SCREENING OF ANTIBACTERIAL ACTIVITY FROM MANGROVE PLANTS (RHIZOPHORA MUCRONATA) LEAF ASSOCIATED ACTINOBACTERIUM OF STREPTOMYCES SPECIES

Running title: Screening of antibacterial activity from mangrove plants (Rhizophora mucronata) leaf associated Actinobacterium of *Streptomyces species*

ABSTRACT:

INTRODUCTION:

Mangrove is a shrub or small tree that grows in coastal saline water mostly. The term is also used for tropical coastal vegetation consisting of special species. Mangroves occur worldwide mostly in the tropics and subtropics, mainly between latitudes 25° N and 25° S. The total mangrove forest area of the world was 137,800 square kilometres spanning 118 countries and territories

MATERIALS AND METHODS:

The fresh leaves of *Rhizophora mucronata* were collected from Pichavaram area, Tamilnadu. The leaves were washed thoroughly with tap water then surface sterilized with 1% of hydrogen peroxide. Then the sample was macerated in mortar and pestle.

RESULTS AND DISCUSSION:

The antibacterial activity of the rhizophora extract was analysed using two types of assays, namely disc diffusion method and Minimum Inhibitory Concentration (MIC) assay. The disc diffusion test was done for different concentrations for the selected clinical isolates (*Klebsiella,streptococcus and vibrio).*

CONCLUSION :

From this study ,we can conclude that Rhizophora Mucronata has good antibacterial activity against these microorganisms (*Klebsiella,streptococcus and vibrio)* and further studies can be done in future.

KEYWORDS:

Mangrove, antibacterial , actinobacteria , rhizophora mucronata,xenobiotics, wetlands.

INTRODUCTION

Mangrove is a shrub or small tree that grows in coastal saline water mostly. The term is also used for tropical coastal vegetation consisting of special species. Mangroves occur worldwide mostly in the tropics and subtropics, mainly between latitudes 25° N and 25° S. The total mangrove forest area of the world was 137,800 square kilometres spanning 118 countries and territories.Mangrove swamps found in tidal areasMangrove ecosystems play an important role as refuge, feeding, breast feeding areas and sustain an extensive food web based on detritus. Marine invertebrates contain a variety of therapeutically bioactive compounds that have shown promise in the treatment of a variety of ailments(1). High toxic compounds were isolated from ascidians that have the ability to alter resting membrane potential but not the formation of toxin-formed channels(2). Additionally, mangroves export nutrients to adjacent marine ecosystems such as seagrass communities and coral reefs(3). Because of washing of land-derived wastes containing metals primarily from industrial and agricultural sites, domestic wastes, and weathering action triggered by monsoonal floods, the bioaccumulation of metals in mangroves and salt marshes showed the highest accumulation in the monsoon season, followed by other seasons(4).

In the mangrove environment, numerous bacterial classes are usually present (5).Mangroves of sea areas are generally nitrogen deficient but are nonetheless highly productive.Endophytes are major microorganisms that live inside of plants without causing any harm to their hosts which have been isolated from root nodules and leaves, stem and fruits of a wide variety of plant species(6). Isolated endophytic bacteria taken from the branches of the two major species of mangrove plants, Avicennia nitida (white mangrove) and Rhizophora mangle (Red mangrove), are mostly found in two different mangrove areas of Brazil. Actinobacteria particularly, the sort Streptomyces, are significant makers of bioactive auxiliary metabolites Following quite a while of screening, it has gotten progressively hard to find new antimicrobials from actinobacteria separated from normal soil conditions. These days, an ever increasing number of investigates are centered around extraordinary territories and outrageous conditions (7)., for example, desert , marine , and mangrove since microorganisms in uncommon conditions need to create special guard component against the pressure from their living spaces and can develop versatile biosynthetic pathways for orchestrating novel natural mixtures (8) be told, countless new bioactive mixtures delivered by actinobacterial strains living in exceptional conditions have been

found as of late (9)Actinobacteria in the sea are one of the most productive classes of secondary metabolite producers, and they're essential for industry(10) (11) (12) (13). Thousands of biologically active secondary metabolites are produced by many members of the Actinomycetales group (14).Since the 1950s, several essential antibiotics, anticancer, antitumor, and immunosuppressive agents have been studied and screened in Actinobacteria from terrestrial sources. However, in recent years, the repeated rediscovery of the same compounds from terrestrial actinobacteria has made them less appealing for screening programmes.At the same time, due to the structural diversity and unusual biological activities of their secondary metabolites, actinobacteria isolated from the marine environment are currently attracting a lot of attention.They are effective sources of secondary metabolites of antibacterial, antifungal, anticancer, antitumor, cytotoxic, cytostatic, anti-inflammatory, anti-parasitic, anti-malaria, antiviral, antioxidant, anti-angiogenesis, and other biological activities. In this review, an evaluation is made on the current status of research on marine actinobacteria yielding pharmaceutically active secondary metabolites(15). Bioactive compounds derived from marine actinobacteria have distinct chemical structures that could be used to develop new medicines to fight resistant pathogens. Our team has extensive knowledge and research experience that has translate into high quality publications(16–20),(21),(22),(12),(23),(24),,(18,25,26),(27–31),(32),(33).With the advancement of science and technology, new bioactive compounds synthesised by actinobacteria from different marine sources will be in higher demand in the future.

MATERIALS AND METHODS

Collection of plant material and preparation

The fresh leaves of *Rhizophora mucronata* were collected from Pichavaram area, Tamilnadu. The leaves were washed thoroughly with tap water then surface sterilized with 1% of hydrogen peroxide. Then the sample was macerated in mortar and pestle.

Isolation of Actinobacteria : Isolation and enumeration of actinobacteria were carried out in Kuster's agar medium (KUA) supplemented with 0.5% (W/v) NaCl. To minimize the fungal and bacterial contamination, KUA medium was supplemented with cycloheximide (10 µg/ml) and nalidixic acid (10 µg/ml) respectively (Kathiresan *et al.,* 2005). Freshly macerated samples were serially diluted and inoculated on KUA medium and incubated at 36°C for 7 days. The colonies were counted and the population density has been expressed as colony forming units per gram (CFU/g) of sediments. Morphologically distinct colonies were selected and pure cultures were obtained.

Identification of marine actinobacteria

Aerial mass colour: The colour of the mature sporulating aerial mycelium was recorded in naked eye. When the aerial mass colour fell between two colours series, both the colours were recorded. If the aerial mass colour of a strain to be studied showed intermediate tints, then also, both the colour series were noted. The media used were Yeast Extract-Malt Extract Agar and Inorganic-Salt Starch Agar.

Melanoid pigments: The grouping was made on the production of melanoid pigments (*i.e.*greenish brown, brownish black or distinct brown, pigment modified by other colours) on the medium. The strains were grouped as melanoid pigment produced (+) and not produced (-). In a few cases, the production of melanoid pigments was delayed or weak, and therefore, it was not distinguishable. This is indicated as variable (V). This test was carried out on the media ISP-1 and ISP-7 (Appendix I), as recommended by the International *Streptomyces* Project (Shirling and Gottlieb, 1966).

Reverse side pigments*:* Reverse side pigment production of the isolate was determined on ISP7 medium. The pigment production was noted as distinctive (+) and not distinctive or none (-). In case, a colour with low chroma such as pale yellow, olive or yellowish brown occured, it was included in the latter group (-).

Soluble pigments: Soluble pigment production of isolate was observed on ISP7 medium. The diffusible pigment production other than melanin was considered positive (+) and not produced (-). The colour was recorded (red, orange, green, yellow, blue and violet).

Spore chain morphology: Spore morphological characters of the strains were studied by inoculating a loopful of one week old cultures into solidified agar medium containing sterile glass slides. The cultures were incubated at 28 ± 2^0 C and examined periodically for the formation of aerial mycelium, sporophore structure and spore morphology.

Chemotaxonomical characteristics

Hydrolysis

Hydrolysis was done for releasing amino acids. Harvested cells of each strain weighing 20 mg (fresh) were placed in an ampo bottle and 1 ml of 6 N HCl was added and sealed with an alcohol blast burner. The samples were kept at 121^0 C for 20 h in a sand bath. The bottles were cooled by keeping them at a room temperature of 28 ± 2^0C. Hydrolysis was also done for releasing sugars. Harvested cells of each strain weighing 50 mg (fresh) were placed in an ampo bottle and 1 ml of 0.5N HCl was added and sealed with an alcohol blast burner. The samples were kept at 110^0 C for 2 h. The bottles were then cooled by keeping them at a room temperature of 28 ± 2^0 C.

Thin Layer Chromatography (TLC): Spotting of the whole cell hydrolysates was made carefully on TLC plate using a microliter pipette. Spots were 5-10 mm in diameter. This was done by multiple applications on the same spot of very small portions of the sample, which were dried by a hand dryer.

Amino acids: Each sample (3 µl) was applied on the baselines of the TLC plate (20 cm x 20 cm). Adjacent to this, 1µl of DL-diaminopimelic acid (an authentic material mixture of DAP isomers) and 1µl of amino acetic acid (glycine) were spotted as standards. The TLC plate was developed with the solvent system containing methanol: pyridine: glacial acetic acid: H_2O (5: 0.5: 0.125: 2.5 v/v). It took approximately more than 4 h for development. The spots were visualized by spraying with 0.4% ninhydrin solution in water-saturated n-butanol, followed by heating at 100^0 C for 5 min. Spots of amino acids ran faster than DAP. The sample spots were

immediately compared with the spots of the standards since spots gradually disappeared in a few hours.

Whole-Cell sugars: On a cellulose TLC plate (20 cm x 20 cm), 5µl of samples was spotted along with 3 µl of sugar solutions as standards on the same plates. Galactose, arabinose, xylose and madurose were the sugars, which were used as standards. The TLC plate was developed with the solvent mixture containing ethyl acetate: pyridine: acetic acid: distilled water (8: 5: 1: 1.5 v/v). The development time was more than 4 h. Spots were visualized by spraying with aniline phthalate reagent (3.25 g of phthalic acid dissolved in 2 ml of aniline and made upto 100 ml with water saturated n-butanol). The sprayed plate was heated at 100^0 C for 4 min. Hexoses appeared as yellowish brown spots and pentoses, as maroon coloured spots.

Assimilation of carbon source: The ability of the actinobacterial strain in utilizing various carbon compounds as source of energy was studied, following the method recommended by International Streptomyces Project (Shirling and Gottlieb, 1966). Chemically pure carbon source certified to be free of admixture with other carbohydrates and contaminating materials were used for this purpose. Carbon sources for this test were Arabinose, Xylose, Inositol, Mannitol, Fructose, Rhamnose, Sucrose and Raffinose. These carbon sources were sterilized by ether sterilization without heating. The media and plates were prepared and inoculated according to the convention of ISP project (Shirling and Gottlieb, 1966). For each of the carbon sources, utilization is expressed as positive (+), negative (-), or doubtful (±). In the 'doubtful ' strains, only a trace of growth slightly greater than that of the control was noticed.

Preparation of actinobacterial extraction: The isolated actinobacteria were inoculated on the nutrient broth and incubated at room temperature for 5-7 days. The culture was centrifuged (10000 rpm for 10 min) and the supernatant was extracted with equal volume of ethyl acetate (Naik *et al.,* 2001). After the evaporation of ethyl acetate, it was re-extracted with the equal amount of chloroform and concentrated under vacuum at room temperature.

Bacterial Suspension: The pathogenic bacteria *Klebsiella pneumonia, Streptococcus* sp. and *Vibrio cholerae* was collected from the Department of Microbiology, Saveetha medical college and hospital, Tamilnadu. The bacterial pathogens were cultured in Muller –Hinton Broth for 24 hr at room temperature. From this bacterial suspension was prepared with saline and the optical density was measured at 600 nm. The concentration of microbial suspension was fixed as 10^8 CFU/ml. 1ml of suspension was spread over on Muller Hinton agar plate and incubated for 24hrs at ambient temperature.

Antibacterial activity: The antibacterial activity of actinobacterial extract was performed with disc diffusion method. Whatman filter paper discs (5mm) were impregnated with various concentrations (0.5, 1, 1.5, 2, 2.5 and 3mg/ml) of leaf extract using ethanol and methanol solvent. The inoculated plates were incubated for 24 hr at room temperature and the inhibition zones around the discs were measured. All the results were expressed from an average of three with standard deviation.

Minimum Inhibitory Concentration:

Minimal Inhibition Concentration of actinobacterial extract on ethanol and methanol was determined in 5 concentrations (0 -50 µg/ml / 0.001 to 0.1 mg /ml) with blank (extract in Muller Hinton broth). The inoculated bacteria in test tubes are incubated for 24hr in ambient temperature then the optical density was observed.

RESULTS:

Table 1: Morphology of *streptomyces species* species isolated from Rhizophora mucronata

Colour of aerial mycelium	gray
Melanoid pigment	-
Reverse side pigment	-
Soluble pigment	+
Spore chain	spirale
Assimilation of Carbon source	
Arabinose	+
Xylose	-
Inositol	+
Mannitol	-
Fructose	+
Rhamnose	-
Sucrose	+
Raffinose	+

Table 2: This table shows the zone of inhibition for different concentrations for the clinical isolated species (*Klebsiella,streptococcus,vibrio)*

Zone of inhibition			
µg/ml	Klebsiella (mm)	Streptococcus (mm)	Vibrio (mm)
0	0	0	0
50	5±1.04	3±2.1	4±1.8
100	11±2.6	8±1.9	9±2.7
150	14±1.4	12±2.4	13±2.4
200	20±1.3	16±1.3	17±2.1
250	24±2.4	20±1.7	22±2.4
300	28±1.8	24±2.1	26±2.6

Table 3: This table shows the Minimum Inhibitory Concentration (MIC) for the selected clinical isolates (*klebsiella,streptococcus and vibrio)* with positive control as tetracycline.

Minimum Inhibitory Concentration							
	0	10	20	30	40	50	MIC (µg/ml)
Klebsiella	+	+	+	-	-	-	30 µg/ml
Tetracycline	+	+	-	-	-	-	20 µg/ml
streptococcus sp	+	+	-	-	-	-	20 µg/ml
Tetracycline	+	-	-	-	-	-	10 µg/ml
Vibrio sp	+	+	-	-	-	-	20 µg/ml
Tetracycline	+	-	-	-	-	-	10 µg/ml

The antibacterial activity of the rhizophora extract was analysed using two types of assays, namely disc diffusion method and Minimum Inhibitory Concentration (MIC) assay. The table 1 shows a disc diffusion test was done . for different concentrations for the selected clinical isolates (*Klebsiella,streptococcus and vibrio sp.)*. For one gram of klebsiella for 0 µg/ml concentration of the crude extract the zone of inhibition is 0, for 50 µg/ml concentration the zone of inhibition was 5 with standard error of ±1.04mm for 100 µg/ml concentration the zone of

inhibition is 11±2.6mm ,150 µg/ml the zone of inhibition is 14±1.4mm for 200 µg/ml the zone of inhibition is found to be ,16±1.3mm and for 250 µg/ml the zone of inhibition seen is 20±2.4mm for 300 µg/ml the inhibition was 28±1.8mm

Similarly was done for streptococcus, for 50 µg/ml the zone of inhibition is 3±2.1mm , for 100 µg/ml the inhibition seen was 8±1.9mm and for 150 µg/ml the zone of inhibition seen is 12±2.4mm for 200 µg/ml of the crude extract the zone of inhibition seen is16±1.3for 250 µg/ml the zone of inhibition is 20 ±1.7mm and for 300 µg/ml the inhibition is 24±2.1mm For one gram of vibrio, for 50 µg/ml of the crude extract, the zone of inhibition seen is 4±1.8mm for 100 µg/ml the zone of inhibition seen is 9±2.7mm,for 150 µg/ml the zone of inhibition seen is around 13±2.4mm and for 200 µg/ml of the crude extract it shows the zone of inhibition of 17±2.1mm, for 250 µg/ml the zone of inhibition seen is 22±2.4mm and for 300 µg/ml the inhibition seen is 26±2.6mm. From the disc diffusion susceptibility test done for the highest concentration of 300 µg/ml of the crude extract, *Klebsiella Showed* the highest zone of inhibition of the selected clinical isolates and *vibrio sp.* showed the second highest zone of inhibition and followed streptococcus..

Table 3 shows the Minimum Inhibitory Concentration (MIC) test was also done on the selected extracts with positive control as tetracycline . For *klebsiella* the MIC was found to be 30 µg/ml of the crude extract of the seaweed and for tetracycline the MIC is seen to be 10 µg/ml. For *vibrio* and streptococcus species the MIC was found to be 20 µg/ml of the crude extract of the seaweed and for tetracycline the MIC for *E.coli* is seen to be 20 µg/ml. For the MIC was found to be 40 µg/ml of the crude extract of the seaweed and for tetracycline the MIC for *E.coli* is seen to be 20 µg/ml.

DISCUSSION:

Overall gram-positive microorganisms are viewed as more delicate than gram-negative microscopic organisms towards various antimicrobial mixtures on account of the distinction in the design of their cell dividers (34)yet our outcome showed that the concentrates are successful against both gram-positive and gram-negative microbes. Previous research suggests that the unsaturated fat methyl ester concentrations of E. agallocha leaves possess important antibacterial properties. Antimicrobial properties of substances are alluring apparatuses in the control of undesired bacteria, especially in the treatment of contaminations and in the treatment of food waste(35). The dynamic constituents of plants generally meddle with development and digestion of microorganisms in a negative (36).In the previous study , ethanol removal showed the presence of a few phytochemicals, making it more dynamic against bacterial strains in contrast with the fluid concentrate. The antibacterial activity of the mixtures distinguished are now depicted by Patra et al.A study by G Sahoo showed that it had maximum zone of inhibition against streptococcus aureus(37)(38) (39) (40) (41) (42) (43) (44) (45) (46) (47) (48) (49) (50) (51)

Staphylococcus aureus, another MDR, was likewise hindered by the ethanol leaf concentrates of the relative multitude of three mangroves though in the event of ampicillin, the restraint zone was similarly less. The current study discovered more inhibition of Staphylococcus aureus by ethanol concentrate of R. mucronata and E. agallocha leaves (52). Sivaperumal et al. screened the antibacterial movement of chloroform unrefined concentrate and segment fractionated compounds from leaves of E. agallocha and uncovered that contrasted with the unrefined concentrate, segment fractionated compounds were more dynamic against S. aureus and S. pneumonia .Albeit in his investigation, no endeavor was made with unadulterated concentrates, very encouraging outcomes were gotten against the MDR strains of Staphylococcus aureus and Salmonella typhi. The potential for using marine-resistant actinobacteria to treat radionuclides was discovered in previous research. The morphing of big functional categories of organic pollutants that have been handled. Interactions between radionuclides and marine actinobacteria were also confirmed using actinobacterial cells(4).Other studies like antibacterial activity, antifungal activity, anti inflammatory activity on both marine as well as using nanoparticles from plant extracts are done (53), (53–58)(59–61).

The previous examination additionally features the anti-toxin capability of S. alba and E. agallocha against P. vulgaris and P. mirabilis which are astute microbes showing opposition and moderate action, individually, to ampicillin. In general, the mangroves of Goa have enormous potential for the planning of novel anti-infection agents against the pathogenic microbes and particularly on multidrug-safe strains. Additionally, their antimicrobial likely should be reached out against a board of microorganisms answerable for most basic microbial infections. Further examination is important to recognize those bioactive mixtures, which will be a stage for clinical applications.actinobacteria are very notable to deliver a wide assortment of auxiliary metabolites like colors and antimicrobials, particularly streptomyces species produces numerous metabolites, for example, polyketides, peptides and polyketide-peptides half and halves was checked for antifungal and safe concealment

CONCLUSION:

In this study, Rhizophora Mucronata associated actinobacteria *Streptomyces* species was checked and concluded that is showed potent antibacterial activity and further studies can be done by isolating its individual component and can be checked for various properties like antifungal, antioxidant and insecticidal activity(62)-(63).These results can be compared with other actinobacterial species to enhance the property and can be used for pharmaceutical purpose

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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