INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com Volume-3, Issue-4, Oct-Dec-2012 Coden : IJABPT Copyrights@2012

ISSN : 0976-4550

Received: 19th Oct-2012 *Revised: 27th Oct-2012* *Accepted: 28th Oct-2012*

Research Article

ANTIBACTERIAL ACTIVITY AND PHYTOCHEMICAL SCREENING OF LEAVES AND STEM EXTRACTS OF *AVICENNIA ALBA* BLUME

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ABSTRACT: The present work was attempted to study the Antibacterial activity and phytochemical analysis of Mangrove plant *Avicennia alba.* Leaf and stem extracts of *A. alba* were prepared in Hexane, Benzene, Chloroform, Ethyl acetate, Acetone and Methanol solvents. The resulted extracts of the plant were screened for antibacterial activity against *Micrococcus luteus* MTCC 106, *Arthrobacter protophormiae* MTCC 2682, *Rhodococcus rhodochrous* MTCC 265, *Alcaligens faecalis* MTCC 126, *Proteus- mirabilis* MTCC 425, *Enterobacter aerogenes* MTCC 10208, *Proteus vulgaris* MTCC 426, *Bacillus megaterium* MTCC 428, *Enterococcus faecalis* MTCC 439, *Streptococcus mutans-* MTCC 497, *Salmonella enterica* MTCC 3858, *Staphylococcus aureus* MTCC 737, *Pseudomonas aeruginosa* MTCC 1688 and *Bacillus subtilis* MTCC 441. The extracts were also screened for phytochemicals like Carbohydrates, Tannins, Steroids, Terpenoids, Saponins, Flavanoids, Alkaloids and Soluble starch. Of the six solvent extracts of *A. alba*, ethyl acetate and acetone extracts of leaf and stem, with few exceptions, showed relatively high antibacterial activity. Benzene and chloroform extracts of *A. alba* showed a larger zone of inhibition against *Salmonella enterica* than other bacteria. *A. alba* leaf and stem extracts of different solvents showed good antibacterial activity against Gram negative bacteria than the Gram positive bacteria tested. Most of the solvent extracts of leaf and stem are effective on many bacteria tested than the positive control. The acetone and methanol extracts of leaf and stem showed maximum positive results towards the phytochemical constituents.

Key words: Leaf extract, Stem extract, *A. alba*, Antibacterial activity, Phytochemicals.

INTRODUCTION

Mangrove forests are among one of the world's most productive tropical ecosystems and are highly potential because the ecosystem is always under stress which leads to the production of certain compounds for their survival. India harbors some of the best mangrove forests of the world which are located in the alluvial deltas of the major rivers such as the Ganga, Mahanadi, Godavari, Krishna, Cauvery and also on the bay of Andaman and Nicobar Islands (Mishra et al., 2005; Kathiresan and Rajendra, 2005; Thatoi and Biswal, 2008; Upadhyay et al., 2008; Upadhyay and Mishra, 2008; Mandal and Naskar, 2008). It covers about 6,749 sq km along the 7,516.6 km long coast line, including Island territories (Mandal and Naskar, 2008). *Avicennia alba* is a species of tropical mangrove belonging to the family *Acanthaceae*. The common local name for this plant is "Ilva mada". This species is found far away from salt water, unlike other species. *Avicennia alba* is a rich source of naphthoquinones (Ito et al., 2000) and leaves of *A.alba* tested for antioxidant activities showed positive result (Banerjee et al., 2008). The bark and seeds of *A. alba* are used as a fish poison and resin used in birth control, ulcers treatment, skin diseases and also used to cure tumors (Bandaranayake, 1998). It provides a rich source of steroids, triterpenes, saponins, flavonoids, alkaloids and tannins (Patra et al., 2010). This work aimed at screening of the plant, *Avicennia alba*, for its phytochemical constituents and antibacterial activity and to look into the possibility to explore it as a potential source of bioactive principle.

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MARTERIALS AND MEDHODS

Avicennia alba was collected from Coringa forest, Kakinada, Andhra Pradesh. After thorough washing, the leaves and stems were dried completely under shade and ground into coarse powder. The leaf and stem powders were successively extracted in different solvents viz., hexane, benzene, chloroform, ethyl acetate, acetone and methanol in a soxlet apparatus. The resulted extracts were concentrated by using roto evaporator. The crude extracts collected were preserved in air tight containers for further use.

Bacterial strains used

The antibacterial activity of the crude extracts was determined on eight Gram positive and six Gram negative bacteria. Strains viz., *Micrococcus luteus* MTCC 106, *Arthrobacter protophormiae* MTCC 2682, *Rhodococcus rhodochrous* MTCC 265, *Alcaligens faecalis* MTCC 126, *Proteus- mirabilis* MTCC 425, *Enterobacter aerogenes* MTCC 10208, *Proteus vulgaris* MTCC 426, *Bacillus megaterium* MTCC 428, *Enterococcus faecalis* MTCC 439, *Streptococcus mutans* MTCC 497, *Salmonella enterica* MTCC 3858, *Staphylococcus aureus* MTCC 737, *Pseudomonas aeruginosa* MTCC 1688 and *Bacillus subtilis* MTCC 441 were used in the study.

Antibacterial screening:

Antibacterial activity of the samples was screened by using agar well diffusion method (Sharief and Umamaheswara Rao, 2011). Bacterial suspensions of different bacteria were prepared by using 24 hours old bacterial cultures. About 0.3 ml of the each bacterial suspension was mixed in separate 15 ml aliquots of sterilized molten state nutrient agar medium and poured into oven sterilized Petri dishes to prepare nutrient agar plates. After solidification, wells were bored in each plate by using sterile cork borer of 6 mm diameter. A minute quantity of sterile agar suspension was added to the well before dispensing 100 µl of the sample which was prepared by dissolving 100mg of sample in 1 ml of DMSO. In a separate well, DMSO was also dispensed to maintain the control. The plates were incubated at 37^{o} C for 24 hrs. After incubation, the diameter of the zone of the inhibition was measured. For each sample and bacterial species, triplicates were maintained. Streptomycin standard antibiotic was used as positive control in the concentration of 10µg/ml DMSO.

Phytochemical analysis:

The crude extracts were subjected to preliminary phytochemical analysis by following standard protocols from Pharmacopia for identification of different phytochemical constituents.

1) Test for flavanoids

a) Ferric chloride test

About 2ml of the test solution was boiled with distilled water and then filtered. Then, few drops of 10% ferric chloride solution was added to the 2 ml of filtrate. A green-blue or violet coloration indicates the presence of a phenolic hydroxyl group.

b) Shinoda's test

About 0.5gm of each extract was dissolved in ethanol, warmed and then filtered. Small pieces of magnesium chips were then added to the filtrate followed by few drops of conc. HCl. The pink, orange, or red to purple coloration indicates the presence of flavonoids.

c) Sodium hydroxide test

About 0.2gm of the each extract was dissolved in water and filtered; to this 2 ml of the 10% aqueous sodium hydroxide was added to produce a yellow coloration. A change in color from yellow to colourless on addition of dilute hydrochloric acid was the indication for the presence of flavonoids.

d) Lead acetate test

About 0.5gm of the each extract was dissolved in water and filtered. To the 5 ml of each filtrate, 3 ml of lead acetate solution was then added. Appearance of a buff-colored precipitate indicates the presence of flavonoids.

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2) Test for alkaloids

About 0.5 g of crude powder was stirred with 1% aqueous HCl on water bath and then filtered. To the 1 ml filtrate few drops of dragendroff's reagent was added. Orange- Red precipitate was taken as positive. To another 1 ml filtrate few drops of Mayer's reagent was added and appearance of buff- colored precipitate will be taken as presence of alkaloids.

3) Test for soluble starch

Small quantity of crude extract was boiled with 1 ml of 5% KOH, cooled and acidified with H_2SO_4. Yellow coloration indicates the presence of soluble starch.

4) Test for Saponins

About 0.5 g of crude powder was shaken with water in a test tube and it was warmed in a water bath. The persistent froth indicates the presence of saponins.

5) Test for terpenoids

Small quantity of crude extract was dissolved in ethanol. To this, 1 ml of acetic acid was added followed by the addition of conc.H_2SO_4. A change in color from pink to violet confirms the presence of terpenoids.

6) Test for steroids

a) Salkowskii test

About 0.2 g of each extract was dissolved in 2 ml of chloroform, followed by the addition of conc. H_2SO_4 to form reddish brown color at interphase indicates the presence of steroids.

b) Keller-Killiani test

To 0.5 ml of test solution, 2 ml of 3.5% $FeCl_3$, small amount of glacial acetic acid and 2 ml of conc. H_2SO_4 were added carefully. Appearance of reddish brown ring at inter phase is a positive indication for the presence of steroids.

c) Liebermann-Burchard test

To 0.2 g of each extract, 2 ml of acetic acid was added and the solution was cooled well in ice followed by the addition of conc. H_2SO_4 carefully. Color development from violet to blue or bluish-green indicates the presence of a steroidal ring (i.e. aglycone portion of cardiac glycoside).

7) Test for carbohydrates

a) Molisch's test

About 0.2 ml of *Molisch*'s reagent was added to each of the extract dissolved in distilled water and 1 ml of conc. H_2SO_4 was dispensed along the walls of the test tube. The mixture was allowed to stand for two minutes and then diluted with 5 ml of distilled water. Formation of a dull violet color at the interphase of the two layers indicates the positive test for carbohydrates.

b) Fehling's test

The crude extracts were treated with 5.0 ml of Fehling's solution (A & B) and kept in boiling water bath. The formation of yellow or red color precipitate indicates the presence of free reducing sugars.

c) Fehling's test (for Combined Reducing Sugars)

About 0.5 g of each extract was hydrolyzed by boiling with 5 ml of dilute hydrochloric acid and the resulting solution neutralized with sodium hydroxide solution. To this, few drops of Fehling's solution were added and then heated on a water bath for 2 minutes. Appearance of a reddish-brown precipitate of cuprous oxide indicates the presence of combined reducing sugars.

d) Barfoed's test (for monosaccharides)

About 0.5 g of each extract was dissolved in distilled water and filtered. To 1 ml of the filtrate, 1 ml of Barfoed's reagent was added and then heated on a water bath for 2 minutes. Reddish precipitate of cuprous oxide formation is the positive test for the presence of monosaccharides.

8) Test for tannins

About 0.5 g of each extract was stirred with about 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate. Occurrence of a blue-black, green or blue-green precipitate indicates the presence of tannins.

a) Borntrager's Test

About 0.2 g of each extract to be tested was shaken with 10 ml of benzene and then filtered. To the filtrate, 5 ml of the 10% ammonia solution was added and then shaked the tube well. Appearance of pink, red or violet color in the ammoniacal (lower) phase was taken as the presence of free anthraquinones.

b) Phlonatanins test

About 0.2g of each extract to be tested was added with 1% HCl solution. Formation of red precipitate indicates the presence of tannins.

RESULTS & DISCUSSION

The plant analyzed in this study is commonly used as medicinal plant from longer time in India and some parts of the world. Agar well diffusion method was performed in the present study for antibacterial activity screening, as diffusion method allows better diffusion of the extracts into the medium than disc method. Paper discs may act as a barrier between the extract and the organisms, thus preventing total diffusion of active components absorbed by the discs into the medium and may reflect in antibacterial activity. In the present study, the antibacterial activity of the extracts of leaves, and stem of *A. alba* in different solvents was tested against 14 bacteria including 8 Gram positive and 6 Gram negative by using Agar well diffusion method. The data related to the antibacterial activity of *A. alba* leaves and stem are presented in Tables-1 and 2 and the results revealed that all the extracts exhibited anti bacterial activity against all bacteria tested except *M. luteus.* Hexane and benzene extracts of *A. alba* leaves showed better activity against *A. protophormiae* than the positive control. All the extracts of *A. alba* leaves expressed good growth inhibitory activity against *R. rhodochrous* compared to positive control. Positive control (streptomycin) did not show any activity against *R. rhodochrous* and *E. faecalis*.. Ethyl acetate and acetone fractions gave good results than the positive control against *E. faecalis.* Benzene extract and chloroform extracts of *A. alba* leaves showed a larger inhibition zone against *S. enterica* with almost 3-fold and 2-fold more activity, respectively than the positive control. Hexane, chloroform, ethyl acetate, acetone and methanol extracts of *A.alba* leaves showed larger inhibition zone than the positive control against *A. faecalis.* Benzene, ethyl acetate and acetone extracts of *A. alba* leaves showed greater activity than positive control against *Proteus mirabilis.* The leaf extracts of *A. alba* in chloroform, ethyl acetate, acetone and methanol solvents exhibited more antibacterial activity than the positive control against *P. aeruginosa.* Different solvent extracts of *A. alba* leaves showed more antibacterial activity on Gram negative bacteria than the Gram positive bacteria. Benzene extract of *A. alba* stem showed more antibacterial activity than the positive control against *A. protophormiae*, *B. subtilis*,*R. rhodochrous* and *P. vulgaris*, and same activity to that of positive control against *S. mutans* and *S. aureus.* All the solvent extracts of the *A. alba* stem showed the activity against *Rhodococcus rhodochrous* than the positive control. Benzene, chloroform, ethyl acetate and acetone extracts were effective equally and/or more against *B. subtilis* and *S. aureus*. *A. faecalil*, *P.mirabilis* and P.aeruginosa were found to be more susceptible to chloroform, ethyl acetate, acetone and methanol extracts of *A. alba* stem than positive control. In this present investigation, *A. alba* leaf and stem extracts of different solvents showed good antibacterial activity on majority of Gram negative bacteria than Gram positive bacteria. This antibacterial property could be attributed to the presence of bioactive phytochemicals in *A. alba* plant.

The data on phytochemical analysis of *A. alba* leaf and stem extracts are shown in Tables-3 and 4. Results revealed the presence of different classes of phytochemicals viz., Carbohydrates, Tannins, Steroids, Terpenoids, Saponins, Flavanoids, Alkaloids and Soluble starch in the leaf and stem extracts of *A. alba* in different proportions. Acetone and Methanol extracts of both leaf and stem displayed more phytochemicals tested for than the other extracts. Ethyl acetate extracts of both leaf and stem were found positive only for flavonoids.

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Table-1. Antibacterial activity of Avicennia *alba* Leaf extracts in different solvents

S.No.	Test Organism	Diameter of Zone of inhibition (mm)						Positive Control
		H	B	C	E	A	M	
1.	*Micrococcus luteus* MTCC 106	--	--	--	4.3	5.3	--	13
2.	*Arthrobacter protophormiae* MTCC 2682	9.3	8	4.6	4	4.6	3	7
3.	*Rhodococcus rhodochrous* MTCC 265	5	4.3	7.6	9	7	5	--
4.	*Bacillus megaterium* MTCC 428	3	2	2	5	5	4	7
5.	*Bacillus subtilis* MTCC 441	4	3.6	4.6	5.6	5.3	3	7
6.	*Enterococcus faecalis* MTCC 439	4.3	5	5.3	7.3	7.3	6.3	7
7.	*Streptococcus mutans* MTCC 497	3.3	7	6.6	6.3	6	3.6	8
8.	*Staphylococcus aureus* MTCC 737	4	3.3	6.3	5	5	4	6
9.	*Alcaligens faecalis* MTCC 126	5	4.3	5.6	7	6.7	6	5
10.	*Proteus mirabilis* MTCC 425	4.6	7	4.6	7	6.3	5	6
11.	*Proteus vulgaris* MTCC 426	5.3	4	8	9	7	6	7
12.	*Enterobacter aerogenes* MTCC 10208	4	3	3.6	5	5.3	3.6	--
13.	*Salmonella enterica* MTCC 3858	3.3	14	10.7	4.3	4	3.6	5
14.	*Pseudomonas aeruginosa* MTCC 1688	6	--	8.3	8.3	7.3	9	7

H - Hexane; B - Benzene; C - Chloroform; E - Ethyl Acetate; A - Acetone; M - Methanol

Table-2. Antibacterial activity of Avicennia *alba* Stem extracts in different solvents

S.No.	TEST ORGANISM	Diameter of Zone of inhibition (mm)						Positive Control
		H	B	C	E	A	M	
1.	*Micrococcus luteus* MTCC 106	--	4.6	6.3	6	9	4	13
2.	*Arthrobacter protophormiae* MTCC 2682	5.6	8.3	3	4	5	6	7
3.	*Rhodococcus rhodochrous* MTCC 265	3.6	6.6	6	7.3	9	4.3	--
4.	*Bacillus megaterium* MTCC 428	3	3	4.3	5	6	3.6	7
5.	*Bacillus subtilis* MTCC 441	4	8.6	7	7	9	4	7
6.	*Enterococcus faecalis* MTCC 439	3.3	3.7	7	6.6	8.6	6	7
7.	*Streptococcus mutans* MTCC 497	2.6	8	5.3	6.6	6.6	3	8
8.	*Staphylococcus aureus* MTCC 737	4	6	6	5.6	7	4	6
9.	*Alcaligens faecalis* MTCC 126	3	4.6	8	7	7.6	5.6	5
10.	*Proteus mirabilis* MTCC 425	2	4	8	6.3	8	5.3	6
11.	*Proteus vulgaris* MTCC 426	3.7	7.3	6.3	6	7	4.3	7
12.	*Enterobacter aerogenes* MTCC 10208	3	4.3	4.6	5.3	4.3	3.3	--
13.	*Salmonella enterica* MTCC 3858	2.3	2.3	2.6	6	7.3	3.6	5
14.	*Pseudomonas aeruginosa* MTCC 1688	3	4.6	8	7	7.6	5.6	7

H - Hexane; B - Benzene; C - Chloroform; E - Ethyl Acetate; A - Acetone; M - Methanol

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Table-3. Phytochemical analysis of *Avicennia alba* leaf extracts in different solvents

S.No.	Phytochemicals	H	B	C	E	A	M
1.	Carbohydrates	+	+	+	--	+	+
2.	Monosaccharides	--	--	--	--	++	++
3.	Free reducing sugars	+	--	--	--	++	++
4.	Combined reducing sugars	+	--	--	--	--	--
5.	Tannins	--	--	--	--	+	+
6.	Free anthraquinones	--	--	+	--	--	--
7.	Steroids	++	--	+	--	++	++
8.	Cardiac glycosides	++	--	++	--	++	++
9.	Terpenoids	--	+	+	--	++	++
10.	Saponins	--	--	--	--	--	--
11.	Flavonoids	+	++	+	+	++	++
12.	Soluble starch	--	+	+	--	+	--
13.	Alkaloids	+	+	--	--	+	--

H - Hexane; B - Benzene; C - Chloroform; E - Ethyl Acetate; A - Acetone; M - Methanol

Table-4. Phytochemical analysis of *Avicennia alba* stemextracts in different solvents

S.No.	Phytochemicals	H	B	C	E	A	M
1.	Carbohydrates	+	+	+	--	+	+
2.	Monosaccharides	--	--	--	--	--	++
3.	Free reducing sugars	+	--	--	--	--	++
4.	Combined reducing sugars	+	--	--	--	++	--
5.	Tannins	--	--	--	--	+	+
6.	Free anthraquinones	--	--	--	--	--	+
7.	Steroids	+	--	--	--	++	++
8.	Cardiac glycosides	--	--	--	--	--	+
9.	Terpenoids	+	--	--	--	++	++
10.	Saponins	--	--	--	--	++	++
11.	Flavonoids	+	--	+	+	++	++
12.	Soluble starch	+	--	--	--	+	--
13.	Alkaloids	+	+	+	--	+	+

H - Hexane; B - Benzene; C - Chloroform; E - Ethyl Acetate; A - Acetone; M – Methanol

Tannins were found only in acetone and methanol extracts of both leaf and stem. Steroids and cardiac glycosides were found in all leaf extracts, except in benzene and ethyl acetate. Free anthraquinones were observed only in methanol extract of stem and chloroform extract of leaf. On the whole, there is some variation in presence or absence and concentrations of various phytochemicals among the different solvent extracts of leaf and stem. This variation of phytochemicals may reflect the antibacterial potency of different solvent extracts of the plant. It is the known fact that phytochemicals of plants have been a good source of antibacterial agents. But, still many plants remained enexplored or under-explored (Rios and Recio, 2005). The profound chemical diversity within the plants gives an opportunity for the discovery of new drugs (Noor et al., 2010; Mandal et al., 2010). The present work on this plant needs further extension to characterize and identify the bioactive principle of this plant.

ACKNOWLEDGEMENT

Both the authors are thankful to University Grants Commission, New Delhi for the financial support.

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