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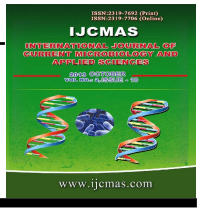

Original Research Article

Assessment of antimicrobial potential of endophytic bacteria isolated from *Rhizophora mucronata*

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Antimicrobial activity; *Rhizophora mucronata*; bacterial strains.

A B S T R A C T

The present study was carried out to find out the antimicrobial activity of endophytic bacteria isolated from *Rhizophora mucronata*. A total of 26 endophytic bacterial strains were identified from the tissues of plant. Of them, 5 strains showed broad-spectrum of antimicrobial activity against fungal and bacterial pathogens and were identified as genus of *Serratia, Bacillus, Pseudomonas, Micrococcus* and *Enterobacter* through biochemical characterization.

Introduction

Mangroves are salt-tolerant, marshy plants, which act as primary producers in the estuarine food chain. They are the most fragile but highly productive ecosystems along the coastal areas forming an economic, ecological, scientific and cultural resource. Mangroves have very special adaptations that enable them to live in salt waters. They produce novel metabolites unique to the environment with various important economic and environmental functions (Bandarnayake, 2002).

Microbes that inhabit asymptomatically in the living tissues of plants without causing any substantive negative effect are known as endophytic microbes (Bacon *et al*., 2000). Each plant species that exists on earth is considered to be a host for one or more endophytes. Diversity also associated with the colonizing bacterial taxa. The study of endophytic bacteria is a challenging field of research even though the attempts to use endophytic bacteria for the production of bioactive compounds have been promising.

Many bioactive compounds are produced from mangroves but reports on the usefulness of the mangroves as the source of microbial endophytes are scarce. Mangrove fungi constitute the second largest ecological group of marine fungi (Sridhar, 2004). More than 200 species of endophytic fungi have been isolated and identified from mangrove and these belong mainly to the genera *Alternaria,*

Aspergillus, Cladosporium, Fusarium, Pencillium and *Trichoderma* (Liu *et al.*, 2007). Since the bioprospecting of microbial endophytes from mangroves is at the stage of infancy efforts should be taken for judicious and gainful utilization of mangrove endophytes.

It is the need of hour to search for new antimicrobial agents because infectious diseases are still a global problem because of the development and spread of drug-resistant pathogens. Major active compounds used in medicines are obtained from microbes. The endophytic microbes were well studied in terrestrial plants which are found to possess antibacterial, antifungal, anticancer, antimalarial, antiviral, antioxidant and antidiabetic activities. Mangrove endophytic fungi are known to produce metabolites such as antraquinones, eniatin G, and xyloketals. But, isolation effort of endophytic bacteria for antimicrobial activity from mangrove plants is limited. Medicinal properties of different parts of mangrove plant may be fully or partially dependent on the endophytes.

Present study illustrates the details of the isolation and characterization of endophytic bacterial strains of *Rhizophora mucronata* for antimicrobial activity.

Materials and Methods

Sample Collection and Location

Samples were collected from mangrove forest situated in chettuva backwaters, Thrissur, Kerala. Healthy leaf, stem and root samples of mangrove species *Rhizophora mucronata* were collected in sterile bags and transported to the laboratory aseptically.

Isolation of endophytic bacteria

Collected samples were washed in running tap water followed by 70% ethanol, 2% Sodium hypochlorite and distilled water respectively and air dried under a laminar-flow hood for surface sterilization. The outer tissues were removed and the inner tissues were macerated with distilled water using mortar and pestle. 1ml of macerated sample was serially diluted and 100µl of each dilution was inoculated on nutrient agar medium by spread plate method (Gayathri *et al*., 2010). Plating was done in triplicates and incubated at 37°C for 48 hours. After attaining visible growth, the heterotrophic bacterial colonies were selected. All the isolated colonies were sub cultured in nutrient agar plates and stored at 4°C.

Assay of Antimicrobial Activity

Test organisms

Bacterial and fungal pathogens used in the study were given in the table (1), obtained from the Culture Collection Centre, Department of Microbiology, PSG Medical College Hospital, Coimbatore, Tamil Nadu. The bacteria were maintained in Nutrient agar (NA) and Sabouraud's dextrose agar (SDA) slants are used for the maintenance of fungi.

Preparation of extracts

Endophytic bacterial strains were inoculated into 100 ml of sterile nutrient broth and kept at 37±2°C for 24 hrs with continuous shaking. Then 20 ml of grown culture was transferred into 1000 ml of sterile nutrient broth and incubated at 37±2°C for 5 days under continuous shaking at 150 rpm/min.

Mass cultivated cultures were centrifuged and the supernatant was mixed with equal volume of ethyl acetate (1:1) in a separating funnel and after vigorous shaking, the organic phase was collected and subjected for evaporation.

Assay of antibacterial activity

The antimicrobial activity was screened by agar well diffusion method (Azoro *et al*., 2002). Mueller Hinton agar plates (for bacteria) and SDA plates (for fungi) were prepared and swabbed with 24 hours old broth culture of respective test organism. Using the sterile cork borer, the well (6mm) was made in to each Petri plate and added 10µl of ethyl acetate extract. Then the plates were incubated at 37±2°C for 24hours (for bacteria) and 48 hours (for fungi). After the incubation period, the diameter of inhibition zones of each well was measured and the values were noted. Triplicates were maintained in each extract and the average values were calculated for the eventual antimicrobial activity.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of all the extracts was determined for each of the test organisms in triplicates at varying concentrations of 1.0, 0.5, 0.25, 0.125, 0.0625mg/ml. To obtain these concentrations, 1 ml of varying concentrations of the extracts containing double strength of the concentrations (2.0, 1.0, 0.5, 0.25, 0.125 mg/ml) and 1 ml of nutrient broth was added into test tubes and a loopful of the test organism was introduced to the tubes. A tube containing nutrient broth only was seeded with the test organism to serve as control. All the tubes were then incubated at 37^{o}C for 24 hrs and then examined for growth by observing turbidity (Ajaiyeoba *et al.*, 2003). The MIC endpoints were the lowest drug concentrations that showed no growth.

Determination of minimum microbicidal concentration (MMC)

The MMC of the extracts were determined as described by Espinel-Ingroff *et al.*, (2002). After 72 hrs of incubation, 20 µl was sub cultured from each tube that showed complete inhibition onto NA and SDA plates for bacteria and fungi respectively. The plates were incubated until growth was seen in the positive control. The MMC was the lowest drug concentration that showed either no growth or fewer than three colonies to obtain approximately 99 to 99.5% killing activity.

Characterization of Bacteria

Endophytic bacterial strains BL6, BS7, BR7 BR1 and BS1 were identified based on gram staining, endospore staining and biochemical tests according to Cappucino and Sherman (2004).

Results and Discussion

Mangroves are salt tolerant plants existing at the interface between land and sea in the tropical and subtropical latitudes. Although presence of endophytic fungi in mangroves is known their endophytic bacteria and their bioprospecting potential is largely unknown for Indian mangroves. Present study was carried out to isolate and characterize endophytic bacteria for antimicrobial activities. In the study total of 26 heterotrophic endophytic bacterial colonies were isolated from the mangrove species *Rhizophora mucronata* (Table.2). Gayathri *et al.* (2010) reported isolation of

102 endophytic bacterial colonies from 5

Table.1 Bacterial and fungal pathogens used in the study

Sl.No	Test organisms	
	Bacteria	**Fungi**
1	*Escherichia coli*	*Aspergillus niger*
2	*Shigella* sp	*Candida albicans*
3	*Yersinia enterocolitica*	*Candida tropicalis*
4	*Vibrio cholerae*	*Rhizopus* sp
5	*Salmonella typhimurium*	*Trycophyton rubrum*
6	*Streptococcus pyogenes*	*Malassesia furfur*
7	*Pseudomonas aeruginosa*	
8	*Klebsiella pneumoniae*	

Table.2 Number of endophytic bacterial strains from *Rhizophora mucronata*

Plant part	**Leaf**	**Stem**	**Root**
No of colonies	10	7	9

Table. 3 Assay of antimicrobial activity

Sl.No	Test organism	Zone of inhibition in mm				
		BL6	**BS7**	**BR7**	**BR1**	**BS1**
1	*E.coli*	15±0.0	-	15±0.0	10±0.6	-
2	*Shigella* sp	-	6±0.0	-	-	-
3	*Y. enterocolitica*	-	-	-	-	-
4	*V.cholerae*	12±0.0	-	14±0.0	14±0.0	8±0.3
5	*S. typhimurium*	12±0.0	9±0.2	14±0.3	13±0.0	6±0.0
6	*S.pyogenes*	10±0.0	-	17±0.8	18±0.0	6±0.4
7	*P.aeruginosa*	18±0.0	6±0.0	14±0.4	16±0.0	8±0.0
8	*K.pneumoniae*	15±0.0	-	13±0.8	9±0.2	10±0.0
9	*A. niger*	14±0.5	8±0.3	10±0.6	14±0.8	19±0.3
10	*C. albicans*	20±0.7	6±0.6	17±0.0	19±0.6	-
11	*C. tropicalis*	14±0.0	-	16±0.6	18±0.6	-
12	*Rhizopus* sp	21±0.0	-	17±0.6	17±0.8	-
13	*T. rubrum*	23±0.0	7±0.0	20±0.5	20±0.5	14±1.5
14	*M. furfur*	18±0.5	-	26±0.0	19±0.0	17±0.0

Table.4 MIC and MMC of selected extracts against test organisms

Sl.No	Test organism	MIC (mg/ml)			MMC (mg/ml)		
		BL6	BR7	BR1	BL6	BR7	BR1
1	*E.coli*	0.5	0.25	-	0.5	0. 5	-
2	*Shigella* sp	-	-	-	-	-	-
3	*Y. enterocolitica*	-	-	-	-	-	-
4	*V.cholerae*	0.5	0.5	0.5	0.5	0.5	0.5
5	*S. typhimurium*	0.5	0.25	0.5	1.0	0. 5	0.5
6	*S.pyogenes*	0.5	0.25	0.125	0.5	0.25	0.25
7	*P.aeruginosa*	0.125	0.5	0.25	0.12	0.5	0.25
8	*K.pneumoniae*	0.5	0.5	1.0	1.0	1.0	1.0
9	*A. niger*	0.5	0.5	0.5	0.5	0.5	0.5
10	*C. albicans*	0.125	0.125	0.125	0.25	0.125	0.25
11	*C. tropicalis*	0.5	0.5	0.25	0.5	0.5	0.25
12	*Rhizopus* sp	0.125	0.50	0.5	0.25	0.50	0.5
13	*T. rubrum*	0.125	0.25	0.125	0.25	0.5	0.5
14	*M. furfur*	0.25	0.0625	0.25	0.25	0.062	0.25

Table.5 Differential characteristics of selected strains

Characteristics studied **BS1**	**BL6**	**BS7**	**BR7**	**BR1**	
Gram staining	-	+	-	+	-
Shape	rod	rod	rod	cocci	rod
Endospore	-	+	-	-	-
Motility	+	+	+	-	+
Urea hydrolysis	-	-	-	-	-
Catalase	+	+	+	+	+
Methyl red	-	-	-	-	-
VP test	+	+	-	-	+
Citrate	+	-	+	+	+
Indole	-	-	-	-	-
Utilization of carbohydrates					
i Glucose	+	+	-	-	+
v. Sucrose	+	+	v	v	v
vii. Lactose	+	+	-	-	+
viii. Mannitol	-	+	+	v	+

mangrove and salt marsh plants. Out of 26 colonies 5 strains (BL6, BR1, BR7, BS1 and BS7) were showed antimicrobial activity and they were characterized biochemically.

Ethyl acetate extracts of 5 bacterial isolates (BL6, BR1, BR7, BS1 and BS7) showed broad spectrum antimicrobial activity (Table.3). The mean zones of inhibition produced by the different extracts were found to be 05–20 mm (bacteria) and 11–25 mm (fungi). Extracts of BL6, BR7 and SR1 showed high activity against most of test pathogens compared to extracts of BS7 and BS1.

Maximum antibacterial activity was shown against *Streptococcus pyogenes* by BR1 (18mm) and BR7 (17mm). MIC and MMC of extracts ranged from 0.0625-1.0mg/ml (Table.4). The strain BL6 has MIC value at 0.125 mg/ml against *pseudomonas aeruginosa* and BR1 has 0.125 mg/ml against *S.pyogenes*. Recently, one study reported 2 endophytes of 14 isolated bacterial strains showed promising antibacterial activity against human pathogens. The strain MB4 has MIC values at 250 µg/ml against *Bacillus subtilis, Vibrio harveyi* and the strain MB8 showed MIC value of 250 µg/ml against *Bacillus subtilis* and *Serratia sp.* (Sundaram Ravikumar *et al.*, 2010). Another study reported that, the endophytic strain ENS3 from the seagrass *Syringodium isoetifolium* and the endophytic strain ENC5 from the seagrass *Cymodocea serrulata* posses MIC value 125 µg/ml against *Pseudomonas aeruginosa*. The MIC of *M. jodocodo* against *E. coli* was 2.75 mg/ml while that of *T. robustus* against *M. bourtardi* was 15.75 mg/ml.

Present study showed high activity of endophytic bacterial strains against fungal pathogens compared to bacterial pathogens. The isolate BR7 and BL6 were more active compared to other extracts. Extract of BR7 showed lowest values of MIC and MMC against *M.furfur* (MIC 0.0625mg/ml, MMC 0.0625mg/ml) followed by extract against *T.rubrum* (MIC 0.125mg/ml, MMC 0.25mg/ml) and *Rhizopus* (MIC 0.125mg/ml, MMC 0.25mg/ml). Jalgaonwala *et al*. (2010) reported that bacterial isolates KB4 from roots of *P.glabra*, NB6 from stem *of E.globulus* and HB3 from rhizomes of C.*longa* have strong antifungal activity. In the study the MMC and MIC values were comparatively less that will be helpful in the development of drugs with economic feasibility.

Zarin and Sharon (2009) isolated endophytic bacteria from sweet potato plants and identified as Enterobacter, Rahnella, Rhodanobacter, Pseudomonas, Xanthomonas and Phyllobacterium. In the present study, the isolated endophytic bacteria were identified as *Serratia* (BL6), *Bacillus* (BS7), *Pseudomonas* (BR7), *Micrococcus* (BR1) and *Enterobacter* (BS1) by biochemical characterization.

In the present study endophytic bacterial strains were isolated from mangrove species *Rhizophora mucronata.* Antimicrobial activity of ethyl acetate extract of endophytic bacteria was assessed by disc diffusion method. All the extracts showed a broad spectrum activity against most of the tested bacterial and fungal pathogens. Based on the previous supportive works carried out by many other researchers, the present study can conclude that endophytic bacteria from mangrove plants have potential ability in drug discovery. Detailed investigations on

mangrove endophytic bacteria are needed to prove its potential further and it will leads to the discovery of numerous high value metabolites.

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