Phytochemical Constituents And Antibacterial Activity Of Leaf Extract Of White Mangrove, *Avicennia marina* (Forsk.) Vierh.

Surya Shekhar Das
Assistant Professor of Botany
Department of Botany
Bolpur College, West Bengal, India

ABSTRACT

Studying plant-based antimicrobial properties provides additional information in developing natural antibiotics and discovering the alternative of antimicrobial drugs for the treatment of infectious diseases. Traditionally, mangrove plants have been used medicinally in diverse cases like to treat infections, relief pain and purify blood and as antioxidant. Avicennia marina is commonly used for treatment of ulcers, rheumatism, small pox and other ailments Phytochemical constituents from Avicennia marina leaf extracts were determined qualitatively. Crude extracts of the plants under study were screened for the presence of alkaloids, phenolics, flavonoids, tannins, diterpenes, triterpenes, sterols, saponins, glycosides, proteins, carbohydrates and reducing sugars. Avicennia marina was tested for bioactivity against seven bacteria.

Key words: *Avicennia marina*; mangrove; phytochemicals; bioactivity.

INTRODUCTION

Plants produce many bioactive compounds, alkaloids, phenolic compounds and terpenoids being the most important ones. These chemicals constitute the defense system against various diseases and stress conditions.

Currently, the indiscriminate use of commercial antimicrobial drugs has caused multiple drug resistance in human pathogenic microorganisms (Aliero *et al*., 2008). In addition to this problem, antibiotics are sometimes associated with adverse effects including hypersensitivity, immune-suppression, and allergic reactions on the host (Nebedum *et al*., 2009). This situation forced scientists to search for new and effective antimicrobial agents to replace the current practice (Jacquelyn, 2002). Plants remain the most common source of traditional health remedies and is reported to have minimal side effects (Safary *et al*., 2009).

Traditional records and ecological diversity indicate that Indian plants represent an exciting resource for possible lead structures in drug design. Mangrove plants possess novel chemical compounds many of which are biologically active and have medicinal values (Bandaranayake, 2002). Recently scientists are veering in search of effective remedies from mangroves for diseases such as diabetes, asthma, cancer, ulcer, wounds and AIDS (Itigowa et al., 2001). Extracts from different mangrove plants are reported to contain diverse medicinal properties (Agoramoorthy *et al*., 2007) and are active both against human pathogens and plant pathogens (Chandrasekaran *et al*., 2009). However, the majority of these plants have not yet undergone chemical, pharmacological and toxicological studies to investigate their bioactive compounds (Singh A. *et al*., 2009).

Avicennia marina is commonly used for treatment of ulcers (Subashree *et al*., 2010), rheumatism, small pox and other ailments (Bandaranayake *et al*., 2002). Some studies were done about the antiparasitic, antifungal and antibacterial activity of *Avicennia marina* (Abeysinghe, 2010; Khafagi *et al*., 2003; Sheela Devi *et al*., 2012). *A. marina* have been shown to exhibit marked inhibitory effect on mouse skin tumor promotion (Itigowa,2001).

MATERIALS AND METHODS

Sample Collection Areas

The plant samples were collected from different sites of the mangrove forest of Indian Sundarban (between 21˚40'04"N - 22˚09'21"N latitude, and 88˚01'56"E - 89˚06'01"E longitude) located in the South 24-Paragnas District of West Bengal, India.

Plant material

Leaves of the plant mentioned in Table 1 were collected randomly from different parts of the above mentioned sample collection areas and were subsequently used for conducting tests.

Table 1: Some attributes of the plant species used in the present study

Scientific Name	Family	Habit	Flowering Period	Fruiting Period	Local Name
Avicennia marina (Forsk.) Vierh.	Avicenniaceae	Tree	April to June	June to August	Peyara Baen

Sample Collection and Processing

Fresh and healthy leaves of the plants mentioned in table 1 were collected from the above mentioned sample collection areas and used for the present investigation. The leaves were then washed with sterile distilled water to make it free from dirt and dust, quickly mopped and dried on blotting sheets. These leaves were then shade dried at room temperature for 10 days. Shade dried leaves were then cut into small pieces and crushed to obtain powder with the help of a mechanical grinder.

Extraction

50 grams of mangrove leaf powder was added to 250 ml. of methanol. After 48 h of incubation in the shaker, the supernatant was collected and freed from solvent by evaporation under reduced pressure. The residues (crude extracts) obtained were finally dried under vacuum, stored in sterile dark containers and were tested for their phytochemical profile and antimicrobial activities.

Preliminary Qualitative Phytochemical Analysis

Phytochemical constituents from the mangrove sample extracts were determined qualitatively. Crude extracts of the plants under study were screened for the presence of alkaloids, phenolics, flavonoids, tannins, diterpenes, triterpenes, sterols, saponins, glycosides, proteins, carbohydrates and reducing sugars using the methods adopted from different authors (Hanaa et al., 2008; Panda et al., 2012).

Figure 1: Leaves of *Avicennia marina* after seven days of shade drying.

Evaluation of Antibacterial Activity

Microorganisms, culture media and incubating temperatures

All of the different extracts were individually tested against a panel of bacteria including Gram negative *Erwinia herbicola* (MTCC NO. 3609) incubated at 37^0C, *Escherichia coli* (MTCC-443) incubated at 37^0C, *Serratia marcescens* (MTCC NO. 7298) incubated at 30^0C, *Xanthomonas* sp. (MTCC NO. 7444) incubated at 30^0C and Gram positive *Arthrobacter chlorophenolicus* (MTCC NO. 3706) incubated at 28^0C, *Bacillus subtilis* (MTCC-441) incubated at 37^0C, *Staphylococcus aureus* (MTCC-96) incubated at 35^0C. All the bacterial strains were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The reference strains of bacteria were maintained on nutrient agar medium and LB medium slants at 4^0C with a subculture period of 30 days.

Preparation of McFarland standard

The turbidity standard was prepared by mixing 0.5 ml of 1.75% (w/v) $BaCl_2.2H_2O$ with 99.5 ml of 1% H_2SO_4, $BaSO_4$ (v/v). The standard was taken in screw cap test tube to compare the turbidity. The bacterial culture of selected strains were grown for 48- 72 hours and subsequently mixed with physiological saline. Turbidity was corrected by adding sterile saline until McFarland 0.5 $BaSO_4$ turbidity standard 10^8 Colony Forming Unit (CFU) per ml was achieved. These inoculates were used for seeding of the nutrient agar medium, LB medium respectively.

Disc diffusion assay

1 mg of each sample was separately dissolved in 1 ml of propylene glycol and then the volume was adjusted to 10 ml by adding sterile water. The ultimate concentration reaches to 10^2 µg/ ml and sterilized by filtration (0.22 µm filter). From the solution of each concentrated sample(s) final concentrations were made from 500 µg/ ml to 100 µg/ ml by adding sterile double distilled water. The sterile paper discs (6 mm diameter) were saturated with 10 µl of the solution of the respective sample(s) at a concentration of 500 µg/ ml to 100 µg/ ml and placed on the inoculated agar of 10^8 CFU/ml. Antibacterial tests were then carried out by disc diffusion method (Sokmen et al., 2004) using 100 µl of suspension containing 10^8 CFU/ml of bacteria on nutrient agar medium, LB medium respectively. Negative controls were prepared using propylene glycol. Gentamicin (10 µg/ disc) was used as positive reference standards to determine the sensitivity of each bacterial species tested. The inoculated plates were incubated at 30^0 C, 37^0 C and 28^0 C respectively for 48 h, 24 h and 72 h. Antibacterial activity was evaluated by measuring the zone of inhibition and the diameters of these zones were measured in millimeters against the test organisms.

Determination of Minimum inhibitory concentration

The minimal inhibitory concentration (MIC) values were studied for the bacteria strains, being sensitive to the sample(s) in disc diffusion assay. The inoculates of the bacterial strains were prepared from 24-72 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The samples were dissolved in sterile propylene glycol, first diluted to the highest concentration (500 µg/ml) to be tested, and then serial dilutions were made in order to obtain a concentration range from 500 to 100 µg/ml in 10 ml sterile test tubes containing nutrient broth and LB broth medium respectively. MIC values of the sample(s) against bacterial strains were determined based on a micro well dilution method. The plate was covered with a sterile plate sealer and then incubated at appropriate temperatures for 24 - 72 h at 30^0 C, 37^0 C, 30^0 C and 28^0 C respectively. Bacterial growth was determined by absorbance at 600 nm and confirmed by plating 10 µl samples, forming clear wells on nutrient agar medium or LB medium respectively. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms. Each test in this study was repeated, at least, thrice.

RESULTS AND DISCUSSIONS

Results of Phytochemical Analysis and Discussions

The results of the preliminary phytochemical analysis of crude extracts of the mangrove plant under study to reveal the presence or absence of alkaloids, phenolics, flavonoids, tannins, diterpenes, triterpenes, sterols, saponins, glycosides, proteins, carbohydrates and reducing sugars are summarized in Table 2. *Avicennia marina* extract contains alkaloids, phenolics, flavonoids, tannins, diterpenes, triterpenes, saponins and carbohydrates but lacks glycosides, proteins reducing sugars and sterol.

Plants produce a large diverse array of organic compounds that appear to have no direct function in their growth and development. These compounds are known as secondary metabolites or natural products. They have no generally recognized direct roles in the process of photosynthesis, respiration, solute transport, translocation, protein synthesis, nutrient assimilation or differentiation or the formation of the primary metabolites – carbohydrates, proteins, nucleic acids and lipids. Secondary metabolites also differ from primary metabolites in having a restricted distribution within the plant kingdom. That is, certain secondary metabolites are only found in one plant species or related group of species, whereas primary metabolites are found throughout the plant kingdom.

These compounds, for many years, were thought to be simply functionless end product of metabolism, or metabolic wastes. Today we know the adaptive significance and ecological function of most secondary metabolites.

From the study, it is clear that *Avicennia marina* is phytochemically diverse. These metabolites protect plants against mammalian herbivores, insect herbivores, bacterial and fungal pathogens. Some of them mediate plant-microbe symbiosis. Some are responsible for allelopathy. Again, some attract pollinators and seed disperser. The ability of plants to compete and survive is therefore profoundly affected by these phytochemicals.

Table 2: Qualitative phytochemical profile of the plant under study

Sl. No.	Phytochemicals screened	Tests done	Present(+) /Absent(-)
1	Alkaloids	a.Mayer's test b.Wagner's Test c. Hager's Test d. Dragendroff's test	+ + + +
2	Phenolics	.Ferric chloride test	+
3	Flavonoids	a.Ferric chloride test b.Lead acetate test	+ +
4	Tannins	a.Ferric chloride test b.Gelatin test	+ +
5	Diterpenes	a. Copper acetate test	+
6	Triterpenes	a.Salkowski test b. Lieberman Burchardt test c.Tschugajen test	+ + +
7	Sterols	a .Salkowski Test b. Lieberman Burchardt test	– –
8	Saponins	a. Foam test b. Haemolysis test	+ +
9	Glycosides	a.Sodium hydroxide reagent b.Kellar Killani's test	- -
10	Protein	Millon’s test	-
11	Carbohydrate	Molisch’s test	+
12	Reducing sugar	Fehling’s test	-

Results of Antibacterial Assay and Discussions

Different secondary metabolites from the groups namely terpenoids, phenolics and alkaloids are known for their antibacterial activity and the phytochemical analysis of extract of the studied plant species showed the presence of one or the other group(s). So, we can expect some antibacterial properties in the extract. This study revealed the preliminary evidence of antibacterial ability of leaf extracts in methanol.

The details of antibacterial activity of the plant extracts are given in the following tables (Table 3 and Table 4) and Figure 2 & 3.

Table 3: Antibacterial activity of the methanol extracts of *Avicennia marina* against the bacteria tested based on disc diffusion method

Name of the bacterium	Antibacterial potentiality of the crude extract
Arthrobacter chlorophenolicus	No
Bacillus subtilis	Yes
Staphylococcus aureus	Yes
Erwinia herbicola	Yes
Escherichia coli	No
Serratia marsescens	Yes
Xanthomonas campestris	Yes

Mangrove species with medicinal properties are also harvested as herbal remedies by coastal communities in some countries including India. Traditionally, mangrove plants have been used medicinally in diverse cases like to treat infections (TriSiam, 2011), relief pain and purify blood (Singh & Suttee, 2009) and as antioxidant (Chan et al., 2011). Alkaloids, terpenoids, sterols and phenolics were reported to exhibit different biological activities (Kubmarawa *et al*., 2008). For example, saponins are known to associate with hypercholesterolemia, hyperglycemia (Rupasinghe *et al*., 2003), anticancer, anti-inflammatory activities. Plant steroids are known for their anti-inflammatory (Akindele and Adeyemi, 2007), analgesic (Malairajan *et al*., 2006), anti-microbial activities and ability to act on central nervous system (Argal and Pathak, 2006). Tannins have been widely recognized for their pharmacological properties. These are well studied for anti-diabetic, anti-inflammatory and anti-bacterial activities. Flavonoids are known for their antifungal and antibacterial properties.

Table 4: Assessment of antibacterial potentiality of the crude extract of *Avicennia marina*

Name of the bacterium	Concentration of extract (µg/ml)				
	100	**200**	**300**	**400**	**500**
Arthrobacter chlorophenolicus	No	No	No	No	No
Bacillus subtilis	No	No	No	No	3.2mm*#
Staphylococcus aureus	3.2mm*#	4.1mm*	4.8mm*	5.3mm*	5.7mm*
Erwinia herbicola	No	4.3mm*#	4.7mm*	5.0mm*	5.2mm*
Escherichia coli	No	No	No	No	No
Serratia marsescens	No	5.2mm*#	8.2mm*	10.7mm*	12mm*
Xanthomonas campestris	No	4mm*#	5.6mm*	7.1mm*	8.1mm*

***Diameter of inhibition zone around the discs impregnated with extracts.**
No- means no antibacterial activity of the extract.
#the corresponding concentration of extract represents the minimal inhibitory concentration (MIC) value.

Figure 2: *Avicennia marina* extract against *Bacilus subtilis*

Figure 3: *Avicennia marina* extract against *Xanthomonas campestris*

The leaf extract was tested for antimicrobial activity against seven bacterial species used in the study. The extract of *Avicennia marina* showed very broad-spectrum activity and was effective against five of the seven bacteria tested namely *Bacillus subtilis*, *Staphylococcus aureus*, *Erwinia herbicola*, *Serratia marsescens* and *Xanthomonas campestris* with the MIC values of 500 µg/ml, 100 µg/ml, 200 µg/ml, 200 µg/ml and 200 µg/ml, respectively. The difference in sensitivity and MIC values (which is a measure of degree of effectiveness) may be due to the qualitative difference of the phytochemicals present and/or concentration of the active principles present in different plant extracts. This result is very promising in that the extract was active against most of the tested bacteria and more importantly against both Gram positive and Gram negative bacteria. The plant extracts were unable to exhibit antibacterial activity against two of the tested bacteria. These bacteria may have some kind of resistance mechanisms e.g. enzymatic inactivation, target sites modification and decrease intracellular drug accumulation or the concentration of the extract used may not contain sufficient amount of active principle(s).

In recent years, screening of mangrove plants has been done to search for a variety of biologically active compounds. This work is a successful attempt of phytochemical characterization and antimicrobial efficiency of the mangrove plant studied. Further attention should be paid to develop the novel drugs from these resources of natural products. The plant studied here can be seen as a potential source of useful drugs. It also justifies the folklore medicinal uses and the claims about the therapeutic values of this plant as curative agent and I, therefore, suggest further the isolation, identification, purification, characterization and elucidation of the structure of the bioactive compounds of the plants that would be obtained with a view to obtain useful chemotherapeutic agent.

ACKNOWLEDGEMENTS

I am grateful to the Head, Department of Botany, University of Kalyani, West Bengal for providing central equipment facility funded by DST-FIST.

REFERENCES

1. Abeysinghe, P,D.,(2010). Antibacterial activity of some medicinal mangroves against antibiotic resistant pathogenic bacteria. Indian Journal of Pharmaceutical Sciences, 72, 167-172.
2. Agoramoorthy G, Chandrasekaran M, Venkatesalu V, Hsu MJ. (2007). Antibacterial and antifungal activities of fatty acid methyl esters of the blind-your eye mangrove from India. Braz J Microbiol. 38:739–42.
3. Akindele AJ and Adeyemi OO. (2007). Antiinflammatory activity of the aqueous leaf extracts of *Byrsocarpus coccineus.* Fitoterapia. 78, 25–28.
4. Aliero A, Aliero BL, Buhari U. (2008). Preliminary phytochemical and antibacterial screening of *Scadoxus multiflorus*. Int J Pure Appl Sci. 2: 13-17.
5. Argal A and Pathak AK. (2006). CNS activity of *Calotropis gigantea* roots. J.Ethnopharmacol. 106, 142-145.
6. Bandaranayake WM. (2002). Bioactivities, bioactive compounds and chemical constituents of mangrove plants. Wetlands Ecology and Management. 10:421–52.
7. Chan, E.W.C., Lye, P.Y. & Tan, L.N. (2011): Analysis and evaluation of the antioxidant properties of Thai herbal teas. *International Journal for the Advancement of Science and Arts*, **2**, 8–15.
8. Chandrasekaran M, Kannathasan K, Venkatesalu V, Prabhakar K. (2009). Antibacterial activity of some salt marsh halophytes and mangrove plants against methicillin resistant Staphylococcus aureus. World J Microbiol Biotechnol. 2:155–60.
9. Hanaa, H Abd El- Baky., Farouk, K. El Baz and Gamal, S. El. Baroty. (2008), Evaluation of marine algae *Ulva lactuca*. L as a source of natural preservative ingredient. American- Eurasian J. Agric & Environ. Sci., 3 (3): 434- 444.
10. Itigowa M, Ito C, Tan HT, Okuda M, Tokuda H, Nishino H, Furukawa H (2001): Cancer chemopreventive activity of naphthoquinones and their analogs from *Avicennia* plants. Cancer Lett. 174: 135-139.
11. Jacquelyn GB. (2002). Microbiology principles and exploration. 5th ed. USA: John Wiley and Sons Inc.
12. Khafagi I, Gab-Alla A, Salama W, Fouda M, (2003). Biological activities and phytochemical constituents of the gray mangrove *Avicennia marina* (Forsk.) Vierh. Egypt Journal of Botany, 5: 62-69.
13. Kubmarawa D, Khan ME, Punah AM and Hassan. (2008). Phytochemical screening and antibacterial activity of extracts from *Parkia clappertoniana* keay against human pathogenic bacteria. J. Med. Pl. Res. 2(12), 352-355.
14. Malairajan P, Geetha G, Narasimhan S and Jessi Kala Veni K. (2006). Analgesic activity of some Indian medicinal plants. J. Ethnopharmacol. 19, 425-428.
15. Nebedum J, Ajeigbe K, Nwobodo E,Uba C, Adesanya O, Fadare O, et al. (2009). Comparative study of the ethanolic extracts of four Nigerian plants against some pathogenic microorganisms. Res J Med Plant. 3: 23-28.

16. Panda, S.K., Pati,D., Mishra, S.K., Sahu, S., Tripathy, B., Nayak, L. (2012), Phytochemical investigation and antimicrobial activity of methanolic extract of *Sonneratia apetala* aerial parts. International journal of Pharmaceutical and biological archives, 3(1) : 79-83.

17. Rupasinghe HP, Jackson CJ, Poysa V, di Berado C,Bewley JD and Jenkinson J.(2003). Soyasapogenol A and B distribution in Soybean (*Glycine max (*L.) Merr.) in relation to seed physiology, genetic variability and growing location. J. Agric.Food Chem. 51, 5888-5894.
18. Safary A, Motamedi H, Maleki S and Seyyendnejad SM. (2009). A preliminary study on the antibacterial activity of Quercus brantii against bacterial pathogens, particularly enteric pathogens. Intl. J. Botany. 5(2),176–180.

19. Sheela Devi, A., Johanna Rajkumar, M Rajathi D Modilal, R.Ilayaraja (2012), Antimicrobial activities of *Avicennia marina*, *Caesalpinia pulcherrima* and *Melastoma malabathricum* against clinical pathogens isolated from UTI. Int J Pharm Bio Sci 3(3): 698 – 705.

20. Singh, A., Duggal, S. & Suttee, A. (2009): *Acanthus ilicifolius* Linn. – Lesser known medicinal plants with significant pharmacological activities. *Ethnobotanical Leaflets*, 13, 431–436.

21. Subashree, M., Mala, P., Umamaheswari, M., Jayakumari, M., Maheswari, K.,Sevanthi, T., and Manikandan, T., (2010).Screening of the antibacterial properties of *Avicennia marina* from Pichavaram mangrove. International Journal of Current Research, 1, 016-019.

22. TriSiam (2011): Herbal tea. The Home of Siamese Herbal Products. http://www.trisiam.com/herbaltea.htm.