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DETERMINATION OF SECONDARY METABOLITES AND ANTIOXIDANT ACTIVITY OF *AVICENNIA GERMINANS*

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ABSTRACT
To quantify the major secondary metabolites and the antioxidant potential of ethanolic leaf extract of *Avicennia germinans*. The ethanolic leaf extract of *Avicennia germinans* 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. germinans* was found to contain alkaloids, terpenoids, phenols and flavonoids. The major flavonoid detected was quercetin and rutin. The *Avicennia germinans* was found to possess significant radical scavenging activity against DPPH, NO and superoxide anions the IC_{50} value of 42.0 µg/ml, 42.0µg/ml and 42.6µg/ml respectively and comparable to that of their corresponding IC_{50} value. The medicinal property of *A. germinans* 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*.

KEYWARDS: *Avicennia germinans*, Phytochemicals, free radical scavenging activity.

INTRODUCTION
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.,[1] Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects.[2] 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.[3] Inflammation is a disorder characterized by invasion of leucocytes and production of proinflammatory cytokines.[4]

Medicinal plants are the source of many potent and powerful drugs. The plant derived drugs are healthier and safer alternate to the synthetic drugs.[5] 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.[6] Even today compounds from plants continue to play a major role in primary health care as therapeutic remedies in many developing countries.[7] Standardization of plant materials is the need of the day. Several pharmacopoeia containing monographs of the 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.[8] 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.

Avicennia germinans L. is a mangrove plants belongs to the family Rhizophoraceae. Mangrove forest can decay into peat deposits because of fungal and bacterial processes as well as by the action of termites. It becomes peat in good geochemical, sedimentary and tectonic conditions. *Avicennia germinans* or black mangroves occupy different ecological niches and have slightly different chemical compositions so the carbon content

various between the species as well as different tissues of the plant leaves and roots. The plant useful to diuretic, febrigual and anti-inflammatory effects, cure swellings of the skin, leprosy, laxative, sore eyes, sore throats leaves are used as human food, as medicine for infected wounds.[9] Traditionally, it has been used in anemia but there is no scientific proof to support this claim. Keeping this in view, the present study has been undertaken to investigate the phytoconstituents present in ethanolic leaves extract of *Avicennia germinans*.

MATERIALS AND METHODS

Collection and Authentication of Experimental Plant

The mangrove plant of *Avicennia germinans* leaves were collected from Muthupet mangrove, Tamil Nadu, South India. The leaves were identified with the help of flora of presidency, Tamil Nadu and Karnatic flora[10-11] and standard references.[12]

Preparation of Extract

The dried and powdered leaves of *Avicennia germinans* (500 g) were extracted using soxhlet extractor by evaporating with 75% ethanol. The soxhlet extraction was carried out for 3 days and the extract was collected. The excess ethanol was evaporated by using vacuum evaporator. The sample is evaporated to dryness under boiling water bath at 55°C.

Phytochemical Analysis

The preliminary phytochemical evaluation of leaves was carried on extract prepared by successive extraction method in Soxhlet. The previously dried powdered (50 gm) were extracted in a Soxhlet apparatus with ethanol successively. The resultant extracts were evaporated to dryness under vacuum. These extract 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[13-15] Alkaloids, carbohydrates, tannins and phenols, flavonoides, gums and mucilage, fixed oils and fats and saponins were qualitatively analyzed.

HPLC – UV analysis (Total Phenols)

Avicennia germinans was subjected to solid phase extraction using column 5mm (4.6.mm) & 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 *Avicennia germinans* 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 germinans was detected using monobasic Phosphate as mobile phase (270ml. of Acetonitril). The liquid Chromatography is equipped with 235 nm detector & 4.6nm x 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 germinans* 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.

Free radical scavenging activity

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 37^0C 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[16]

The method was applied for the measurement of *Avicennia germinans* superoxide anion scavenging activity, Briefly 312µm Nitroblue tetarzolium in 120 µm phosphate buffer 74 were added to an aliquots of *A. germinans* (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 germinans* 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 25^0C 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.[17]

RESULT AND DISCUSSION

The result of phytochemical screening of the alcoholic extracts of *Avicennia germinans* revealed that the presence of alkaloids, flavanoids, phytosterols, tannins and phenols (Table 1). The plant extract of *Avicennia germinans* 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.[18]

This 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 several workers.[13] In the present study, it was clearly understood that the alcohol extracted maximum amount of the different type of metabolites present in the *A. germinans*. Boominathan and Ramamurthy[19] reported that the phytochemical analysis of the *H. indicum* and *C. procumbens* extracts showed the presence of tannins, alkaloids, flavonoids and phenolic compounds. Tannins have been found to form irreversible complexes with proline-rich proteins.

Table 1: Qualitative Phytochemical screening on extracts of *Avicennia germinans*

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

-, absents; +, present;

Preliminary quantities of phytochemical screening of ethanolic extract of *A. germinans* revealed the presence of alkaloids, flavonoids, terpenoids and phenolic compounds which are essential to prevent diseases and to maintain a state of well being. 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 *A. germinans* contains[20] rich amount of bioactive compounds which exhibit antioxidant property the quantitative analysis revealed that *A. germinans* 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: Quantiative Phytochemical Analysis

S. No.	Phytochemicals	Quantity mg/gm of dry material
1.	Alkaloids	1.45
2.	Terpenoids	0.88
3.	Total phenols	5.27
4.	Gallic acid	4.34
5.	Cinnamic acid	0.41
6.	Coumaric acid	0.32
7.	Flavonoids	1.07
8.	Rutin	0.224
9.	Quercetin	0.662

HPLC analysis reveals that the extract was found to be rich in Alkaloids (1.45 mg/g) terpenoids (0.88mg/g) and phenols phenolcs (5.27 mg/g). *A. germinans* also contain flavonoids such as Rutin (0.224mg/g) and quercetin (0.662 mg/g) many reports demonstrate that antioxidant principle present in medicinal plants are responsible for their therapeutic potential.[21] The flavonoic compound such as quercetin and Rutin are formed to be responsible for anti-inflammatory and anticancer properties proliferate by their terminating action of free radicals.[22] Alkaloids have many pharmacological activities including anti cancer & anti arthythmic effect.[23] Alkaloids are known to reduce the inflammation level significantly. The present study the extract of *A. germinans* may responsible for the antioxidant and anti-inflammatory effects.

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. germinans* 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.[24] These highly reactive free radicals if not counteracted and inactivated by cellular antioxidants. The DPPH is decolourised nature it receives electron or hydrogen atom from antioxidants and extend of decolorisation represents the antioxidant potential of the test compounds. The result obtained in these investigation shows that *A. germinans* possess a potent scavenging activity against DPPH radicals. The scavenging activity was comparable to that of standard ascorbic acid.

The IC_{50} value of *A. germinans* (42.0µg/ml) was found to be nearer to that of standard ascorbic acid (55 µg/ ml) super oxide anion scavenging activity. Calculate the percentage inhibition = (optical density of Control- Optical density of Test / Optical density of Control) x 100.

Super oxide anion scavenging activity of *A. germinans* was found to possess comparable free radical scavenging activity against super oxide anions when compared to that of standard quercetin. The IC_{50} value was found to be (42.0 ug / ml and in 61.9ug/ml) for MIT respectively. Calculate the percentage inhibition = (optical density of Control- Optical density of Test / Optical density of Control) x 100.

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.[25]

Nitric Oxide scavenging activity is an important chemical mediator generated by endothelial cells, macrophages, neuron & it is involved in the regulation of various physiological processes like control of arthritis, cytotoxice effects Alzhemer's disease.[26] No formation is toxic to living organism and it was found that *A. germinans* significantly scavenges the nitric oxide and the effect was comparable to that of standard Ascorbic acid at similar concentration with IC_{50} value (42.6µg/ml and 56.8 µg/ml) of and respectively. Nitric oxide scavenging activity of *B. oleracea* and standard Ascorbic acid are calculated the percentage inhibition = (optical density of Control- Optical density of Test / Optical density of Control) x 100.

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. germinans* 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.

Table 3: Free Radicals scavenging activity in *Avicennia germinans*

Free radicals		Concentration of Standard and extract in (µg/ml)				
		20	40	60	80	100
DPPH	**Ascorbic Acid**	48.5	54.2	75.7	85.3	92.1
	B.oleracea	38.8	44.3	57.1	70.5	77.9
Nitric Acid	**Ascorbic Acid**	42.9	48.7	54.4	67.6	77.3
	B.oleracea	43.1	48.2	59.3	65.8	71.1
Superoxide	**Quercetin**	25.2	38.4	46.1	52.9	69.3
	B.oleracea	38.7	40.2	58.1	62.3	89.5

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