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Antimicrobial activity of mangrove leaves against drug resistant pathogens

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Abstract: In the present study, aqueous and solvent extracts of leaves collected from nine mangrove plants were screened against Methicillin resistant *Staphylococcus aureus* (MRSA), Vancomycin resistant *Staphylococcus aureus* (VRSA) and Extended spectrum beta lactamase (ESBL) strains. Out of nine mangrove plants *Avicennia sp., Rhizophora sp., Ceriops decandra* and *Thillai species* showed antimicrobial activity against test pathogens. Methanolic extracts of *Avicennia sp.,* and *Rhizophora sp.,* showed 10 mm,9mm and 10mm, 13mm against *S. aureus* and *Pseudomonas aeruginosa,* respectively. Methanol extract of *Ceriops decandra* showed 12 mm inhibition against *S. aureus* and *Pseudomonas aeruginosa*. Methanol extracts of *Thillai sp.,* showed 10mm activity against *Pseudomonas sp*. The crude extract of *Avicennia sp.,* produced two spots in thin layer chromatography (TLC) when chloroform: methanol (60:40) used as a solvent system. In bioautography, the first spot (Rf value 0.65) showed activity. The active compound purified by preparative TLC showed maximum activity (15 mm inhibition) against MRSA. The methanolic crude extract of *Rhizophora sp.,* and *Ceriops decandra* produced Rf value 0.58 and 0.6 respectively. The active compound of *Rhizophora sp.,* and *Ceriops decandra* purified by TLC showed maximum activity of 18 and 16 mm activity against MRSA. Further isolation and characterization of active compounds is in progress.
Keywords: Antimicrobial, Mangrove, Extraction, Activity, Bioautography.

Introduction

There is a continuing change in the spectrum of pathogens that cause infection in humans. One among the various reasons for the increase in the incidence of infectious diseases is the emergence of multi-drug resistance among the pathogens. Among the various drug resistant pathogens, MRSA (Methicillin Resistant *Staphylococcus aureus*), VRSA (Vancomycin resistant *Staphylococcus aureus)* and ESBL (Extended spectrum β-lactamase) strains are in major concern. Extended spectrum β-lactamase (ESBL) is enzymes that mediate resistance to extended spectrum cephalosporin, cefotaxime, ceftriazone and ceftazidime and the monobactum aztreonam. Such enzymes are commonly found in *Escherichia coli*, *Klebsiella sp*., *Pseudomonas aeruginosa* and *Acinetobacter baumanii*[1].

To solve this problem all over the world scientists are searching various living resources for lead compounds for the development of novel drugs against multidrug resistant pathogens. A large portion of the world population depends mainly on traditional system of medicine for variety of diseases. Several hundreds of plants are used as of medicine and are a source of very potent and powerful drugs which is used for a long time and still being in use today[2]. Mangroves forests are among one of the world's most productive tropical systems.

Recently it has been strongly recommended that mangroves should be considered as a valuable source for chemical constituents with potential medicinal and agricultural values.

Although the chemical constituents of most mangrove plants still have not been studied, investigations have led so for to the discovery of several novel compounds with prospective medicinal value for the discovery of new chemotherapeutic agents[3]. Mangroves have been used in fisher folk medicine to treat diseases. Sixteen plants are possible source of anticancer drugs, a few mangrove species especially belongs to botanical family Rhizophoraceae are known to have high antiviral activity[4]. Acid polysaccharides extracted from *Rhizophora mucronata* are active at a very low concentration to suppress the drug resistant HIV strains[5]. From the available literature, it was known that countable number of works is reported on antimicrobial activity of mangrove extracts, but there are no reports on antimicrobial activity of mangrove extracts against drug resistant pathogens such as MRSA, VRSA and ESBL strains. With this view the present investigation was initiated for screening of selected mangrove plants for antimicrobial compounds against Multi Drug Resistance (MDR) bacterial pathogens.

Materials and Methods

Sample collection and preparation

In this present investigation, leaves samples were collected from 9 different mangrove plants (*Avicennia sp., Ceriops decandra, Brugeria s., Suaeda monica, Rhizophora sp., Susuvium sp., Thillai sp., Aegicerous sp.,* and *Lumnitgera racemosa)* to study their antimicrobial activity. All the plants were collected from Pichavaram mangrove (Lat. 11°20' to. 11°30'; Long. 79°45' to 79°55'). The freshly collected leaves were washed and dried in shade at room temperature for 10-15 days. The dried leaves were used for powdering by using mortar and pestle. The larger plant debris was removed and powdered leaves were used for extraction for antimicrobial compounds[6].

Antibiotic resistant pattern of test bacterial pathogens

In the present study *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* were obtained from Christian Medical College, Vellore, Tamil Nadu Antibiotic resistant pattern of test pathogens were confirmed by Kirby-Bauer disc diffusion method using different antibiotics.

Preparation of aqueous extracts

One gram of fresh leaves were taken and washed with sterile distilled water. The leaves were crushed by using mortar and pestle. The crushed leaf paste was mixed with 20ml of sterile distilled water in 50ml beaker. The aqueous leaf mixture was covered with aluminium foil and kept at room temperature for 24 hours. This procedure was adopted for the preparation of aqueous extracts from all the plants[6].

Preparation of solvent extracts:

Five different solvents such as methanol, n-hexane, ethyl acetate, dichloromethane and acetone were used for the extraction of antimicrobial compounds from leaf powder. One gram of dried plant powder was taken in a 50 ml beaker and 20ml of methanol was added into it. This content was mixed well and the beaker was covered with aluminium foil and kept for extraction at room temperature for 24 hours. The procedure was adopted for all the plants and extractions were done for other remaining solvents.

Separation of crude extracts:

After extraction, the aqueous and solvent mixture of leaf powder was filtered by passing through muslin cloth to remove debris. Further the filtered liquid portion is centrifuged at 5000 rpm for 15 minutes to remove fine debris which are not removed by filtration. The aqueous and solvent extracts were transferred to clean 25ml beaker. Crude compounds were concentrated by evaporation at room temperature and stored in small vials at 4°C until further use.

Antimicrobial activity of aqueous extracts:

The antimicrobial activity of mangrove aqueous extracts was studied by well diffusion method using Muller Hinton agar (MHA) plates. About 18 hours old bacterial culture was prepared and inoculated into MHA plates. 5 mm diameter well was cut on plates. Each 10µl of aqueous plant extracts were added in wells using micropipette. Ten µl sterile distilled water was used as a control well. All the plates were incubated at 37°C for 24 hours and plates were observed for zone of inhibition[6].

Antimicrobial activity of solvent extracts:

The antimicrobial activity of solvent extracts was studied by disc diffusion method using MHA plates. About 18 hours old bacterial cultures were inoculated into MHA plates. 0.25mg of crude extracts were added into sterile filter paper disc (5 mm diameter) and allowed to dry at room temperature for few minutes. Crude plant extract impregnated discs were placed on MHA plates inoculated with test bacterial strains. Sterile empty disc was used as a control. All the plates were incubated at 37°C for 24 hours. After incubation the plates were observed for zone of inhibition.

Partial purification of active compound by Thin Layer Chromatography

The crude compound was purified by using silica gel thin layer chromatography[7]. To find out the best solvent system to separate the crude compound, solvents such as methanol, chloroform, acetic acid, n-butanol, n-hexane and water were used in different proportions. After running, the sheet was kept at room temperature for the complete drying of the plate and the separated spots were visualized in iodine chamber. Rf value of the spots on the TLC plate was determined by;

$$\text{Rf value} = \frac{\text{Movement of the solute from the origin}}{\text{Movement of solvent from the origin}}$$

Autobiography

The bioautography method was for the detection of active compound separated in TLC[8]. Chromatogram developed was placed in a sterile bioassay petri dish containing nutrient agar medium inoculated with *S. aureus*. Active compound from methanol extract was further purified from preparative TLC and tested for antimicrobial activity by disc diffusion method as described earlier.

Result and Discussion

Microorganisms develop resistant to many antibiotics due to incriminate use of antibiotics. The emergence of MRSA, VRSA and ESBL producing strains of *E. coli, Klebsiella sp*., and *Pseudomonas aeruginosa*. This has created immense clinical problem in the treatment[9]. Nearly 2000 different plants are used for the medicinal preparations in India alone in which at least 100 species are serving as regular source of medicine[2]. The emergence of bacterial strains resistant to many currently used antibiotics make the need for fresh approaches to the treatment of infections. To solve this problem all over the world scientists are searching various living resources for lead compounds for the development of drugs against multidrug resistant pathogens.

Recently it has been strongly recommended that mangroves should be considered as a valuable source for chemical constituents with potential medicinal values[3]. In this present study 9 different mangrove plants were collected from Pichavaram mangrove ecosystem and tested against drug resistant bacteria pathogens (MRSA, VRSA and ESBL strains of *E.coli, Klebsiella sp.*, *Pseudomonas aeruginosa*).Antibiotic susceptibility of five test bacterial pathogens against selected antibiotics was represented in Table 1.

Table: 1: Antibiotic susceptibility of test bacterial pathogens

Antibiotics	S.aureus		E.coli		Klebsiella sp.		P. aeruginosa	
	IZ(in mm)	S	IZ (inmm)	S	IZ(in mm)	S	IZ(in mm)	S
Methicillin	-	R	-	R	-	R	-	R
Vancomycin	-	R	-	R	-	R	-	R
Amoxyclav	16	S	13	S	10	S	12	S
Ceftriaxone	13	S	16	S	-	R	-	R
Cefotoxmine	-	R	-	R	-	R	-	R
Carbapenem	-	R	-	R	-	R	-	R

IZ – inhibition of zone; S- sensitive; R- resistant

Based on the observation of antibiotic susceptibility pattern, *Staphylococcus aureus* was found to be resistant against methicillin and vancomycin. *Escherichia coli* and *Pseudomonas aeruginosa* was found to be resistant against fourth generation antibiotics like cefatoxime and carbapenem. Based on the observation, both *Escherichia coli* and *Pseudomonas aeruginosa* are considered to be as ESBL producing pathogens.

Antibacterial activity of aqueous and methanol extracts:

Antimicrobial activity of mangrove *Avicennia marina* aqueous and ethanol extracts against pathogens (*E. coli*, *S. aureus*, *Klebsiella sp*., *Pseudomonas sp*.).[10]Among the aqueous extract prepared from leaves of 9 mangrove plants, extracts from four plants such as *Avicennia sp., Brugaria sp., Ceriops sp*., and *Rhizophora sp*., showed activity against tested drug resistant pathogens especially against *S. aureus* and *Pseudomonas aeruginosa*.

In this present study, solvents such as ethyl acetate, methanol, n-hexane, acetone and dichloromethane) were tested for extraction of crude compound. Among the various solvents tested, the crude compounds were extracted only in methanol but not in other solvents.

In this present study, all the crude extracts were tested for antibacterial activity by disc diffusion method. Methanol extract of *Avicennia* and *Rhizophora sp.,* showed 10 mm,9mm and 10mm, 13mm against *S. aureus* and *Pseudomonas aeruginosa,* respectively. Methanol extract of *Ceriops decandra* showed 12 mm inhibition against *S. aureus* and *Pseudomonas aeruginosa*. Methanol extracts of *Thillai sp*., showed 10mm activity against *Pseudomonas sp*. Methanol extracts from remaining plants does not showed activity against any of the bacterial strains tested. In most of the previous studies, it was reported that methanol extract of mangrove plants showed good antimicrobial activity. Methanolic extract of *Aegceras*, *Aeginiliris* and *Cynometra* showed good activity against fish pathogens[11].

Partial purification of active compound by Thin Layer Chromatography and Autobiography

Among the different solvent systems tested in various proportions, chloroform: methanol in 60:40 showed good separation and two spots were observed when exposed into iodine vapor for *Avicennia* species. The Rf value of the separated spots were calculated as 0.65 and 0.85, respectively for Avicennia. Rf values for other extracts were represented in table 2. In bioautography, the first spot (Rf value 0.65) showed activity against the test organisms.

Table. 2. Partial purification of crude extraction and its autobiography

Methanol extraction	Rf values	Active spot
Avicennia species	0.65 and 0.85	0.65
Rhizophora species	0.58	0.58
Ceriops decandra	0.6	0.6

Bioautography allows localizing the antimicrobial activity of an extract on the chromatogram, it supports quick search of new antimicrobial agent through bioassay guided fractionation. This method also avoids the previous purification of the substance and reducing the cost of initial screening[12]. The active substance purified by preparative TLC showed maximum activity (15-18 mm) against test pathogens when compared to crude extract. (Table 3)

Table.3. Antimicrobial activity of crude extract and partially purified compound

Mangrove Plant	Zone of inhibition in mm for MRSA	
	Crude extract	**Partially purified by TLC**
Avicennia species	10	15
Rhizophora species	13	18
Ceriops decandra	12	16

Conclusion

The findings of the present study was found that mangrove plants will be a potential source for production of novel bioactive compounds against drug resistant organisms like MRSA, VRSA and ESBL producing pathogens. Chemical characterization, structure elucidation, of active compound are in further studies.

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