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RESEARCH ARTICLE

SCREENING OF ANTIOXIDANT POTENTIAL COMPOUNDS FROM *AVICENNIA MARINA* BARK

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ABSTRACT

The present study has been conducted to evaluate the antioxidant activity of medicinal plant *Avicennia marina*. Stem bark of *Avicennia marina* was powdered and extracted with 95% ethanol and water by cold extraction method. The water and ethanol extract of air dried stem bark was estimated by using spectrometric method. Antioxidant activity of *Avicennia marina* was determined in the presence of different methods namely reducing power assay, DPPH assay, Superoxide radical scavenging assay, Nitric oxide radical scavenging assay ect,.the determinations are conducted by in vitro methods the water and ethanolic plant extraction achieved a maximum antioxidant activity in a different concentration.

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 are act as a 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 contains phenolic compounds and its possess 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.

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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 a antioxidants and it is evaluated by various screening methods (Kokate, 1999; Nikhat *et al*., 2009). 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. *Avicennia marina* bark Aqueous and alcoholic extracts were subjected for the antioxidant screening using six different methods.

MATERIALS AND METHODS

Plant collection and extraction

The bark of *Avicennia marina* were collected from Pitchavramm 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 (Kokate, 1999). The solvent was removed under pressure to obtain a total extracts.

In vitro Antioxidant Activity

Reducing power assay (Nikhat *et al*., 2009)

Reducing power assay works under the following principle. Substances, which have reduction potential, react with

potassium ferricyanide (Fe3+) to form Potassium ferrocyanide (Fe2+), 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 (Yildrim *et al* 2001). 1 ml of plant extract solution (final concentration 100-500 mg/l) was mixed with 2.5 ml phosphate buffer (0.2M, pH 6.6) and 2.5 ml potassium ferricyanide [K3Fe(CN6)] (10g/l), then mixture was incubated at 50degree C for 20 minutes. 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 Fecl3 (1g/l) and absorbance measured at 700nm in UV-Visible Spectrophotometer (Systronics UV-Visible Spectophotometer 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 expresses as mean ± standard deviation. Increased absorbance of the reaction mixture indicates stronger reducing power.

% increase in Reducing Power = $A_{test/} A_{blank}$ - 1 X 100

A_{test} is the absorbance of test solution; A_{blank} is absorbance of blank. The antioxidant activity of the root extract was expressed as EC50 and compared with standard.

DPPH assay: (2, 2-diphenyl-1-picrylhydrazyl)

The scavenging reaction between (DPPH.) and an antioxidant (H-A) can be written as :

(DPPH) + (H-A) →DPPH-H + (A)
(Purple) (Yellow)

Antioxidants react with DPPH, which is a stable free radical and is reduced to the DPPH-H and as consequence the absorbance's decreased from the DPPH radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

Antioxidant activity by DPPH staining (Dot assay)

An aliquot of each sample and standard (Quercetin 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 (4µL to 1µL) along the row. Water sample was used as a control. After 5 minutes 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). (Figure –1).

DPPH assay by 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 (CHCl3-MeOH-H2O (61:32:7)) after application of 5 μl of each test compound solution (1 mg/ml), dried and sprayed with DPPH solution (0.2%, MeOH). 5 min later active compounds appeared as yellow spots against a purple background. The purple stable free radical 2,2-diphenyl-1-picrylhydrazyl was reduced to the yellow coloured diphenylpicryl hydrazine. Quercetin was used as positive control (Takao *et al*., 1994). (Figure 2).

DPPH Radical Scavenging Activity (Spectrophotometry)

The free radical scavenging capacity of the extracts of *Avicennia marina* aqueous and alcoholic extract was determined using DPPH. DPPH solution (0.004% w/v) was prepared in 95% methanol. Extracts of *Avicennia marina* was mixed with 95% methanol to prepare the stock solution (10 mg/100mL). The concentration of Extract solution was 10 mg /100 ml or 100µg/ml. From stock solution 2ml, 4ml, 6ml, 8ml & 10ml of this solution were taken in five test tubes & by serial dilution with same solvent were made the final volume of each test tube up to 10 ml whose concentration was then 20µ g/ml, 40µg/ml, 60µg/ml, 80µg/ml & 100µg/ml respectively. Freshly prepared DPPH solution (0.004% w/v) was added in each of these test tubes containing extracts (20 µg/ml, 40µg/ml, 60µg/ml, 80µg/ml, 100µg/ml) and after 10 min, the absorbance was taken at 517 nm using a spectrophotometer (Systronics UV-Visible Spectrophotometer 119, INDIA). Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (10 mg/100mLor 100µg/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

% DPPH scavenging activity = A control-A test/A control X 100 (Blois, 1958).

Where a control is the absorbance of the control reaction and a test is the absorbance in the presence of the sample of the extracts. The antioxidant activity of the root's extract was expressed as IC50 and compared with standard. The IC50 value was defined as the concentration (in µg/ml) of extracts that inhibits the formation of DPPH radicals by 50.

Nitric oxide scavenging activity (Marcocci *et al*., 1994).

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the Griess reagent. 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. Sodium nitroprusside (5 mmol L–1) in phosphate buffered saline pH 7.4, was mixed with different concentrations of the extract (250–2500 mg mL–1) 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.

% Nitric oxide scavenging activity = A control-A test/A control X 100

Superoxide radical scavenging activity (PMS-NADH System) (Nishimiki *et al*., 1972)

Superoxide anions were generated using PMS / NADH system. The superoxide anions are subsequently made to reduce nitroblue tetrazolium which yields a chromogenic product, which is measured at 560 nm. Test solution (0.1 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. Linear graph of concentration vs percentage inhibition was prepared and IC50 values were calculated.

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

ABTS radical scavenging assay (Arnao *et al*., 2001)

ABTS (2, 2'-azinobis-3-ethylbenzothiozoline- 6-sulphonic acid) assay is based on the scavenging of light by ABTS radicals. 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. 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 ± 0.01 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 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.

% ABTS radical scavenging activity = A control-A test/A control X 100

ABTS radical scavenging activity where $Abs_{control}$ is the absorbance of ABTS radical in methanol; Abs sample is the absorbance of ABTS radical solution mixed with sample extract/standard. All determinations were performed in triplicate ($n = 3$).

Hydroxy radical scavenging activity (Elizabeth and Rao, 1990)

Hydroxy radical scavenging activity was determined using 2-deoxyribose oxidative degradation as described previously. The principle of the assay is the quantification of the 2-deoxyribose degradation product, malonaldehyde, by its condensation with thiobarbturic acid (TBA). The reaction mixture contained deoxyribose (2.8mM); FeCl3 (100 mM); KH2PO4–KOH buffer (20mM, pH 7.4); EDTA (100 mM); H2O2 (1.0 mM); ascorbic acid (100m M), and various concentrations of the test compounds in a final volume of 1 ml. Ferric chloride and EDTA (when added) were premixed just before addition to the reaction mixture. The reaction mixture was incubated at 37°C for 60 min. After incubation at 37°C for 1 h, 1.0 ml of 2.8% trichloroacetic acid and 1.0 ml of 1% aqueous solution of TBA were added to the sample; test tubes were heated at 100°C for 20 min to develop the color. After cooling, TBARS formation was measured spectrophotometrically at 532 nm against an appropriate blank. The hydroxyl radical-scavenging activity was determined by comparing absorbance of the control with that of test compounds.

Estimation of total phenolic content (Yu *et al*., 2008)

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 the higher the total phenolics content). 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_2CO_3) solution and 0.1 ml of 1 N 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.

Statistical analysis

The Results are given as mean ± standard deviation. The IC50 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 (IC50) was calculated.

RESULTS AND DISCUSSION

Figure 1 depicts IC50 values of different antioxidant assay. 34.29µg/mL to 112.7µg/mL concentrations of aqueous and alcoholic extracts needed to ascertain 50% of radical scavenging activity. Various methods were employed to assess antioxidant properties of *Avicennia marina* 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). 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 colour fades rapidly (Khushad *et al*., 2003; Buhler and Miranda, 2000; Gooijer *et al*., 1997). In DPPH radical scavenging assay, *Avicennia marina* extracts showed dose dependent scavenging of DPPH radical with the reference ascorbic acid (Figure 2); DPPH radical with the IC50 value of water extract was 112.7 µg/mL and alcoholic extract was 95.18 µg/mL while the IC50 value for the reference ascorbic acid was 12.77µg/mL.

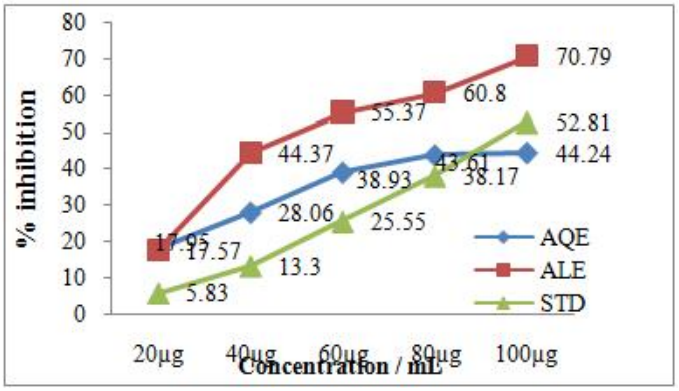

Fig. 2. DPPH activity of *Avicennia marina*AQE-Aqueous extract; ALE-Alcoholic extract; STD- Standard

Fig. 1 - DPPH Dot assay

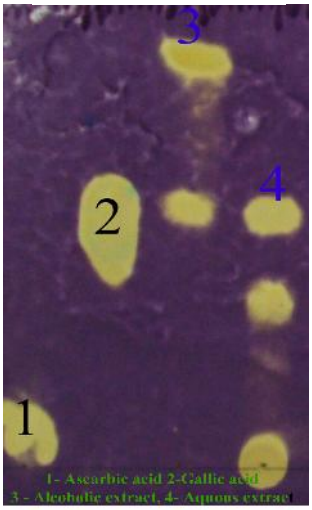

Fig. 2 - DPPH TLC assay

Reducing capacity of extracts by measuring 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 (25.8%), alcoholic extract (18.96%) produced lower ferric reducing power when compared to standard which produced 87.5% inhibition at 100µg/mL concentration. The IC50 value of aqueous extract of *Avicennia marina* bark is 37.692 µg/mL alcoholic extract is 34.82 µg/mL the values were compared with the standard IC50 value of 42.5.

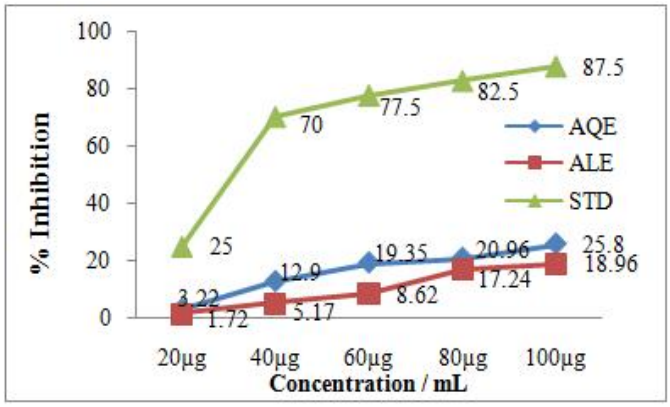

Fig. 3. Reducing power assay of *Avicennia marina*AQE-Aqueous extract; ALE-Alcoholic extract; STD- Standard

Scavengers of nitricoxide compete with oxygen and leads to reduced production of nitricoxide. In general physiological pH sodium nitropruside spontaneously generates nitricoxide which interacts with oxygen to produce nitrite ions. These nitrite ions are estimated by making use of Griess reagent. Figure 6 illustrate the percentage of inhibition of nitric oxide generation by the extracts of *S asoca*. All the extracts and standard chemicals produced 46.04 – 46.76 % of nitric oxide production inhibition at 100 µg/mL concentration.

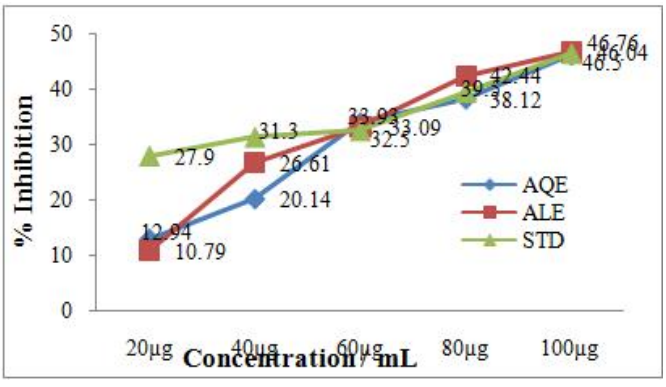

Fig. 4. Nitricoxide activity of *Avicennia marina*AQE-Aqueous extract; ALE-Alcoholic extract; STD- Standard

In Superoxide radical scavenging assay, superoxide derived from dissolved oxygen by PMS/NADH coupling reaction and it reduces NBT. Colour reduction indicated the consumption of superoxide anion. Extracts of *Avicennia marina*have significant inhibition of superoxide anion generation. In general superoxide anion is generated by neutrophils during host microbe interaction. Extracts of *Avicennia marina*showed best superoxide radical scavenging power. About 65% radical

Antioxidant activity of Aqueous extract of *Avicennia marina* bark

S.No	Concentration	DPPH	Reducing power	Nitricoxide	SOD	DMSO	ABTs	Hydrogen peroxide
1	20	18.56±0.822	2.87±0.54	11.62±1.17	9.48±0.82	38.48±0.46	66.85±0.52	13.77±1.26
2	40	27.87±1.42	12.206±0.69	19.2±0.92	17.45±0.87	40.06±1.62	78.3±1.67	28.076±1.12
3	60	38.003±1.50	18.323±0.90	32.836±1.03	27.60±1.32	43.59±0.83	84.52±1.16	29.13±1.38
4	80	41.656±1.94	19.526±1.36	36.966±1.08	29.32±0.82	63.42±0.89	88.37±1.28	53.46±1.47
5	100	43.38±1.41	25.063±0.84	45.296±1.00	63.42±1.26	67.89±1.46	90.82±0.38	63.70±1.41
6	Ic50	83.17	216.1	100.03	108.8	56.286	35.25	80.03

Antioxidant activity of Alcoholic extract of *Avicennia marina* bark

S.No	Concentration	DPPH	Reducing power	Nitricoxide	SOD	DMSO	ABTs	Hydrogen peroxide
1	20	18.32± 0.85	1.43± 0.25	9.62± 1.03	19.46± 0.77	4.90± .75	78.28±1.16	12.88± 1.00
2	40	41.95± 2.17	5.01± 0.30	26.64± 0.79	24.37± 1.24	18.85± 1.39	80.37±1.19	65.99± 0.68
3	60	54.08± 1.70	7.47± 1.06	32.37± 0.83	30.19± 0.80	34.46± 1.29	82.42±1.002	77.58± 0.58
4	80	59.57± 1.07	16.34± 0.80	41.61± 0.86	42.10± 1.70	41.64± 0.92	85.97± 1.75	80.43± 1.14
5	100	70.07± 1.69	17.87± 0.93	45.82± 0.93	65.55± 1.02	65.02± 1.51	93.50± 0.84	97.21± 1.05
6	Ic50	59.24	272.82	95.38	80.81	114.03	34.8	49.55

Standard values for Antioxidant activity

S.No	Concentration	DPPH	Reducing power	Nitricoxide	SOD	DMSO	ABTs	Hydrogen peroxide
1	20	18.32± 0.85	1.43± 0.25	9.62± 1.03	19.46±0.77	4.90± 0.75	78.28± 1.16	12.88± 1.00
2	40	41.95± 2.17	5.01± 0.30	26.64± 0.79	24.37±1.24	18.85±1.39	80.37± 1.19	65.99± 0.68
3	60	54.08± 1.70	7.47± 1.06	32.37± 0.83	30.19±0.80	34.46±1.29	82.42± .00	77.58± 0.58
4	80	59.57± 1.07	16.34± 0.80	41.61± 0.86	42.10±1.70	41.64±0.92	85.97± 1.75	80.43± 1.14
5	100	70.07± 1.69	17.87± 0.93	45.82± 0.93	65.55±1.02	65.02±1.51	93.50± 0.84	97.21± 1.05
6	Ic50	59.24	272.82	95.38	80.81	114.03	34.8	49.55

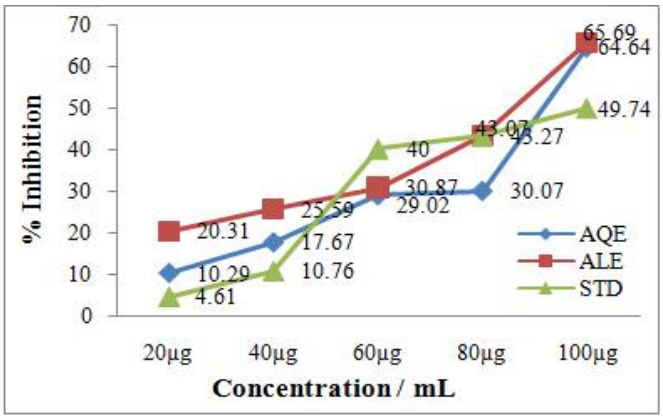

Fig. 5. Superoxide Radical Scavenging Assay of *Avicennia marina*AQE-Aqueous extract; ALE-Alcoholic extract; STD-Standard

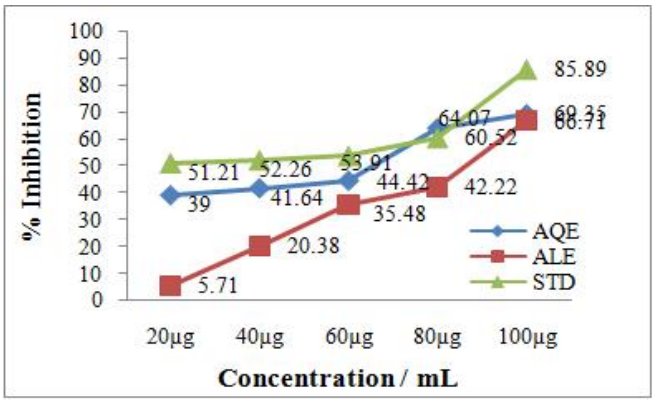

Fig. 6. Alkaline DMSO Radical Scavenging Assay of Avicennia marinaAQE-Aqueous extract; ALE-Alcoholic extract; STD-Standard

inhibition power was exhibited by both the extracts at 100 µg/mL concentrations with 104.79 and 78.62 IC50 values for aqueous and alcoholic extracts and 134.23 for Ascorbic acid. Superoxide scavenging activities of the products / extracts were assessed by making use of DMSO method. Aqueous extract produced 69.35%, alcoholic extract produced 66.71% and standard (Ascorbic acid) exhibited 85.89% superoxide scavenging activity at 100 µg/mL concentrations with 55.14 for aqueous extract, 105.536 for Alcoholic extract and 47.54 for standard IC50 values.

Figure 7 illustrates 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 (91.24% for aqueous extract, 94.35% for alcoholic extract) IC50 values of extracts ranging from 34.29 to 34.88µg/mL concentration.

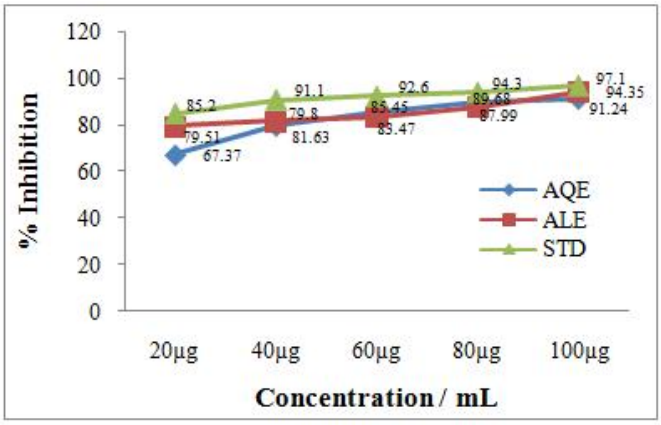

Fig. 7. ABTs Assay of *Avicennia marina*AQE-Aqueous extract; ALE-Alcoholic extract; STD- Standard

Hydrogen peroxide itself is not very reactive, but it may be toxic to cell due to increase in hydroxyl radical concentration

in the cells (Halliwell, 1991). Thus, removal of H_2O_2 as well as O_2 leads to survival of the cell life and its components. The scavenging ability of *Avicennia marina* bark extract on hydrogen peroxide is comparable with that of standard ascorbic acid. Aqueous extract (64.96%), alcoholic extract (98.29%) produced lower ferric reducing power when compared to standard which produced 73.12% inhibition at 100µg/mL concentration The IC50 value of aqueous extract of *Avicennia marina* bark was 77.41µg/mL and alcoholic extract was 47.99µg/mL for inhibitory activity of hydrogen peroxide radical, while its standard ascorbic acid showed IC50 113.56 µg/mL. These finding showed the potent hydrogen peroxide radical inhibitory activity.

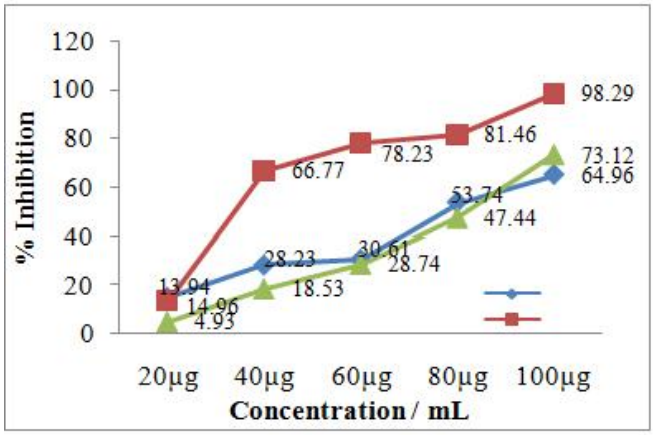

Fig. 8. Hydrogen Peroxide Assay of *Avicennia marina*AQE-Aqueous extract; ALE-Alcoholic extract; STD- Standard

Preliminary phytochemical natures of the extracts were analysed using standard methods. Flavonoids, Terpenoids, Saponin, Phenol, Tannins, Lignin, and Cardioglycoside were present in aqueous extract. Alcoholic extracts showed the availability of flavonoids, Terpenoids, Tannins, Phenol, Lignin, Inulin and protein.

Phenolic compounds are well known powerful chain breaking antioxidant [15] 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 *Avicennia marina* aqueous extract was 8.0% and alcoholic extract was 36% which are equivalent to gallic acid. This is one of the compounds responsible for antioxidant activity. Flavonoids are present in food origin of plant which are also act as a potential antioxidants (Salah *et al*., 1995; Van Acker SABE *et al*., 1996). Beneficial effects of flavonoids are attributed to their antioxidant and chelating ability. The total flavonoids content in *Avicennia marina* water extract was found to be 26.80% and Alcoholic extract was found to be 30%. Tannins act as a chemo preventing agent against free radical scavengers and it stopped the cell damages (Sudha and Gnanamani, 2008; McDonald *et al*., 2001; Polshettiwar *et al*., 2007). The total tannin content of *Avicennia marina* bark water extract was found to be 45.60% and alcoholic extract was found to be 56 %.

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