at 695 nm. Total antioxidant activity is expressed as the number of equivalents of ascorbic acid.

2.4. Free radical scavenging assays

2.4.1 Total DPPH assay

The scavenging effects of samples for DPPH radical were determined by the method [29]. 2.0 ml of aliquot of test samples was added to 2.0 ml of 0.16 mM DPPH methanolic solution. The mixture was vortexed for 1 min and then left to stand at room temperature for 30 min in the dark. The absorbance of all the sample solutions was measured at 517 nm. The scavenging effect (%) was calculated by using the formulae.

Scavenging effect (%) = [1-(A sample -A sample blank)/A control] × 100

2.4.2. ABTS radical scavenging

The ABTS scavenging capacity of the extract was compared with that of BHT and/or ascorbic acid and percentage inhibition calculated as For ABTS assay, the procedure followed with some modifications. The antioxidant effect of the leaf extracts was studied using ABTS (2,2'-azino-bis- 3-ethyl benzthiazoline-6-sulphonic acid) radical cation decolourisation assay according to the method [30]. ABTS radical cations (ABTS+) were produced by reacting ABTS solution (7mM) with 2.45mM ammonium per sulphate. The mixture was allowed to stand in the dark at room temperature for 12-16 hours before use. Aliquots (0.5ml) of the different extracts were added to 0.3ml of ABTS solution and the final volume was made up to 1ml with ethanol. The absorbance was read at 745nm in a spectrophotometer and the per cent inhibition was calculated using the formula

% ABTS radical scavenging activity = control - test/ control x 100

2.4.3. Hydrogen peroxide radical scavenging assay

The ability of the crude extract to scavenge hydrogen peroxide was determined by the standard procedure [31]. Hydrogen peroxide 10 mM solution was prepared in the phosphate buffer saline of 0.1 M, pH 7.4, 1 ml (0.25 mg) of the extract was rapidly mixed with 2 ml of hydrogen peroxide solution. The absorbance was measured at 230 nm in the UV spectrophotometer against a blank (without hydrogen peroxide) after 10 min of incubation at 37ºC.

2.4.4. Scavenging of superoxide radical

Scavenging of superoxide radical was studied using the method elaborated [32] Assay tubes contained 0.2 ml of the extract (corresponding to 20 mg extract) with 0.2 ml EDTA, 0.1 ml Nitro blue tetrazolium, 0.05 ml riboflavin and 2.64 ml phosphate buffer. The control tubes were set up with DMSO (Dimethyl sulfoxide) solution instead of the extracts. The initial optical densities of the solutions were recorded at 560 nm and the tubes were illuminated uniformly with the fluorescent lamp for 30 mins. A 560 was measured again and the difference in O.D was taken as the quantum of superoxide production. The percentage of inhibition by the sample was calculated by comparing with O.D of the control tubes.

2.4.5. Superoxide dismutase radical scavenging activity

The Superoxide Dismutase Radical Scavenging Activity was measured [33]. Superoxide anions were generated using PMS / NADH system. The superoxide anions are subsequently made to reduce nitro blue tetrazolium (NBT) which yields a chromogenic product, which is measured at 560 nm. Test solution (20100 mg/ml) in 0.1M phosphate buffer pH 7.4, 625 µl of 468 µM NADH solution, 625 µl of 150 µM NBT solution and 625µl of 60 µM PMS solution were added to a test tube and incubated at room temperature for 5 min. The absorbance was read at 560 nm.The percentage of inhibition by the sample was calculated.

% Superoxide dismutase radical scavenging activity = control- test/ control X 100

2.4.6. Ferric Reducing Antioxidant Power Assay

Reducing power of different crude extract was determined by the method [34]. 1.0 ml of different solvent extract containing different concentration of samples was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferric cyanide (1%). Reaction mixture was kept in a water bath at 50ºC for 20 min. After incubation, 2.5 ml of Trichloroacetic acid (10% of TCA) was added and centrifuged at 650 rpm for 10 min. From the upper layer, 2.5 ml solution was mixed with 2.5 ml distilled water and 0.5 ml of FeCl3 (0.1%). Absorbance of all the solution was measured at 700 nm. Ferric reducing antioxidant Power is expressed as the number of equivalents of ascorbic acid.

2.4.7. Deoxyribose Radical Scavenging Assay

It was used to determine the hydroxyl radical scavenging activity in an aqueous medium [35].The reaction mixture containing $FeCl_3$ (100 µM), EDTA (104 µM), H_2O_2 (1 mM) and 2-deoxy- D-ribose (2.8 mM) were mixed with or without sample at various concentrations (10-250 µg) in 1 ml final reaction volume made with potassium phosphate buffer (20 mM, pH 7.4) and incubated for 1 hr at 37ºC. The mixture was heated at 95 0C in water bath for 15 min followed by the addition of 1 ml each of TCA (2.8%) and TBA (0.5% TBA in 0.025 M NaOH containing 0.02% BHA). Finally the reaction mixture was cooled on ice and centrifuged at 5000 rpm for 15 min. Absorbance of supernatant was measured at 532 nm. All readings were corrected for any interference from brown colour of the extract or antioxidant by including appropriate controls. The negative control without any antioxidant or sample was considered 100% Deoxyribose oxidation. The % hydroxyl radical scavenging activity of test sample was determined accordingly in comparison with negative control. Ascorbic acid was taken as the positive control.

2.4.8. Lipid Peroxidation by Egg Yolk Method

Inhibitions of lipid peroxidation in the egg of hen were determined [36]. Thiobarbituric acidreactive species (TBARS) as- say as previously described. Egg homogenate (0.5 ml, 10% in dis- tilled water, v/v) and 0.1 ml of each fraction were mixed separately in a test tube and the volume was made up to 1 ml, by adding distilled water. Finally, 0.05 ml FeSO4 (0.07 M) was added to the above mixture and incubated for 30 min, to induce lipid peroxidation. Thereafter, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA (w/v) in 1.1% sodium dodecyl sulfate (SDS) and 0.05 ml 20% TCA was added, vortexed and then heated in a boiling water bath for 60 min. After cooling, 5.0 ml of butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. Also, 10% of liver and brain homogenates obtained from rat were also used in place of egg homogenate for the evaluation of lipid peroxidation.

2.4.9. β- Carotene Bleaching Assay

The antioxidant capacity of each of the sample extracts was estimated by the β-carotene bleaching method [37] with modifications. One milliliter of β-carotene (0.2mg/ml chloroform), linoleic acid (0.02 ml) and Tween 20 (0.2 ml) were added to 0.2ml of sample extracts, standard (α-tocopherol) and control (80% methanol). Thereafter, chloroform was evaporated to dryness under vacuum using rotary evaporator. After evaporation, 100 ml of deionized water was added into the mixture and shaken vigorously until emulsion was obtained. Two milliliters of aliquots of the emulsions were pipetted into the test tubes and immediately placed in water bath at 45°C for 2 hours. The absorbance was read at 20 min interval at 470 nm, using a UV-visible spectrophotometer (Secomam, Anthelie Advanced 5) at

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initial time (t=0). Degradation rate (dr) of the sample was calculated according to the formulae

$$\textbf{dr of sample} = (\ln [A_0 / A_t]) / t$$

Where: ln = natural log; A_o = initial absorbance at time 0; A_t = absorbance at 20 min of incubation; t = 120 min and dr = degradation rate.

Antioxidant activity (AA) was expressed as percent of inhibition relative to the control by using the equation:

$$AA\% = ([dr\ control - dr\ sample] / dr\ control) \times 100$$

3. Results and Discussion

Quantitative determination of phytoconstituents

Total Phenolic Content

Total phenolic content of the different extracts of *Rhizophora mucronata* leaves was determined by using the Folin-Ciocalteu reagent. The total phenolic contents of the test were calculated using the standard curve of gallic acid. Methanol extract of *Rhizophora mucronata* was found to contain the highest amount of phenols **(Figure 1)** .The results strongly suggest that phenolics are important components of the tested extract. That antioxidant activity of plant extract is mainly due to presence of phenolic compounds, which may exerts antioxidant effects as free radical scavengers, as hydrogen donating sources or as single oxygen quenchers and metal ion chelators.

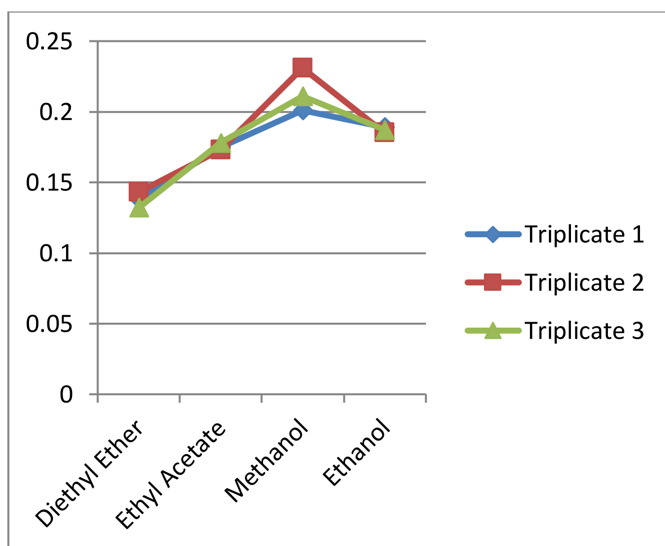

Figure 1 The comparative result for total phenol content

Total Flavonoid Content

The total flavonoid content of the different extracts of *Rhizophora mucronata* was calculated using the standard curve of quercetin. Methanol extract of *Rhizophora mucronata* was found to contain the highest amount of flavonoid **(Figure 2)**. Flavonoids play an important role in antioxidant system in plants. The antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, such as iron and copper and inhibition of enzymes responsible for free radical generation [38]. According to our work, the high contents of flavonoids in *Rhizophora mucronata* can explain its high radical scavenging activity.

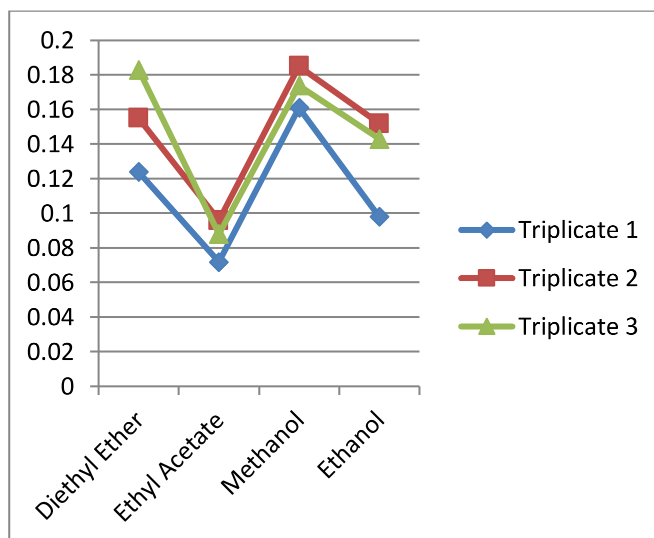

Figure 2 The comparative result for total flavonoid content

Total Antioxidant Capacity

Total antioxidant capacity of the different extracts of *Rhizophora mucronata* was evaluated. Total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid. Methanol extract of *Rhizophora mucronata* was found to possess the highest total antioxidant capacity **(Figure 3)**. The literature reveals a highly positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species [39]. In our study where the methanol extract of *Rhizophora* mucronata showed maximum total phenol and total antioxidant capacity.

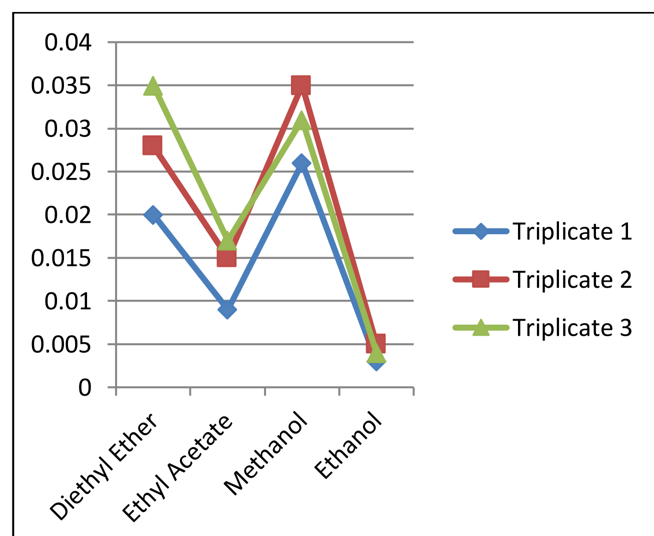

Figure 3 The comparative result for total antioxidant capacity

Free Radical Scavenging Assays

Total DPPH Assay

The DPPH radical scavenging effect of different extracts of *Rhizophora mucronata* were determined. DPPH is a purple colour dye having absorption at 517nm. The percentage inhibition was calculated and the Ethanol extract shows the maximum anti scavenging activity **(Figure 4)** when compared to methanol extract, diethyl ether extracts and ethyl acetate extract. Methanol extract and ethanol extract shows highest antioxidant results. Free radical scavenging activity of the extracts was determined using the free radical 1,1-diphenly-2-picrylhydrazylhydrate (DPPH), which is a molecule containing a stable free radical [40].

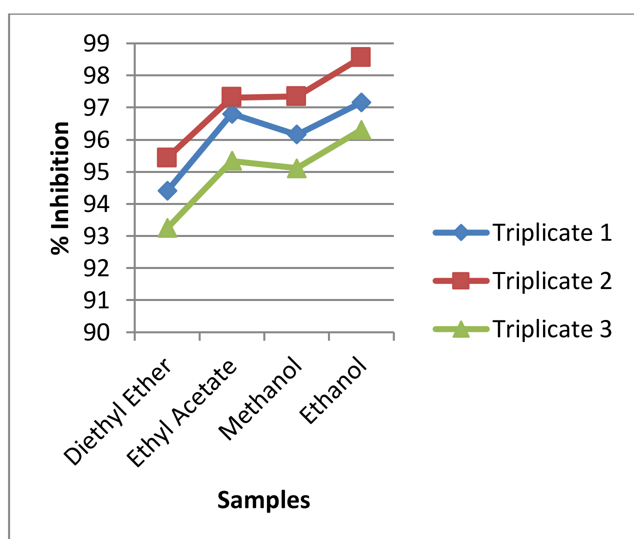

Figure 4 The comparative result for total DPPH activity

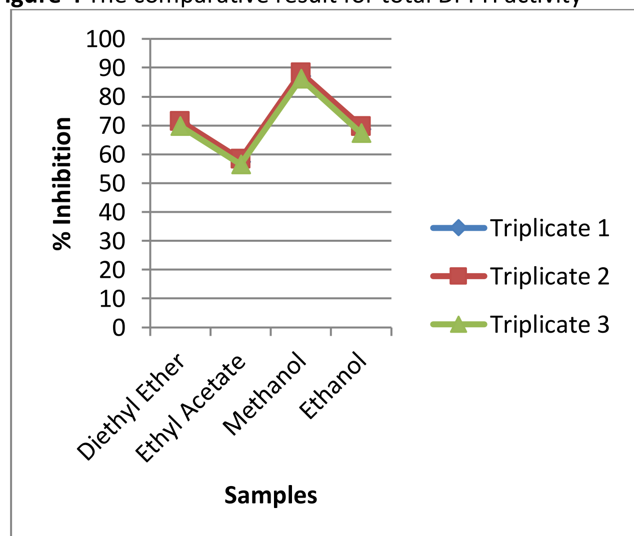

Figure 5 The comparative result for ABTS Radical activity

ABTS Radical Scavenging

The ABTS scavenging capacity of the extract was compared with that of BHT and/or ascorbic acid and percentage inhibition was calculated. The antioxidant effect of four leaf extracts was studied and methanol extracts shows the maximum activity **(Figure 5).** When compared to the other extracts.

Hydrogen Peroxide Radical Scavenging Assay

The ability of the crude extract to scavenge hydrogen peroxide was determined by the standard procedure and absorbance was measured at 230 nm against the blank. And the percentage inhibition results were found to be that diethyl ether extract shows the maximum antioxidant activity than the other extracts **(Figure 6)**.

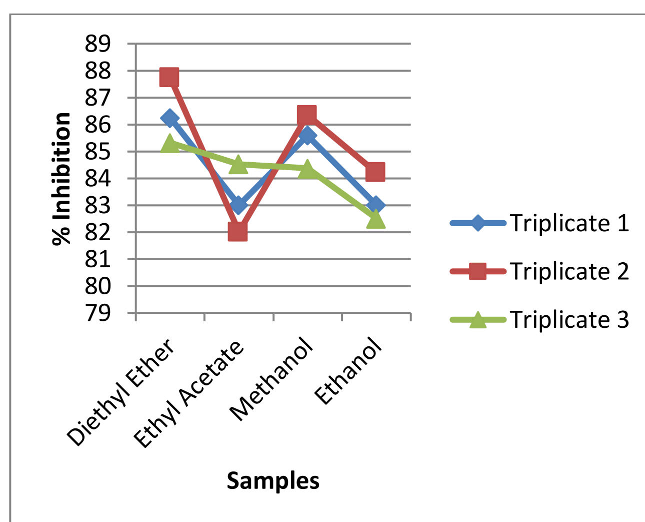

Figure 6 The comparative result for Hydrogen peroxide radical scavenging activity

Scavenging of superoxide radical

Scavenging of superoxide radical was studied and the percentage of inhibition was calculated by comparing with O.D of the control tubes. Out of four extractions, methanol extract shows the maximum activity of scavenging of super oxide radical **(Figure 7)**.

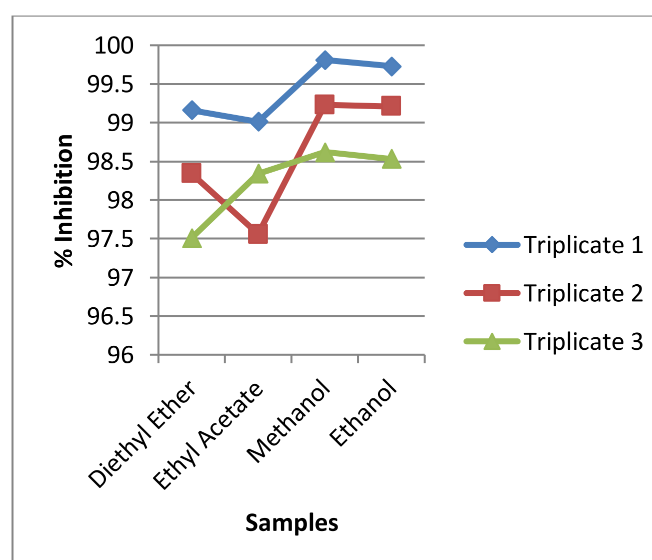

Figure 7 The comparative result for Super oxide radical scavenging activity

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Superoxide dismutase radical scavenging activity

The Superoxide Dismutase Radical Scavenging Activity was measured and percentage inhibition of the extracts was calculated. Ethanol extracts shows the maximum activity of antioxidant when compare methanol extract, diethyl ether extract and ethyl acetate extracts **(Figure 8).**

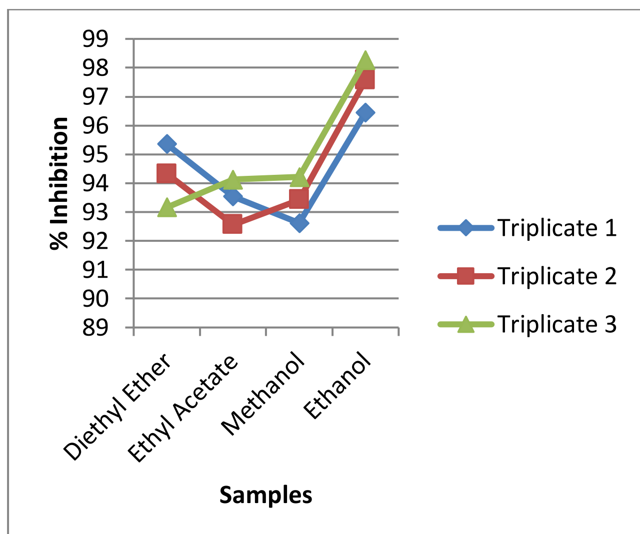

Figure 8 The comparative result for Superoxide Dismutase Radical Scavenging Activity

Ferric reducing antioxidant power assay

Reducing power of different crude extract was determined and the Absorbance of all the solution was measured at 700 nm. Ferric reducing antioxidant Power is expressed as the number of equivalents of ascorbic acid. The diethyl ether extract shows maximum ferric reducing antioxidant capacity where compared to other extracts **(Figure 9)**.

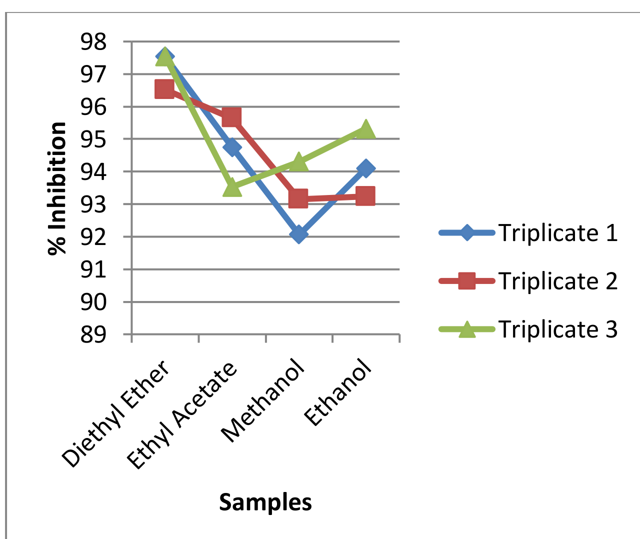

Figure 9 The comparative result for Ferric Reducing Antioxidant Power Assay

Deoxyribose radical scavenging assay

The hydroxyl radical scavenging activity in an aqueous medium was determined accordingly in comparison with negative control. Ascorbic acid was taken as the positive control. Methanol extract shows the activity of deoxyribose radical scavenging when compared to the other extracts **(Figure 10).**

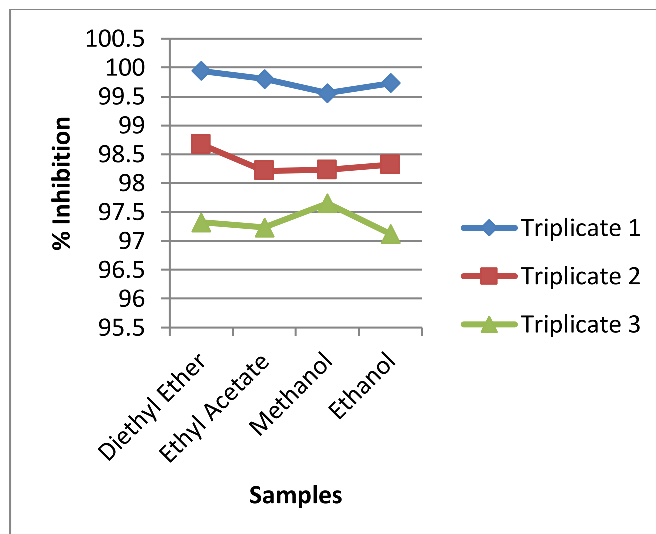

Figure 10 The comparative result for Deoxyribose Radical Scavenging Assay

Lipid per oxidation by egg yolk method

Inhibitions of lipid per oxidation in the egg of hen were determined .The absorbance of the organic upper layer was measured at 532 nm and evaluated for of lipid per oxidation. The ethyl acetate extracts shows the maximum activity of lipid per oxidation when compared to other extracts **(Figure 11).**

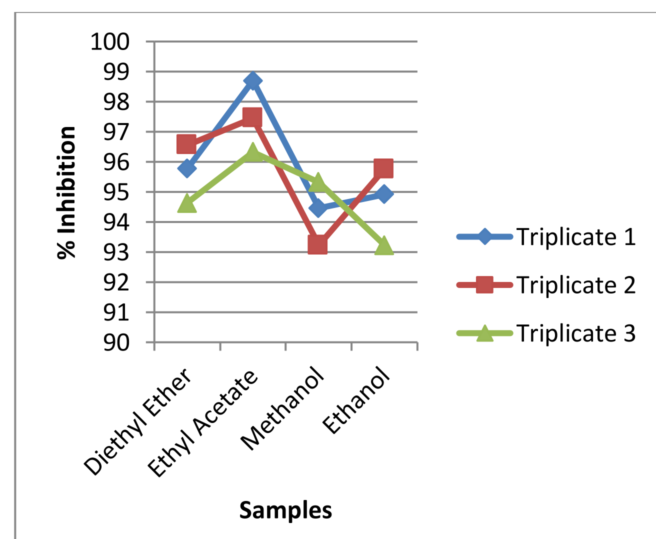

Figure 11 The comparative result for Lipid per oxidation

β-Carotene bleaching assay

The antioxidant capacity of each of the sample extracts was estimated by the β-carotene bleaching method and the

absorbance was read at 470 nm, using a UV-visible spectrometer. The ethanol extracts shows the maximum activity of β carotene bleaching when compared with other extracts **(Figure 12).**

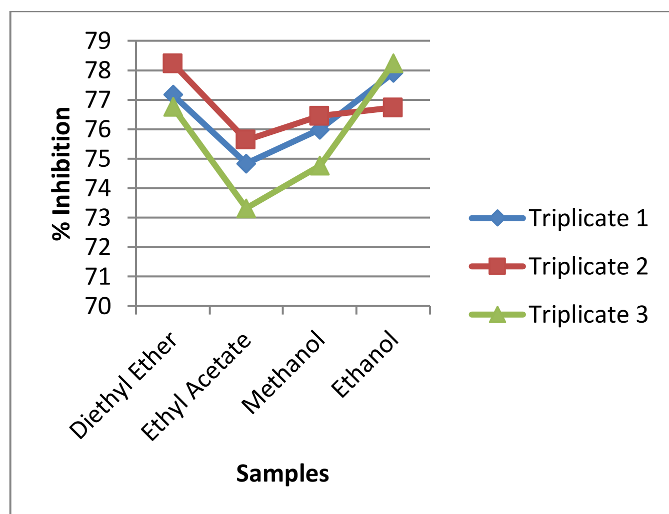

Figure 12 The comparative result for β- Carotene Bleaching Assay

4. Conclusion

The results expressed in this study are the preliminary information on the anti-oxidant activities of *Rhizophora mucronata* . The crude extract expressed free radical scavenging activity in different assays. Among all the extracts, methanol extracts shows maximum activity in most of the antioxidant assays. The scavenging effect on DPPH and superoxide radicals represents the scavenging activity. It is well documented that free radicals are responsible for several diseases. The present result confirms the free radical scavenging activity of mangrove which can be accounted for the traditional uses of the mangrove in treating several diseases. Therefore, there is a need for extensive study to isolate bioactive compounds.

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