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Isolation and Antagonistic Activity of Epiphytic Bacteria Associated with Three Seagrasses

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Abstract: Methicillin resistant *Staphylococcus aureus* (MRSA) and multidrug resistant (MDR) *Escherichia coli* and *Pseudomonas* have been reported as a global health issue. This led to the exploration of new therapeutic agents to treat the infection. This study was designed to explore the ability of seagrasses-associated bacteria as the sources of secondary metabolites which inhibit the growth of pathogens. Three seagrasses (*Cymodaceae serrulata*, *Syringodium isotefolium*, and *Halodule uninervis*) were collected from Mandapam, Ramanathapuram, Tamil Nadu and islated 17 epiphytic bacteria. Out of all the isolates, only three bacteria exhibited antibacterial activity against MRSA and MDR.

Keywords: Anti-MRSA, Anti-*E. coli*, Endophyte, Epiphyte, Antibacterial activity, Seagrass

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Introduction

Infections with multidrug-resistant (MDR) bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* (ESBL), *Pseudomonas* (MDR) have been reported as a global health issue (CDC, 2017). For many years, these bacteria have been the cause for a high number of hospitalizations in various countries and in several cases it has led to death (Olvi *et al*., 2019). The resistance to various antimicrobial agents makes the treatment of MRSA and MDR *E. coli* infections increasingly difficult and multifaceted. Therefore, there is a pressing need to discover new antimicrobial applicants for the remedy of MRSA, MDR *Pseudomonas* and *E. coli* infections.

New developments in drug discovery from herbal sources emphasize on research of the marine ecosystem to explore numerous complex and novel chemical entities. These entities are the source of new lead for treatment of many diseases such as cancer, AIDS, inflammatory condition, arthritis, malaria and large variety of viral,

bacterial, fungal diseases (Ravikumar *et al*., 2009). Because of the exceptionally chemical and physical harsh situation in marine environment, the organisms produce a diffusion of molecules with precise structural functions and exhibit diverse biological activities. Marine plants are known to produce a large number of structurally diverse secondary metabolites (Ravikumar *et al*., 2009).

Sea grasses are the marine flowering plant life. They are the only angiosperms that correctly grow in tidal and sub-tidal marine surroundings. Sea grass belongs to the families Hydrocharitaceae and Potamogetonaceae and they are in no manner associated with the terrestrial grass of Poaceae.

There are 13 genera and 58 species available all over the world. Six genera (Amphibolis, Heterzostera, Phyllospadi, Posidonia, Pseudalthenia and Zostera) are generally restrained to temperate seas and the remaining seven genera (Cymodocea, Enhalus, Halodula, Halophila, Syringodium, Thalasia and Thalassodendeon) are distributed in tropical seas. Several species of sea grass have obligate microbial populations inhabiting their roots, leaves and rhizomes. A variety of medicines and chemical are also prepared from sea grass and their associates (Ravikumar *et al*., 2005).

Seagrasses harbor diverse communities of bacteria including epiphytic and endophytic bacteria symbioses with their leaves and roots. Epiphytic bacteria are nonharmful bacteria that live on the surface of various organs of plants (Tarquinio *et al*., 2019). The epiphytic species provide seagrasses with nitrogen fixation and nutrient cycling. They not only contribute to the seagrass ecosystem but also support other functions to herbivory and transfer of energy to higher trophic levels (Mishra and Mohanraju, 2018). Olvi *et al*. (2019) studied seagrass-associated bacteria from the North Java Sea, Indonesia. Moreover, a recent study highlighted the importance of seagrass ecosystems to the health of humans and other organisms. The study revealed that the seagrass meadows significantly reduce the relative abundance of bacterial pathogens (Lamb *et al*., 2017). It was believed that the seagrass-associated bacteria are involved in this antipathogenic mechanism. The previous report described that some symbiotic bacteria play a protective role by releasing chemicals that protect their hosts (seagrasses) from pathogens and biofouling by other organisms (Tarquinio *et al*., 2019).

The effect of marine secondary metabolites on co-occurring microorganisms remains in huge component undocumented. Perhaps of greater concern than resistance to single antibiotics is the development of bacteria evidence against more than one antibiotic sorts. As antibiotic resistance has developed, clinical researchers have tried again with alternative antibiotics and combination therapy, however, everyday overuse of antibiotics in human beings and livestock way that strains of bacteria resistant to nearly all antibiotics. In the present study three seagrasses (*Cymodaceae serrulata*, *Syringodium isotefolium*, and *Halodule uninervis*) were collected from Mandapam, Ramanathapuram, Tamil Nadu and islated 17 epiphytic bacteria. Antibacterial activity of the above mentioned crude extracts of epiphytes was evaluated

Materials and Methods

Three seagrass samples *Cymodaceae serrulata*, *Halodule uninervis* and *Syringodium isotefolium* were collected at the depth of 0.5-2 m from the intertidal coastal region of Mandapam, South east coast of India (Latitude 9^0 17'N, Longitude $79^0$22 ′E). Soon after the collection, the seagrasses were immediately washed with seawater to remove the foreign particles, sand particles and macro epiphytes. All the samples were stored in a sterile plastic bottle containing sterile seawater and brought to the laboratory in ice box and processed within 3 h of collection. The sea grass samples were appeared to be healthy at the time of collection.

Isolation of marine bacteria:

The sea grass samples were washed thoroughly with sterile sea water to remove loosely bounded

epiphytes, and other attached settlements on the surface of thallus. Leaves were separated and surface sterilized with disinfectant (2% sodium hypochlorite containing 0.1% Tween 20). To remove the disinfectant, samples were again rinsed 5 times with de-ionized distilled water followed by sterile distilled water.

A sample was taken by rubbing the surface of seagrass using sterile swab and inoculated in a sterile seawater and serially diluted using saline water. The serially diluted samples were spread on the Zobell marine medium. The plates were then incubated for 5 days at 32℃ for the growth of epiphytic bacteria.

Crude extract preparation:

Overnight bacterial culture (100 ml) with the O.D. of 0.6-0.9 was centrifuged at 4℃ at 7000 rpm for 20 min. The supernatant was collected and extracted with ethyl acetate. Organic layer was concentrated with vaccum and the concentrated powder was dissolved in deionised water and used for bioassay against several human pathogens such as *E. coli* (ESBL), MRSA, MDR *Pseudomonas*, and ATCC *Staphylococcus aureus*.

Determination of antibacterial activity:

Antibacterial activity of the above mentioned crude extracts of epiphytes was evaluated using agar well diffusion method as described by Patra *et al*. (2009) with some modification. The extracts were reconstituted in Dimethyl sulfoxide (DMSO). Five wells of 6 mm diameter were aseptically made on the assay plates inoculated with target microorganism by using sterile tips. The wells were filled with 100 µl of each extract. The DMSO was used as negative control. The plates were left for 2 h for complete diffusion and then incubated overnight at 37℃. The diameter of inhibition zones, was measured and compared to that of negative control. Each test was made in triplicate and the average of the three replicates for each organism (Elnabris *et al.*, 2013).

Minimum Inhibition Concentration (MIC) Evaluation:

Preparation of Resazurin Solution:

The resazurin solution was prepared at 0.02% (w/v) according to Khalifa et al. (2013). 0.002 g of resazurin salt powder was dissolved in 10 ml of distilled water and vortexed. The mixture was filtered by Millipore membrane filter (0.2 µm). The resazurin solution was kept at 4°C for future use.

The MIC were done using the method described in the guideline of CLSI (2012). The MIC test was performed in 96-well round bottom microtiter plate using standard broth microdilution methods. The bacterial inoculums were adjusted to the concentration of 105 CFU/ml. For the MIC test, 100 µl of the crude extract (3 mg/ml) was added and diluted twofold with the bacterial inoculums in 100 µl of MHB started from column 3 to column 12. Column 3 of the microtiter plate contained the highest concentration of enzyme, while column 12 contained the lowest concentration. Column 1 served as negative control (only medium) and the column 2 served as positive control (medium and bacterial inoculums). The microtiter plate was incubated at 37°C for 24 h. After 24 h, the MIC was determined by adding 30 µl of 0.002% resazurin to each well and incubated for 4 h, any changes in color was observed after 24 h. Blue/purple color indicated no bacterial growth while pink colour/colourless indicated bacterial growth.

Evaluation of minimum bactericidal concentration:

MBC was assessed by inoculating a loopful of culture from the wells that had no bacterial growth. They were then streaked on different sterile Muller-Hinton agar plates. The agar plates were incubated at 37 ℃ for 24 h. The lowest concentration of the extract that exhibited the complete killing of the selected bacterial test species was considered as the MBC.

Results and Discussion

Seagrasses are blooming plants found in marine conditions. The plants total lifecycle in seawater is described by Kuo and McComb (1998). In this study three seagrass species specifically *Cymodaceae serrulata*, *Halodule uninervis* and

Syringodium isotefolium were inspected in Mandapam.

Macroscopic appearance:

Syringodium isotefolium: *Syringodium isoetifolium* (Fig. 1) can grow to a length of 50 cm. The plant has slender underground rhizomes which send up shoots at intervals. The shoots are encased in a sheath at the base and each consists of two or three hollow, tubular leaves with smooth pointed tips. The inflourescence is a cyme, with male and female flowers appearing on separate plants. The fruits are small, hard, beaked nuts. The plant is somewhat fragile; leaves may float when they break off.

Fig. 1: *Syringodium isoetifolium.*

Halodule uninervis: This species (Fig. 2) is a flowering plant spreading via a branching rhizome that roots at the nodes. It produces erect stems and alternately arranged leaves. The narrow, toothed leaf blades are up to 15 cm, leaf width is variable. Each leaf has a sturdy sheath up to 3.5 cm long. The tip of the leaf blade has three teeth. Plants of this family are dioecious.

Fig. 2: *Halodule uninervis.*

Cymodaceae serrulata: Serrated ribbon seagrass (Fig. 3) is considered common and widespread. In reefs, it grow mixed with other seagrasses commonly found in such ecosystems. Long ribbon-like leaves with blunt, rounded tips that have serrations (these are sometimes very tiny). The leaf scars around the upright stem are not continuous. It has thick rhizomes (underground stems). The leaf sheaths around the leaf are flattened. Sometimes seen with reddish bands.

Fig. 3: *Cymodaceae serrulata.*

Isolation of Epiphytic bacteria:

The epiphytic bacterial load in the surface of the seagrass is represented in Table 1. The population load was high on *Cymodaeae serrulata* followed by *Halodule uninervis* and *Syringodium isotefolium*, respectively. In this study three seagrasses were screened and 17 different bacterial strains were isolated, purified and preserved.

Biochemical Characteristics:

The bacterial characteristics of the three seagrasses are shown in Table 2.

Antibacterial activity (*Agar well diffusion method*):

All of the isolated strains were tested for their antibacterial activity by agar diffusion method (Fig. 4). Antibacterial activity was assessed against Multidrug resistant pathogens. The epiphytic bacterial isolate showed clear zone of inhibition against the MDR *Staphylococcus*, MDR *Pseudomonas*, *E. coli* (ESBL) and ATCC *Staphylococcus*. Among 14 epiphytic bacterial strains 3 have showed high activity against the Multidrug resistance organisms. 3 strains have

Table 1: Epiphytic bacterial load in the surface of the seagrass

Name of the seagrass	**Epiphytes**	**No of active strains**	**Percentage of active strains**
Cymodaceae serrulata	**5**	**2**	**40%**
Halodule uninervis	**8**	**2**	**25%**
Syringodium isotefolium	**4**	**1**	**25%**

Table 2: Bacterial characteristics of the three seagrasses

	EP	**Gram staining**	**Indole**	**Methyl red**	**VP**	**Citrate**	**TSI**	**Urease**
Cymodaceae serrulata	1	**Gram – ve rods**	**-**	**+**	**-**	**-**	**RS/YB**	**-**
	2	**Gram +ve rods**	**-**	**-**	**-**	**-**	**RS/YB**	**+**
	3	**Gram – ve rods**	**-**	**-**	**-**	**-**	**RS/RB**	**+**
	4	**Gram – ve rods**	**-**	**+**	**-**	**-**	**RS/YB**	**-**
	5	**Gram +ve rods**	**-**	**-**	**-**	**-**	**RS/YB**	**+**
Halodule uninervis	**1**	**Gram – ve rods**	**-**	**+**	**-**	**+**	**YS/RB**	**-**
	2	**Gram – ve rods**	**-**	**+**	**-**	**-**	**YS/RB**	**-**
	3	**Gram – ve rods**	**-**	**-**	**-**	**+**	**RS/YB**	**+**
	4	**Gram +ve rods**	**-**	**+**	**-**	**-**	**YS/YB**	**-**
	5	**Gram – ve rods**	**-**	**-**	**-**	**-**	**RS/RB**	**-**
	6	**Gram – ve rods**	**-**	**-**	**-**	**-**	**YS/YB**	**-**
	7	**Gram +ve rods**	**-**	**+**	**-**	**-**	**YS/YB**	**-**
	8	**Gram +ve long rods**	**-**	**+**	**-**	**-**	**YS/YB**	**-**
Syringodium isotefolium	**1**	**Gram – ve rods**	**+**	**+**	**-**	**-**	**YS/YB**	**+**
	2	**Gram +ve rods**	**+**	**+**	**-**	**-**	**YS/YB**	**+**
	3	**Gram – ve rods**	**+**	**+**	**-**	**+**	**RS+H2S**	**+**
	4	**Gram – ve rods**	**-**	**+**	**-**	**-**	**YS/YB**	**-**

EP- Epiphytes; VP- Voges–Proskauer; TSI- Triple Sugar Iron; YS-Yellow slant; YB-Yellow Butt; RS-Red slant

showed no activity.

Minimum inhibitory concentration:

MIC ranged from 46.875 µg /ml to 93.75 µg /ml. The profound antibacterial activity of the extract was against the MDR *Pseudomonas*, MRSA *Staphylococcus aureus*, ATCC *Staphylococcus aureus*, and ESBL *E. coli*. MIC value of *E. coli* ESBL and *Staphylococcus aureus* ATCC showed 46.875 µg/ml, MDR *Pseudomonas* and *Staphylococcus aureus* MRSA showed 93.75 µg/ml. Minimum bactericidal concentration (MBC) of the extract was evaluated against the selected bacterial strains. The extract showed 23.875 µg/ml against *Staphylococcus aureus* ATCC and *E. coli* ESBL. The MBC of *Staphylococcus aureus* MRSA and MDR *Pseudomonas* showed 46.875 µg /ml.

Conclusion

In the present study it was observed that higher number of epiphytic bacteria were isolated from *Cymodaceae serrulata* rather than *Halodule uninervis* and *Syringodium isotefolium*. Epiphytic bacteria were isolated on the Zobell medium, Gram staining and Biochemicals have been done to determine morphological and biochemical characteristics. 17 epiphytic bacteria were isolated which were further analyzed for Antibacterial activity. Out of 17, 14 showed antibiotic activity against MDR pathogens (*Staphylococcus aureus* ATCC, MDR *Pseudomonas*, *E. coli* ESBL, and *Staph* ATCC). 3 strains does not have any activity against the above test organisms. In the present study 3 isolates exhibited high

Activity against Staphylococcus (MRSA)

Activity against E. coli (ESBL)

Activity against *Staphylococcus* (ATCC)

Activity against *Pseudomonas aeruginosa* (MDR)

Fig. 4: Antibacterial activity of isolated strains from seagrasses.

Table 3: Zone of inhibition

Seagrass	**EP culture no.**	***E. coli* (ESBL)**	***Staphylococcus* (MRSA)**	***Pseudomonas* (MDR)**	***Staphylococcus* (ATCC)**
Cymodaceae serrulata	1	7 mm	No zone	7 mm	7 mm
	2	No zone	5 mm	6 mm	8 mm
	3	7 mm	7 mm	7 mm	7 mm
	4	9 mm	No zone	7 mm	7 mm
	5	8 mm	7 mm	7 mm	7 mm
Halodule uninervis	6	7 mm	5 mm	No zone	No zone
	7	No zone	7 mm	7 mm	8 mm
	8	**10 mm**	**8 mm**	**8 mm**	**9 mm**
	9	8 mm	No zone	7 mm	8 mm
	10	8 mm	7 mm	7 mm	No zone
	11	9 mm	7 mm	7 mm	No zone
	12	**11 mm**	**11mm**	**13 mm**	**16 mm**
	13	7 mm	7 mm	No zone	7 mm
Syringodium isotefolium	14	9 mm	No zone	7 mm	7 mm
	15	8 mm	7 mm	No zone	7 mm
	16	9 mm	No zone	7 mm	7 mm
	17	**15 mm**	**11 mm**	**16 mm**	**20 mm**

antimicrobial activity compared with other isolates and among three one epiphytic isolate Halodule EP2 shown broad spectrum of activity against MDR bacterial pathogens. In further study secondary metabolites from the epiphytic organism should be extracted and identified using

TLC, GC-MS, and Bioautography.

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