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Fungal Bioproespecting from Sundarban Mangrove Forest with special reference to Antibacterial and Antimycobacterial Activity

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Abstract: Marine fungi were isolated from sediment samples collected from Sundarban mangrove forest. Nine morphologically different fungal isolates were characterised and identified upto genus level. All the isolates belong in to the genus *Aspergillus, Penicillium* and *Fusarium.* Antibacterial activity of fungal isolates was tested against methicillin resistant *S. aureus*, extended spectrum beta lactamase producing *E. coli, K. pneumoniae* and *P. aeruginosa* by agar plug method. Four fungal isolates [SF2, SF5, SF7 and SF8] showed good antibacterial activity. Crude extracts from the four fungal isolates were produced by agar plate method and extracted using n-hexane, methanol and ethyl acetate in which no extract was obtained in n-hexane. In disc diffusion method, the ethyl acetate extract showed maximum inhibition of bacterial pathogens when compared to methanol extract at 100µg/disc concentration. Antimycobacterial activity of fungal extracts was tested against *M. tuberculosis* H37Rv at 100µg/ml concentration by luciferase reporter phage (LRP) assay. Both the ethyl acetate and methanol extracts showed more than 50% RLU reduction in LRP assay which indicated the antimycobacterial activity. Findings of this study evidenced that Sundarban mangrove forest is the potential source for antagonistic fungal populations which have the potential for exploitation in terms of antibacterial and antimycobacterial compounds.
Key words: marine fungi, sundarban mangroves, antibacterial, antimycobacterial, LRP assay.

Introduction

Infectious diseases are the number one causes of death in tropical countries which counting for approximately half of all fatalities and about 335 infectious diseases were emerged between 1940 and 2004. In addition, infectious diseases mortality rates are also increasing in developed countries[1]. The need for new and useful compounds to provide assistance and relief in all aspects of human conditions is ever growing. Natural products have been the traditional path finder compounds, offering an untold diversity of chemical structures. The phrase "Bioprospecting" is today most frequently used to describe the collection and screening of biological material for commercial purposes.

Microorganisms, with its 3.8 billion years of biosynthetic experience, remain nature's best chemists and treasure house for novel biologically active metabolites. So far most of the commercially available antibiotics are isolated particularly from terrestrial microorganisms. Recent days, the rate of discovery of novel antibiotics from microorganisms is decreasing

and the re-isolation of known antibiotics are increasing. The re-discovery of high numbers of previously described metabolites has to some extent precluded the study of terrestrial sources and has led researchers to explore unique habitats such as the marine environment, for potentially new biosynthetic diversity[2].

Recently, among the marine microorganisms, marine derived fungi have been recognized as one of the last barely tapped resources for new biologically active secondary metabolites including antitumor, antibacterial, antiviral, antifungal and enzyme inhibitor compounds. Overall research on marine derived fungi has lead to discovery of some 272 new natural products until 2002 and another 240 new structures from 2002 until 2004, thus, providing evidence that marine derived fungi have a potential to be a rich source of pharmaceutical leads.

Sundarbans is world's largest coastal wetland comprising of mangrove forest which covers about one million hectares in the delta of the rivers Ganga, Brahmaputra and Meghna. This mangrove region is shared between Bangladesh (60%) and India (40%). The biodiversity of Sundarbans includes numerous species of phytoplankton, zooplankton, microorganisms, benthic invertebrates, mollusks, amphibians and mammals. About 350 species of vascular plants, 250 species of fishes and 300 species of birds are reported in Sundarbans region. Little work has been carried out on the bacterial diversity in the Sundarbans sediments[3]. Ramanathan *et al*., (2008) reported the diversity and its interaction with nutrients in the sediments of Sundarbans mangroves. But there is no encouraging report on bioprospecting of marine fungi from Sundarbans sediments. The present study reports antibacterial and antimycobacterial activity of selected marine fungi from Sundarban mangrove forests.

Experimental

The sediment sample was collected from the Sundarbans mangrove forest region of the Bay of Bengal, India and serially diluted (upto 10^5 dilutions) using sterile distilled water. About 0.1 ml of aliquot from 10^3 to 10^5 dilutions was plated on Sabaroud's Dextrose (SDA) Agar prepared with 50% Sea water. Plating was done in duplicate and all the plates were incubated at 28^0C for 14 days. Morphologically different fungal colonies were selected and maintained on SDA slants[4].

All the fungal isolates were inoculated into SDA plates and incubated at 28^0Cfor 14 days. Growth rate, mycelial colour and soluble pigmentation were noted. Microscopic characteristic was studied by slide culture technique. The mycelial structure was observed under light microscope after lactophenol cotton blue straining. Microscopic characteristics recorded include mycelial structure, conidia and its arrangement, sporangia, mycelial fragmentation. Results were recorded after 21 days of incubation. All the fungal isolates were identified at genus level based on their cultural and microscopic characteristics.

Antibacterial activity of fungal isolates was studied by adopting agar plug method[5]. For the preparation of agar plug, the fungal cultures were inoculated into SDA plates. After 14 days of incubation at 28^0C, the mycelial growth was removed from the agar surface using sterile spatula. Agar plugs (5 mm in diameter) which contain the secreted bioactive compounds was cut using sterile gel puncture. Test organisms used in this study include *Staphylococcus aureus* resistant to methicillin (MRSA), extended spectrum beta lactamase (ESBL) producing *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. *S. aureus* was isolated from pus samples and other three pathogens were isolated from urine samples collected from patients with urinary tract infection. Suspensions of test organisms equivalent to 0.5 McFarland standard were prepared using nutrient broth and inoculated into Muller Hinton Agar (MHA) plates using sterile cotton swabs. Then the agar plugs were aseptically placed over test organisms inoculated on MHA plates. Zone of inhibition was measured after 24 hours of incubation at 37^0C. Fungal isolates which showed good antibacterial activity in primary screening was selected for further investigation.

Bioactive metabolite from the selected fungal isolates was produced by adopting agar plate method. The fungal growth was inoculated into each five SDA plates and incubated at 28^0C for 14 days. Then the whole medium was cut into pieces and extracted using n-hexane, ethyl acetate and methanol for 24 hours. The crude extracts were concentrated using eppendorf concentrator at 45^0C. All the fungal extracts were tested against the bacterial pathogens by paper disc diffusion method at 100µg/disc concentration[6].

Antimycobacterial activity of fungal extracts was tested by adopting luciferase reporter phage (LRP) assay[7] at Department of Bacteriology, Tuberculosis Research Centre, Chennai, Tamil Nadu. The standard strain *Mycobacterium tuberculosis* H37Rv was used as test organism and maintained on Lowenstein Jensons (LJ) slopes. About 350 µl of G7H9 broth supplemented with 10% albumin dextrose complex and 0.5% glycerol was taken in cryo vials and added with 50 µl of crude extract in order to get the final concentration of 100 µg/ml. 100 µl of cell suspension was added in to all the vials. 1% DMSO was also added and used as solvent control. All the vials were incubated at 37^0C for 72 hours. After incubation, 40 µl

of 0.1M CaCl2 solution and 50 µl of high titre mycobacteriophage phAE129 was added into all the vials and incubated at 37^0C for 4 hours. After incubation, 50µl of luciferase reporter phage phAE129 and 40 µl of 0.1 M CaCl2 were added to test and control vials. All the vials were incubated at 37^0C for 4 h. After incubation 100µl from each vial was transferred to luminometer cuvette. About 100µl of D-Luciferin was added and relative light unit (RLU) was measured in luminometer. In LRP assay, reduction in RLU by 50% or more compared to control was considered as antimycobacterial activity.

Table 1. Antibacterial activity of fungal isolates by agar plug method

Strain No	Fungal genera	Zone of inhibition (millimetre in diameter)			
		Methicillin resistant *S. aureus*	ESBL producers		
			E. coli	*K. pneumoniae*	*P. aeruginosa*
SF1	*Fusarium* sp.	-	-	-	-
SF2	*Penicillium* sp.	14	-	-	-
SF3	*Aspergillus s*p.		-	-	-
SF4	*Aspergillus* sp.	-	-	-	-
SF5	*Fusarium* sp.	16	10	12	9
SF6	*Penicillium* sp.	-	-	-	-
SF7	*Aspergillus* sp.	12	-	-	10
SF8	*Aspergillus* sp.	12	10	12	10
SF9	*Aspergillus* sp.	-	-	-	-

Table 2. Antibacterial activity of fungal extracts by disc diffusion method

Test organisms	Zone of inhibition (millimetre in diameter)							
	Methanol extract				Ethyl acetate extract			
	SF2	SF5	SF7	SF8	SF2	SF5	SF7	SF8
S. aureus	14	-	11	11	18	12	12	14
E. coli	-	8	-	-	-	10	11	13
K. pneumoniae	-	-	-	-	-	11	18	15
P. aeruginosa	-	12	12	10	-	8	11	11

Table 3. Percentage RLU reduction in LRP assay against *M. tuberculosis* H37Rv

Fungal isolates	% RLU reduction	
	EAE	ME
***Penicillium* sp. SF2**	54.00	60.19
***Fusarium* sp. SF5**	74.37	60.78
***Aspergillus* sp. SF7**	55.00	51.76
***Aspergillus* sp. SF8**	82.11	59.95

Results & Discussion

Fungal colonies were appeared on SDA plates after 72 hours of incubation. During the course of incubation about 22 fungal colonies were recovered, purified and subcultured on SDA plates from which nine morphologically different isolates were selected and identified at generic level. All the fungal isolates were belongs to three genera viz, *Fusarium, Aspergillus* and *Penicillium*. Distribution of fungal species within the mangrove habitat reflects physical conditions and/or habitat preference such as temperature, salinity, humidity and organic contents. Ramanathan et al.,[4] reported that *Aspergillus orchraceous* and *Penicillium frequentus* were the most abundant species in Sundarban mangrove sediments. In the present study also, more number of *Aspergillus* and *Penicillium* colonies were observed on SDA plates when compared to other fungal genera from the same environment.

Antibacterial activity of fungal isolate was given in table 1. Only four out of 9 isolates showed antibacterial activity in which strain SF5, SF7 and SF8 showed broad spectrum activity and strain SF2 showed inhibition only against *S. aureus*. The agar plug method used in this study is a simple and effective method of screening of microorganisms in particular filamentous organisms like fungi and actinomycetes for antagonistic activity. The antibacterial activity of bioactive extracts produced from selected fungal isolates was given in table 2. The ethyl acetate extracts showed maximum inhibition when compared to methanol extract against bacterial pathogens tested. No compound was extracted in n-hexane. In general, filamentous organisms like fungi prefer solid medium for their growth and metabolite production. There are many secondary metabolites and enzymes are produced from fungi by adopting solid state fermentation[8]. In the present study also crude bioactive extracts produced from fungi by solid culture method showed good activity. Further optimization of solid state fermentation using agriculture wastes will be useful for the maximal production of bioactive compounds from the selected fungal isolates.

In LRP assay all the six extracts produced from three fungal isolates showed more than 50% RLU reduction against *M. tuberculosis* H37Rv. In particular methanol extract from the fungal strain SF8 showed maximum of 82.11% RLU reduction. The worldwide problem caused by TB and the lack of new drugs in the market makes it imperative to have novel drugs to fight efficiently against the rapid spread of multi drug resistant TB strain[9]. In this context, findings of the present study showed that mangrove fungi will be a source for the isolation of antiTB compounds.

The unique physic-chemical properties of the marine environment are likely to have conferred marine fungi with special physiological adaptations that could be exploited in biotechnology. This study is an inventory for bioprospecting of fungi from Sundarban mangroves and multifaceted investigations are needed to prove its potential further.

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