#### In vitro antioxidant activity of Avicennia marina (Forssk) Vierh pneumatophore (Avicenniaceae)

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#### ABSTRACT

Antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of the pneumatophore of *Avicennia marina* have been tested using various antioxidant model systems viz., DPPH, hydroxyl, superoxide and ABTS. Ethyl acetate extracts of the pneumatophore showed strong DPPH and hydroxyl radical scavenging activity where as ethanol and methanol extract showed strong superoxide and ABTS radical cation scavenging activity respectively. The  $IC_{50}$  values in all models viz., DPPH, hydroxyl, superoxide and ABTS radical cation scavenging activity of methanol extract of pneumatophore of *A.marina* were found to be 13.24, 19.74, 24.64 and 27.96 µg/ml respectively at 1 µg/ml concentration. This study indicates significant free radical scavenging potential of the pneumatophore of *A.marina* which can be exploited for the treatment of various free radical mediated diseases.

Keywords: Mangrove, Avicennia marina, flavonoid, ABTS, reducing powder

#### INTRODUCTION

Antioxidants are important the in prevention of human diseases. Naturally occurring antioxidants in leafy vegetables and seeds, such as ascorbic acid, vitamin E and phenolic compounds, possess the ability to reduce the oxidative damage associated with many diseases, inducing cancer, cardiovascular disease, cataracts, artherosclerosis, diabetes, arthritis, immune deficiency diseases and aging (Basniwal et al., 2009). Antioxidant means 'Against oxidation' which work to protect lipids from peroxidation by free radicals. Oxidants can damage cells and food substance by starting chain reactions such as lipid peroxidation or by oxidizing DNA or Proteins (Jenecius et al., 2012). Bioactive compounds derived from the plant kingdom have been successfully used to reduce lipid oxidation in food industry products (Dolai et al., 2012; Bernatoniene et al., 2011). Organisms have also evolved complex mechanisms via antioxidants metabolites and enzymes met work in concert to prevent oxidative damage (Sharmila Jose and Radhamani., 2012). These antioxidants are capable of inhibiting the oxidation of biomolecules by removing free radical intermediates and inhibiting other oxidation reactions. Antioxidants could also interrupt peroxidation by donating hydrogen atom rapidly to a lipid radical, forming a new radical, more stable than the initial one oxidative stress occurs when there are low levels of antioxidants or inhibition of the antioxidant enzymes resulting in cell damage or cell death(Awah et al., 2012). Several commercially available synthetic antioxidants such as butylated hydroxyanisole, hydroxytoluene (BHT) butylated and tertbutylhydroquinone (TBHQ) are currently in use but their possible toxic properties for human health and environment are inevitable (Harini et al., 2012). Hence the development of alternative antioxidants from natural origin is the need of the hour. Therefore, it is important to assess antioxidant activity of the plants used in the herbal medicine either to elucidate the mechanism of their pharmacological action or to provide information on antioxidant activity of these herbal plants (Molan et al., 2012) Mangroves are biochemically unique, producing a wide array of novel natural products. Substances in mangroves have long been used in folk medicine to treat diseases. Mangrove and mangrove associates contain biologically active antiviral, antibacterial and antifungal, antiplasmodial and hepatoprotective activities (Ravikumar and Gnanadesigan., 2011; Gnanadesigan et al., 2011).

They provide a rich source of steroids, triterpenes, saponins, flavanoids, alkaloids and tannins (Kanchanapoom *et al.*, 2001; Subasree *et al.*, 2010; Xu *et al.*, 2004; Ravikumar *et al.*, 2010). But, the studies related with *in vitro* antioxidant activity from mangrove plants are too limited. Therefore the main objective of the study is to screen *in vitro* antioxidant activity of *A. marina* pneumatophore. *A. marina* (Forssk) Vierh is commonly known as grey mangrove, belonging to *Avicenniaceae* family.

## MATERIALS AND METHODS

The pneumatophore of *A. marina* was collected from Tuticorin coast, Gulf of Mannar, Tamil Nadu. The collected samples were cut into small fragments and shade dried until the fracture is uniform and smooth. The dried plant material was granulated or powdered by using a blender and sieved 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 PLANT EXTRACT

Freshly collected pneumatophore samples of *A. marina* were dried in shade and then coarsely powdered separately in a willy mill. The coarse powder (100g) was extracted successively with petroleum ether, benzene, ethyl acetate, methanol and ethanol, each 250 ml in a Soxhlet apparatus for 24 hrs. All the extracts were filtered though Whatman No.41 filters paper. All the extracts were concentrated in a rotary evaporator. The concentrated extracts were used for *in vitro* antioxidant activity. The methanol extract was used for the estimation of total phenolics and flavonoids.

## ESTIMATION OF TOTAL PHENOLIC CONTENT

Total phenolic content was estimated using the Folin-Ciocalteu method (Lachman *et al.*, 2000). Samples (100 $\mu$ L) were mixed thoroughly with 2 mL of 2% Na<sub>2</sub>CO<sub>3</sub>. After 2 min. 100  $\mu$ L of Folin-Ciocalteu reagent was added to the mixture. The resulting mixture was allowed to stand at room temperature for 30 min and the absorbance was measured at 743 nm against a blank. Total phenolic content was expressed as gram of gallic equivalents per 100 gram of dry weight (g 100g<sup>-1</sup>DW) of the plant samples.

## **ESTIMATION OF FLAVONOIDS**

The flavonoids content was determined according to Eom *et al* (2007). An aliquot of 0.5ml of sample (1mg/mL) was mixed with 0.1ml of 10% aluminium chloride and 0.1ml of potassium acetate (1M). In this mixture, 4.3ml of 80% methanol was added to make 5mL volume. This mixture was vortexed and the absorbance was measured spectrophotometrically at 415nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

## DPPH RADICAL SCAVENGING ACTIVITY

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H (Shen *et al.*, 2010).

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2picryl-hydrazyl (DPPH) according to the previously reported method (Shen et al., 2010). Briefly, an 0.1mM solution of DPPH in methanol was prepared, and 1mL of this solution was added to 3 ml of the solution of all extracts in methanol at concentration (50,100,200,400 different & 800µg/mL).The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the following formula.

DPPH scavenging effect (% inhibition) =  $\{(A_0 - A_1)/A_0\}^*100\}$ 

Where,  $A_0$  is the absorbance of the control reaction, and  $A_1$  is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged

## HYDROXYL RADICAL SCAVENGING ACTIVITY

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell (1987).

Stock solutions of EDTA (1mM), FeCl3 (10mM), Ascorbic Acid (1mM), H<sub>2</sub>O<sub>2</sub> (10mM) and Deoxyribose (10 mM), were prepared in distilled deionized water. The assay was performed by adding 0.1mL EDTA, 0.01mL of FeCl<sub>3</sub>,0.1mL H<sub>2</sub>O<sub>2</sub>, 0.36mL of deoxyribose 1.0mL of the extract of different concentration (50,100,200,400 &800µg/mL)dissolved in distilled water,0.33mL of phosphate buffer (50mM, pH 7.9), 0.1mL of ascorbic acid in sequence . The mixture was then incubated at 37°C for 1 hour. 1.0mL portion of the incubated mixture was mixed with 1.0mL of 10%TCA and 1.0mL of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532nm. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

## SUPEROXIDE RADICAL SCAVENGING ACTIVITY

The superoxide anion scavenging activity was measured as described by Srinivasan et al (2007). The superoxide anion radicals were generated in 3.0 ml of Tris – HCL buffer (16 mM,  $P^{H}$ 8.0), containing 0.5 mL of NBT (0.3mM), 0.5 ml NADH (0.936mM) solution, 1.0 mL extract of different concentration (50.100.200.400 &  $800\mu$ g/mL), and 0.5 mL Tris – HCl buffer (16mM, P<sup>H</sup> 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12mM) to the mixture, incubated at 25°C for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula

# ANTIOXIDANT ACTIVITY BY RADICAL CATION (ABTS. +)

ABTS assay was based on the slightly modified method of Huang *et al* (2011). ABTS radical cation (ABTS+) was produced by reacting 7mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS + Solution were diluted with ethanol to an absorbance of 0.70+0.02 at 734 nm. After addition of 100 $\mu$ L of sample or trolox standard to 3.9 mL of diluted ABTS+ solution, absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC). The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

# **REDUCING POWER**

The reducing power of the extract was determined by the method of Kumar and Hemalatha (2011). 1.0 mL of solution containing 50,100,200,400 &800µg/mL of extract was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH6.6) and potassium ferricyanide (5.0 mL, 1.0%): The mixture was incubated at 50°C for 20 minutes. Then 5mL of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5°C) in a refrigerator 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 results were averaged.

## STATISTICAL ANALYSIS

Antioxidant activities like DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical activity, ABTS radical cation scavenging activity and reducing powers were estimated in triplicate determinations. Data were analyzed using the statistical analysis system SPSS (SPSS software for windows release 17.5; SPSS Inc., Chicago IL, USA) Estimates of mean, standard error for aforesaid parameters were calculated.

## **RESULTS AND DISCUSSION**

# TOTAL PHENOLIC CONTENT AND TOTAL FLAVONOID CONTENT

In the present study, total phenolic content and total flavonoid content of the methanol extract of A. marina stem was found to be 0.81 g 100g<sup>-1</sup> and 0.74 g 100g<sup>-1</sup>respectively. Phenolic compounds are known for their high antioxidant power. This feature has been attributed to their capacity of reducing oxides, which play an important role in the adsorption or neutralization of free radicals (Sulaiman et al., 2011). Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, activity as scavenger of singlet oxygen and free radicals (Akter and Jaharyir, 2008). Flavonoids are suggested to have many functions like flowers, fruits and seed pigmentation, protection against light; defense against phytopathogens UV (Pathogenic microorganisms, insects, and animals); role in plant fertility and germination of pollen and; acting as signal molecules in plant microbe interactions (Olsen et al., 2010).

Flavonoids are one of the most bioactive plant secondary metabolites. Most flavonoids outperform well known antioxidants, such as ascorbate (vitamin C) and  $\alpha$ -tocopherol (vitamin E), in in vitro antioxidant assays because of their strong capacity to donate electrons or hydrogen atoms. (Hernandez et al., 2009). Flavonoids serve as ROS scavengers by locating and neutralizing radicals before they damage the cell thus important for plants under adverse environmental conditions (Levdal et al., 2010). Flavonoids function by virtue of the number of arrangement of their hydroxyl groups attach to ring structures. Their ability to act as antioxidants depends on the reduction potentials of their radicals and accessibility of the radicals. Flavonoids and other phenolic compounds absorb UV light, and plants able to synthesize these compounds were more tolerant to high UV irradiation than mutants impaired in the flavonoid pathway (Cle et al., 2008).

# DPPH RADICAL SCAVENGING ACTIVITY

Free radicals chemical are species containing one or more unpaired electrons that makes them highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability (Matkowski et al., 2008). Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system, injury, gastritis, cancer and AIDS (Ali et al., 2008). In recent years much attention has been devoted to natural antioxidant and their association with health benefits (Matkowski et al., 2008).

There are several methods available to assess antioxidant activity of compounds. DPPH free radical scavenging assay is an easy, rapid and sensitive method for the antioxidant screening of plant extracts. In presence of an antioxidant, DPPH radical obtain one more electron and the absorbance decreases (Sudhanshu *et al.*, 2012).

The effect of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *A.marina* pneumatophore and standard ascorbic acid on DPPH radical scavenging activity were compared and shown in Figure 1. In the present study, the percentage of scavenging effect on the DPPH radical was concomitantly increased with an increase in the concentration of *A. marina* pneumatophore extracts from 50-800 µg/ml and ascorbic acid. At 800µg/mL concentration of benzene, ethyl acetate and ethanol extracts of A. marina pneumatophore possessed 93.84%, 96.25% and 74.55 % scavenging activity on DPPH respectively. All the concentration of A. marina pneumatophore extracts showed higher activity except petroleum ether and methanol extracts than the standard ascorbic acid. The scavenging ability decreased in the order of ethyl acetate >benzene >ethanol >methanol>petroleum ether respectively. Among the tested extracts, ethyl acetate extracts of A.marina pneumatophore exhibited maximum DPPH radical scavenging activity. The IC<sub>50</sub> value of ascorbic acid was 19.38µg/ml whereas ethyl acetate extract was found to be 21.22µg/ml.

# HYDROXYL RADICAL SCAVENGING ACTIVITY

The hydroxyl radical scavenging activity is measured as the percentage of inhibition of hydroxyl radicals generated in the Fenton's reaction mixture by studying the competition between deoxyribose and extract of hydrogen radicals generated from  $Fe^{3+}/EDTA/H_2O_2$  systems. The hydroxyl radicals attack deoxyribose which eventually results in TBARS formation (Abirami *et al.*, 2012).

The effect of petroleum ether, benzene, ethyl acetate, methanol and ethanol extract of A. marina pneumatophore and standard ascorbic acid on hydroxyl radical scavenging activity were compared and shown in Figure 2. The scavenging effect increases with the concentration of standard and samples. At 800µg/mL concentration of ethyl acetate, methanol and ethanol extracts of A. marina pneumatophore showed 77.33%, 69.12% and 65.84% scavenging activity on hydroxyl radical respectively. All the concentration of A. marina pneumatophore extracts showed higher activity except petroleum ether and benzene extracts than the standard ascorbic acid. Hydroxyl radical scavenging activity of extracts were in following order ethyl acetate> methanol> ethanol> petroleum ether>benzene. Among the tested extracts of A.marina pneumatophore, ethyl acetate extract showed the strongest hydroxyl radical scavenging activity (77.33% at 800µg/ml) while standard ascorbic acid showed 65.39% at 800  $\mu$ g/ml radical scavenging activity. The IC<sub>50</sub> value of ascorbic acid was 21.39µg/ml where as methanol extract was found to be 19.74µg/ml.

#### SUPEROXIDE RADICAL SCAVENGING ACTIVITY

Superoxide radical plays an important role in plant tissues and it is involved in the formation of other cell damaging free radicals.

The A. marina pneumatophore extracts were subjected to be superoxide scavenging assay and the results were shown in Figure 3. It indicates that, ethanol methanol and benzene extract of A. marina pneumatophore (800µg/mL) exhibited the maximum superoxide radical scavenging activity of 92.54%, 89.33% and 84.26% respectively, which is higher than the standard ascorbic acid whose scavenging effect is 80.63%. Superoxide radical scavenging activity of extracts were in following order ethanol> methanol> benzene> ethyl acetate> petroleum ether. The IC<sub>50</sub> value of ascorbic acid was 24.16µg/ml whereas ethanol extract was found to be 28.4µg/ml. It is known that the hydroxyl group of the phenolics contributes to superoxide scavenging activity by their electron donation (Samydurai et al., 2012).

## ABTS RADICAL CATION SCAVENGING ACTIVITY

ABTS radical cation scavenging activity decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids and plasma antioxidants. The preformed radical monocation of 2, 2-azinobis- (3-ethylbenzothiazoline-6-alfonic acid) (ABTS) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen- donating antioxidants (Vasanthi *et al.*, 2012).

The effect of A. marina pneumatophore extracts and standard trolox on ABTS radical cation were compared and shown in Figure 4. The scavenging effect increases with the concentration standard and samples. At 800µg/mL of concentration of methanol, ethanol, benzene, and petroleum ether extracts of Α. marina pneumatophore 93.24%. 86.28%. possessed 65.18% and 54.80% scavenging activity on ABTS. All the concentration of A. marina pneumatophore extracts showed higher activity except ethyl acetate extract than the standard trolox. ABTS radical cation scavenging activity were in following order methanol>ethanol>benzene>petroleum ether>ethyl acetate. The IC<sub>50</sub> value of ascorbic acid was 20.16µg/ml whereas methanol extract was found to be 27.96µg/ml. The scavenging activity of ABTS radical by the plant extracts were found to be

appreciable; this implies that the plant extract may be useful for treating radical related pathological damage especially at higher concentration (Karthika *et al.*, 2012).

#### **REDUCING POWER**

The reducing power of A. marina pneumatophore extracts was compared with the standard ascorbic acid. The reducing power increases with the increasing concentration. The reducing power of the petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of A. marina pneumatophore was shown in Figure 5. At 800µg/mL concentration of methanol and ethanol extracts of A. marina pneumatophore showed higher reducing power than the ascorbic acid. In reducing power assay, the presence of antioxidants in the sample reduced fe3+/ ferricyanide complex to the ferrous form. This reducing capacity of compounds could serve as an indicator of potential antioxidant properties and increase in absorbance could indicate an increase in reducing power (Umamaheswari and Chatterjee., 2008; Aderegun et al., 2009). Among the extracts, methanol extract exhibited higher reducing power activity as compared with ascorbic acids. (Paul priya and Mohan., 2012; Tresina et al., 2012)

#### CONCLUSION:

On the basis of results, in this study, it can be concluded that, all the extracts of *A. marina* pneumatophore is cabable of scavenging a wide range of free radicals. The extracts contain higher quantities of total phenolics and flavonoids, which exhibit antioxidant and free radical scavenging activity. *In vitro* assay systems confirm *A. marina* pneumatophore as natural antioxidants but it is doubtful that specific compounds responsible for antioxidant activity. Further *in vivo* assessment is also needed to confirm antioxidant nature of *A.marina* pneumatophore.

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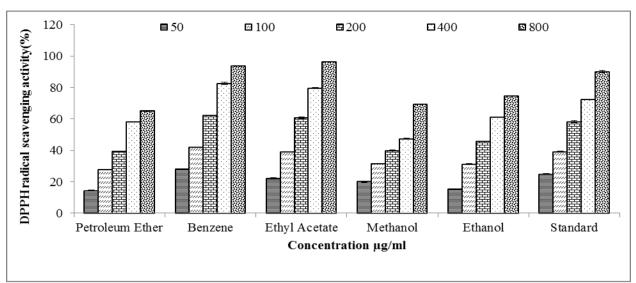


Fig 1: DPPH radical scavenging activity of different extracts of Avicennia marina pneumatophore.

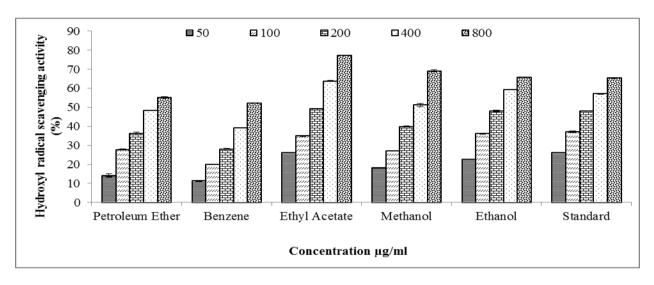
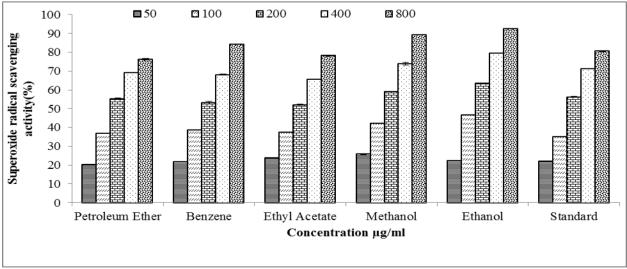


Fig 2: Hydroxyl radical scavenging activity of different extracts of Avicennia marina pneumatophore.





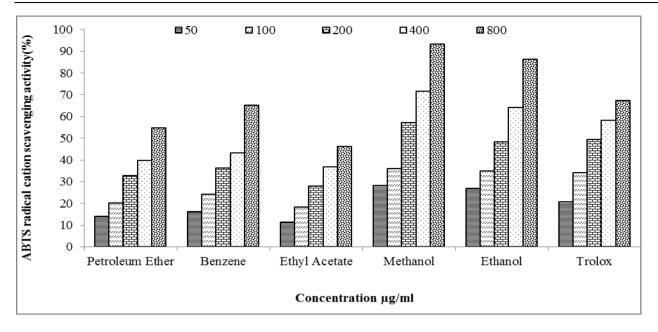


Fig 4: ABTS radical cation scavenging activity of different extracts of Avicennia marina pneumatophore.

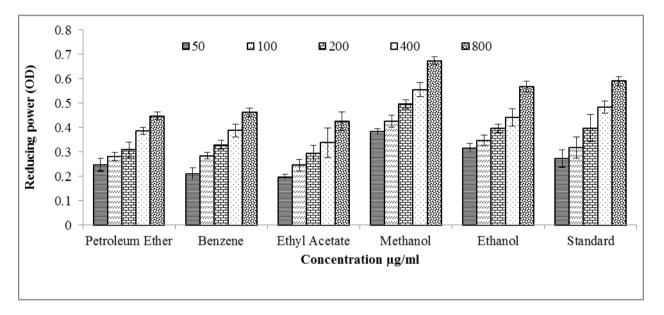


Fig 5: Reducing power ability of different extracts of Avicennia marina pneumatophore.

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