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# Evaluation of Secondary Metabolites and Antioxidant Activity of Ethanolic Leaves Extract of *Avicennia marina*

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## A B S T R A C T

*Avicennia marina* is the marine mangrove found abundantly along the coastal regions of Tamil Nadu. The ethanolic leaf extract of *Avicennia marina* was analyzed by HPLC and GC to determine various Phytochemicals. Free radicals scavenging activity of extract by using DPPH, NO and Super oxide radicals generated *in vitro*. The ethanolic extract of *A. marina* was found to contain alkaloids, terpenoids, phenols and flavonoids. The major flavonoid detected was quercetin and rutin. The *A. marina* was found to possess significant radical scavenging activity against DPPH, nitric oxide and superoxide anions the $IC_{50}$ value of 50.75 µg/ml, 45.85µg/ml and 48.45µg/ml respectively and comparable to that of their corresponding $IC_{50}$ value. The medicinal property of *A. marina* may be attributed to the presence of flavonoids and phenolic compounds with rich antioxidant potential. The therapeutic effect of this plant may be accounted for its counteracting action on free radicals *in vivo*.

***Key words*:** *Avicennia marina*, Phytochemicals, Antioxidant, Free radical scavenging activity

The mangroves are a taxonomically diverse group of halophytic plant communities that are found in the intertidal zones between land and sea of tropical and sub-tropical region of the world [1]. These plants are highly specialized, flourishing under inhospitable environment conditions of extreme tides, high salinity, high temperature, strong winds and anaerobic soil [2]. Exhibition of well-developed morphological and physiological features is the key to their survival in the adverse environmental conditions. Over the years, local communities inhabiting the mangrove forests exploit different mangrove plants for woods and disease treatment [3].

*Avicennia marina* is commonly called white mangrove belongs to the family Avicenniaceae. It is a small medium sized tree (3-11 meter) with many branches. Extensive underground root system with Pencil root (Pneumatophores or breaking roots) up to 90 mm long. The plant has received some attention in determining its important chemical constituents. Phenolic compounds are secondary plant metabolites and are involved in a wide range of specialized physiological function [4-6]. Previous reports suggest that this species was useful in stabilizing banks of estuaries in salty water and that it has tannin rich bark [7]. In this study pharmaceutical properties of *Avicennia marina* was evaluated using certain *in vitro* assays viz. Total antioxidant activity, nitric oxide scavenging activity, protein denaturation inhibition activity and chelating ability of metal ions.

Natural therapy for various human ailments purified with plant products has gained much attention now days, due to various side effects associated with allopathic medicine these can be derived from any part of the plant like bark, leaves, stem, flowers, roots, seeds, etc., [8]. Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects [9]. Free radicals play an important role in various pathological conditions such as tissue injury, inflammation, neurodegenerative diseases, cancer and aging. The Compound that can scavenge free radicals has great potential in ameliorating these diseases [10]. Inflammation is a disorder characterized by invasion of leucocytes and production of proinflammatory cytokines [11].

Medicinal plants are the source of many potent and powerful drugs. The plant derived drugs are healthier and safer alternate to the synthetic drugs [12]. Different parts of medicinal plants like root, stem, flower, fruit, seed etc. are used to obtain pharmacologically active constituents. Medicinal activities of plants can be attributed to the secondary metabolites such as alkaloids, flavonoids, glycosides, tannins, terpenoids and essential amino acids present in these plants. These active principles are isolated for direct use as drugs, lead compounds and or pharmacological agents [13]. Even today compounds from plants continue to play a major role in primary health care as therapeutic remedies in many developing countries [14]. Standardization of plant materials is the need of the day. Several pharmacopoeia containing monographs of the

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plant materials describe only the physicochemical parameters. Hence the modern methods describing the identification and quantification of active constituents in the plant material can be useful for proper standardization of herbals and its formulations. Also, the WHO has emphasized the need to ensure the quality of medicinal plants products using modern controlled technique and applying suitable standards [15]. Nowadays there were a number of dramatic advances in analytical techniques including TLC, UV, NMR and GC-MS that were powerful tools for separation identification and structure determination of Phytochemicals. In GC-MS used to identify the bioactive constituents of long chain hydrocarbons, alcohols, acids, esters, alkaloids, steroids, amino and nitro compounds etc., Keeping this in view, the present study has been undertaken to investigate the phytoconstituents present in ethanolic leaves extract of *A. marina*.

## MATERIALS AND METHODS

*Collection and Identification of plant material*

For the study, the leaves of *Avicennia marina* belongs to Acanthaceae (Avicenniaceae) family were collected from Pichavaram Mangroves, Tamil Nadu, South India. The plant was authenticated by Director, Plant Anatomy and Research Center, Chennai and the voucher specimen is deposited in our laboratory.

*Preparation of plant materials and extract*

The leaves were carefully cleaned, shade dried and powdered. The powdered material was stored in a closed air-tight plastic container at low temperature. The powdered plant material (50 g) was extracted with 300 mL of each solvent ethanol by maceration (3×24 h) at room temperature. The collected solvents were concentrated by rotary vacuum evaporator at 45°C and then dried using a freeze dryer. All extracts and acyclovir (extracted from commercial tablet) were dissolved in dimethyl sulphoxide (DMSO). The final concentration of DMSO was 0.1% v/v in cell culture environment.

*Phytochemical analysis*

The preliminary phytochemical evaluation of leaves was carried on extract prepared by successive extraction method in Soxhlet. The resultant extracts were evaporated to dryness under vacuum. These extracts were subjected to chemical test for different phytoconstituents viz. alkaloids, carbohydrates, phenolics, flavonoids, proteins, amino acids, saponins, mucilage and resins etc. Chemical tests were identifying the phytochemicals as described [16-18]. Alkaloids, carbohydrates, tannins and phenols, flavonoides, gums and mucilage, fixed oils and fats and saponins were qualitatively analyzed.

*HPLC – UV analysis (Total Phenols)*

*Avicennia marina* was subjected to solid phase extraction using column 5mm (4.6.mm) and peptides, small molecules were removed fractionation of neutral and acidic phenolic acids was also carried out simultaneously. The resulting fraction was then subjected to reverse phase high performance liquid chromatography (RP-HPLC). The total phenolics in *A. marina* was detected using, Stationary phase octadecylsil. Silica and mobile phase (A phosphoric acid: water (0.5: 99.5v/v) B acetonitrile). The UV detector was set at 220 nm with the flow rate adjusted to 1.0ms / min. The major peaks were identified and the retention times were compared with these of standards.

*Fractionation of total alkaloids*

*Avicennia marina* was detected using monobasic Phosphate as mobile phase (270ml. of Acetonitril). The liquid Chromatography is equipped with 235 nm detector & 4.6nm × 150 mm column. The flow rate was adjusted to 1.8ml / minute the major peaks were identified and the total alkaloids concentration were determined.

*Fractionation of total flavonoids*

HPLC Chromatography (System Name: LACKROM L-7000 MERCK, Proc Method – HITECHI) total flavonoids. The total flavonoids in the extract was determined by using octadecysil silica gel as stationary phase and acetonitril, sodium dihydrogen phosphate with dilute orthophosphoric acid as mobile phase. UV detector was set at 350nm with flow rate of 0.5ml/min. The major peaks in *Avicennia marina* were determined in comparison to the retention time of standards run at identical conditions.

*Gas chromatography (GC analysis of terpenoids)*

The terpenoids level was measured GC using capillary column coated with macrogol 20000R and nitrogen as carrier gas. The flame ionization detector was set at the flow rate of 0.4ml/min & used as standard.

*Diphenyl – 2- Picrylhydrazyl (DPPH) radical scavenging activity*

DPPH radical scavenging assay is a commonly recommended method for assessment of antioxidant potential of plant extracts. The assay is based on the ability of DPPH, a free radical which get decolorized in the presence of antioxidants. To 200ml of ethanolic solution of DPPH (1µg/ml) various concentration of (20mg –100 µg/ml) in water were added and incubated at 37°C for 30 min in dark and the absorbance was measured at 517nm. Ascorbic acid was used as the reference standard. The percentage scavenging of DPPH free radical was calculated and compared with that of the standard ascorbic acid. The $IC_{50}$ value also determined.

*Superoxide anion scavenging activity*

The method was applied for the measurement of *Avicennia marina* superoxide anion scavenging activity, briefly 312µm Nitroblue tetarzolium in 120 µm phosphate buffer 74 were added to an aliquots of *A. marina* (20-100µg/ml) the reaction was started by adding 100ml of phenazinemethosulphate (120mm prepared in phosphate buffer pH 7.4) and the colour change was monitored at 560nm against water blank quercetin was used as the positive control.

*Nitric oxide scavenging activity*

The nitric oxide scavenging activity of the alcoholic extract was measured by taking various concentrations of *Avicennia marina* and standard. Ascorbic acid (20-100µg/ml) dissolved in phosphate buffer (0.025m, pH 7.4) and incubated with sodium nitroprusside (5mm) in standard phosphate buffer at 25°C for 5 hrs. After the incubation, 0.5ml of the reaction mixture was added with 0.5ml of Griess reagent (equal volume of 1% sulphanilamide in 2% phosphoric acid and 0.1% napthlthyl ethylene diamine dihydrochloride in water). The absorbance of the chromophore formed was read at 540nm. The activity was compared with that of similar concentration of ascorbic acid.

## RESULTS AND DISCUSSION

The result of phytochemical screening of the ethanolic extracts of *Avicennia marina* revealed that the presence of

alkaloids, flavonoids, phytosterols, tannins and phenols (Table 1). The plant extract of *Avicennia marina* used for the present work was choosing on the basis of their medicinal values. Previous study in the naturally the ethanolic extracts of *Avicennia spp*. were subjected for phytochemical analysis. Phytochemical screening of the crude extract revealed that the presence of alkaloids, cardiac glycosides, terpenoids, saponins, tannin, flavonoids and steriods, but reducing sugars, carbonyl (aldehyde) and Phlobatanin show negative results [19].

Table 1 Qualitative phytochemical screening on extracts of *Avicennia marina*

| S. No | Name of Test | Test applied / Reagent used | Ethanol |
|---|---|---|---|
| 1. | Alkaloids | A] Mayer's | +++ |
| | | B] Wagner's | +++ |
| | | C] Hagner's | +++ |
| | | D] Dragndorff's test | ++ |
| 2. | Flavonoids | HCl and magnesium turnings | +++ |
| 3. | Carbohydrate | Molisch's test | ++ |
| 4. | Tannins and Phenols | A] 10% Lead acetate | +++ |
| | | B] $Fecl_3$ | +++ |
| 5. | Test for Steroids | A] Salkowski's Test | ++ |
| | | B] Libermann-Burchard's Test | ++ |
| 6. | Gums and Mucilages | Alcoholic Precipitation | - |
| 7. | Fixed oil and Fats | Spot test | + |
| 8. | Saponins | Foam test | ++ |
| 9. | Phytosterols | LB test | + |
| 10. | Volatile oils | Hydro distillation method | + |
| 11. | Protein and free amino acids. | A] Biuret test | +++ |
| | | B] Ninhydrin test | +++ |
| | | C] Xanthoprotein test | +++ |

These plants growing under natural conditions contain the spectrum of secondary metabolites such as phenols, flavanoids, quinones, coumarins, tannins and their glycosides, alkaloids, essential oils etc., the importance of these substance as microbial agents against the pathogen has been emphasized by Sofowora [20]. In the present study, it was clearly understood that the ethanolic extracted maximum amount of the different type of metabolites present in the *Avicennia marina*. Boominathan and Ramamurthy [21] reported that the phytochemical analysis of the *Heliotropium indicum* and *Chorizanthe procumbens* extracts showed the presence of tannins, alkaloids, flavonoids and phenolic compounds. Tannins have been found to form irreversible complexes with proline-rich proteins.

For instance, the presence of flavonoids suggest that the plant have been reported to exert multiple biological effects including, anti-allergic, anti-inflammatory, anti- microbial antioxidant, anti- cancer activity [22]. It also suggests that the plant might have diuretic properties [23]. The presence of tannins shows that the plant is astringent as documented and suggests that it might have antiviral and anti-bacterial activities and can relief in wound healing and burns [24]. Saponins and glycoside are also very important classes of secondary metabolites as some are cardio-active and used in treatment of heart conditions [25]. Some researchers have also investigated that some saponins have anti-cancer and immune modulatory properties [26]. Volatile oils are used in the industries for various purposes, both as a pharmaceutical/ cosmetic raw material for production of emollients and active ingredient for the respiratory tract infections.

Preliminary quantities of phytochemical screening of ethanolic extract of *Avicennia marina* revealed the presence of alkaloids, flavonoids, terpenoids and phenolic compounds which are essential to prevent diseases and to maintain a state of wellbeing. Recent studies have been focused on finding the natural substance of medicinal plant that decrease the inflammation and reduce oxidative stress and there by counteracting the macromolecular damage. It is well known that reactive oxygen species interact with key bimolecular such as proteins and enzymes which regulate major metabolic path way and decrease their functional efficiency. (Table 2) shows that *Avicennia marina* contains rich amount of bioactive compounds which exhibit antioxidant property the quantitative analysis revealed that *Avicennia marina* contain rich amount of phenolic compounds and flavonoids. It is well known that plant flavonoids and phenols in general are highly effective in scavenging free radical and providing antioxidant defense in living cells. Polyphenols and flavonoids isolated from medicinal plants have been used for the prevention and cure of various diseases which are mainly associated with free radicals.

Table 2 Quantitative phytochemical analysis of *Avicennia marina*

| S. No. | Phytochemicals | Quantity mg/gm of dry material |
|---|---|---|
| 1. | Alkaloids | 2.541 |
| 2. | Terpenoids | 0.785 |
| 3. | Total phenols | 5.615 |
| 4. | Gallic acid | 2.813 |
| 5. | Cinnamic acid | 0.548 |
| 6. | Coumaric acid | 0.436 |
| 7. | Flavonoids | 2.215 |
| 8. | Rutin | 0.298 |
| 9. | Quercetin | 0.528 |

HPLC analysis reveals that the extract was found to be rich in Alkaloids (2.541 mg/g) terpenoids (0.785mg/g) and total phenols (5.615 mg/g). *A. marina* also contain flavonoids such as Rutin (0.298mg/g) and quercetin (0.528 mg/g) many reports demonstrate that antioxidant principle present in medicinal plants are responsible for their therapeutic potential [27]. The flavonoic compound such as quercetin and Rutin are formed to be responsible for pharmacological properties proliferate by their terminating action of free radicals [28]. Alkaloids have many pharmacological activities including anti-cancer and anti arthythmic effect [29]. Alkaloids are known to reduce the inflammation level significantly. The present study the extract of *A. marina* may responsible for the antioxidant and anti-inflammatory effects.

Table 3 Free radicals scavenging activity in ethanolic extract of *Avicennia marina*

| Free radicals | | Concentration of Standard and extract in (µg/ml) | | | | |
|---|---|---|---|---|---|---|
| | | 20 | 40 | 60 | 80 | 100 |
| DPPH | Ascorbic acid | 47.5 | 56.8 | 74.7 | 84.9 | 91.7 |
| | *Avicennia marina* | 39.4 | 45.7 | 55.8 | 72.7 | 79.1 |
| Nitric oxide | Ascorbic acid | 43.8 | 49.2 | 55.7 | 68.6 | 79.5 |
| | *Avicennia marina* | 37.2 | 41.9 | 49.8 | 58.7 | 67.2 |
| Superoxide | Quercetin | 35.8 | 48.9 | 56.2 | 65.8 | 79.4 |
| | *Avicennia marina* | 33.1 | 44.8 | 52.1 | 62.6 | 71.8 |

It may lead to oxidative stress. The Natural phytonutrients presents in leaves vegetables scavenge the free radicals and protect the cells from oxidative damages. The phytonutrients present in *A. marina* which is responsible for the traditional claim by the test drug. Reactive oxygen species and free radicals known as super oxide anions, hydroxy radicals, hydrogen peroxide are the major class of highly reactive species derived from normal all metabolism of major nutrients [30]. These highly reactive free radicals if not counteracted and inactivated by cellular antioxidants. The DPPH is decolorized nature it receives electron or hydrogen atom from antioxidants and extend of decolorization represents the antioxidant potential of the test compounds. The result obtained in these investigation shows that *A. marina* possess a potent scavenging activity against DPPH radicals. The scavenging activity was comparable to that of standard ascorbic acid.

Super oxide anion scavenging activity of *A. marina* was found to possess comparable free radical scavenging activity against super oxide anions when compared to that of standard quercetin. The superoxide onions are toxic intermediates formed during inflammatory process and found to enhance the risk of inflammation related disorders such as arthritis and atherosclerosis. Super oxide anion is a free radio that plays an important role in the formation of reactive oxygen species such as hydrogen periode, hydroxyl / radicals, or singlet oxygen in living organism. Reported that the therapeutic activity of medicinal plants can be determined by superoxide activity were reported by Korycka and Richardson [31].

Nitric Oxide scavenging activity is an important chemical mediator generated by endothelial cells, macrophages, neuron and it is involved in the regulation of various physiological processes like control of arthritis, cytotoxice effects [32]. No formation is toxic to living organism and it was found that *A. marina* significantly scavenges the nitric oxide and the effect was comparable to that of standard Ascorbic acid at similar concentration with $IC_{50}$ value (52.45µg/ml and 45.85 µg/ml) of and respectively.

## CONCLUSION

The result of preliminary phytochemical screening shows the presence of flavonoids such as quercetin and rutin, phenolic compounds and alkaloids in the plant. A large number of these compounds are known to possess strong antioxidant properties. The free radical scavenging activity of *A. marina* revealed that they can be used for the prevention or treatment of human diseases such as cancer, arthritis, diabetes mellitus which are associated with oxidative stress.

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