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Bioactive potential of selected actinobacterial strains against *Mycobacterium tuberculosis* and other clinical pathogens

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Marine actinobacteria produces diverse array of metabolites with novel chemical structures with potential bioactivities. Exploring the understudied ecosystems may increase the chance of getting novel actinobacteria and new metabolites.The present study explores the bioactive potential of actinobacteria isolated from the marine ecosystem of Andaman and Nicobar Islands, Bay of Bengal, against *Mycobacterium tuberculosis* and other clinical pathogens. The crude extracts from 15 marine actinobacterial strains were produced through agar surface fermentation using YEME agar and extracted using ethyl acetate. The crude extracts were tested against the standard strain *M. tuberculosis* H37Rv, clinical drug sensitive *M. tuberculosis*, and MDR *M. tuberculosis* strains by luciferase reporter phage (LRP) assay at 500 µg/ml concentration. The anti-microbial activity against other clinical pathogens, namely, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella paratyphi*, *Klebsiellapneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Cryptococcusneoformans* and non-tubercular mycobacteria, *M. smegmatis* was studied by agar plug method. Among the 15 extracts that were tested for anti-tubercular activity, the crude ethyl acetate extract of the 14 actinobacterial strains showed anti-tubercular activity against at least one of the three *M. tuberculosis* strains. Exceptionally, the ethyl acetate extract of strain SACC 168 inhibited all three *M. tuberculosis* strains tested. In anti-microbial screening, the crude extracts of eight strains showed anti-microbial activity including six strains, which were active against the non-tuberculous mycobacteria. Further purification and characterization of the active molecule from the potential extracts will pave way for the potential natural product candidate for tuberculosis and other microbial infections.

[Keywords: Actinobacteria; Luciferase reporter phage assay; *Mycobacterium tuberculosis*; Anti-microbial activity]

Introduction

Tuberculosis (TB) caused by the bacillus *Mycobacterium tuberculosis* is the second most prime cause of death worldwide. The World Health Organization (WHO, Geneva) has reported almost nine million new cases of TB, out of which 1.3 million succumb to death[1]. Further, the emergence of multidrug resistant (MDR) as well as extensively drug [illegible] urged the global scientists to discover novel antibiotics for TB. Microbial-derived natural products have a long history in the treatment of TB. Among the microbial producers of secondary metabolites, actinobacteria (a group of gram-positive, filamentous bacteria) are supreme secondary metabolite producers[2] which show a range of biological activities, such as anti-bacterial, anti-fungal, anti-cancer, anti-tumor, cytotoxic, cytostatic, anti-inflammatory, anti-parasitic, anti-malaria, anti-viral, anti-oxidant, anti-angiogenesis, and so on. Marine-derived actinobacteria are the emerging source of novel bioactive metabolites. The marine ecosystem in the Andaman and Nicobar Islands is mostly an untapped virgin resource and may provide a rich source of microorganisms producing novel and effectively potent anti-microbial compounds[3]. Actinobacteria from the Andaman marine ecosystems are less explored source for bioactive metabolites. To our knowledge, only a few studies have been reported on the anti-mycobacterial activity of the marine [illegible]daman and Nicobar Islands[4,5]. Of them, many have been obtained from *Streptomyces*[6] and these natural products have been an extraordinary source for lead structures in the development of new anti-microbial drugs[7]. With this view, the present study was attempted to investigate the anti-tubercular activity of actinobacteria isolated from the Andaman mangrove sediments.

Methods and Materials

Isolation of actinobacteria

Sediment samples were collected from the Burmanallah mangrove region in the Andaman

Islands (Lat. 11° 39.478′; Long. 92°.36.264′) and dried at room temperature for two days. About 1 g of sediment sample was taken and treated at 55 °C in a hot air oven for 10 min. The pre-treated sample was serially diluted and aliquots from 10^3 to 10^5 dilutions were plated on Kuster's agar and starch casein agar supplemented with nalidixic acid (20 µg/mL) and nystatin (100 µg/mL). All the plates were incubated at 28 °C for one month. During incubation, colonies with suspected actinobacterial morphology were recovered using ISP2 agar. Morphologically different colonies were selected, subcultured, and maintained on ISP2 agar slants at 4 °C until further study[8]. All the media used in this research work were prepared using50% seawater.

Characterization of actinobacterial strains

Cultural and microscopic characteristics of the actinobacterial strains were studied by adopting the methods described by Shirling and Gottileb[9]. All the actinobacterial strains were cultured on ISP2 agar at 28 °C for 7–14 days. The recorded characteristics include growth level, colony consistency, aerial mass color, reverse side pigment, and soluble pigment. The micromorphological characteristics were studied by slide culture method. The slides were observed under a bright field microscope at 40× magnification on the 14th day of incubation. The micromorphological characteristics that were recorded include the following: (i) Aerial mycelium, (ii) substrate mycelium, and (iii) spore chain morphology.

Bioactive metabolite production

The bioactive metabolites from actinobacterial strains were produced by adopting the agar surface fermentation[10]. The spores of actinobacterial cultures were inoculated into every10 plates of yeast extract–malt extract (YEME) agar and incubated at 28 °C for 10 days. During incubation, the extracellular metabolites were secreted into the agar medium. After incubation, the mycelial growth was scrapped using a sterile spatula. The agar medium, which contains the secreted metabolites, was cut into pieces and extracted using ethyl acetate at 1:2 ratio for 24 h. The solvent portion was collected and concentrated using a rotary evaporator and quantified.

In vitro screening for anti-microbial activity

The anti-microbial activity was evaluated by disc diffusion method against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella paratyphi*, *Klebsiellapneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Cryptococcus neoformans* and non-tuberculous mycobacterium (*M. smegmatis*)[11]. The inoculum of bacterial and fungal cultures with 0.5 McFarlands standard was prepared using sterile nutrient and Sabouraud broth and inoculated onto Muller Hinton Agar (MHA). About 10 mg/ml concentration of actinobacterial extract was prepared as a main stock solution using ethyl acetate and filtered using sterile 0.45 µ syringe filter. About 1 mg/ml of working stock solution was prepared by adding 10 µl of the stock extract into 90 µl of ethyl acetate solvent and mixed well. Antibiotic discs were prepared at 250 µg/disc concentration from working extract using 5 mm diameter disc and allowed to dry. The extracts-impregnated discs were placed over the MHA plates. The zone of inhibition was measured after 24 hours of incubation at 37 °C and expressed in millimeter in diameter[11].

Screening for anti-tubercular activity

The ethyl acetate extract of 15 actinobacterial strains was screened for anti-mycobacterial activity by LRP assay[12]. A reduction of 50% in relative light units (RLU) as measured by a luminometer was considered as sensitive. The standard laboratory strain *M. tuberculosis* H37Rv, Streptomycin Isoniazid Rifampicin Ethambutol (SHRE) sensitive and SHRE-resistant clinical strains of *M. tuberculosis* were obtained from the Department of Bacteriology, National Institute for Research in Tuberculosis (NIRT-ICMR), Chennai. The viability of all the isolates was maintained on LJ slopes. High titer of mycobacteriophage phAE129 used in this study was prepared using *M. smegmatis*mc2155 in Middlebrook 7H9 complete medium[13].

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 to get the final concentration of 100 µg/mL. About 100 µL of *M. tuberculosis* cell suspension was added to all the vials. The above procedure was followed for all the three *M. tuberculosis* isolates. Dimethyl sulfoxide (1%) was also included in the assay as a solvent control. All the vials were incubated at 37 °C for 72 hours. After incubation, 50 µL of high titer phage phAE129 and 40 µL of 0.1 M$CaCl_2$ solution were added to the test and control vials. All the vials were incubated at 37°C for 4 h. After incubation, 100 µL from each vial was transferred to luminometer cuvette. About 100 µL of D-luciferin was added and RLU was measured in aluminometer.

$$\text{Percentage RLU reduction} = \frac{\text{Control RLU} - \text{Test RLU}}{\text{Control RLU}} \times 100$$

Results and Discussion

A total of 15 actinobacterial cultures were isolated from the Andaman marine sediments. All the cultures produced powdery colonies on ISP2 agar with aerial and substrate mycelium formation. Based on the morphological features, all the cultures were tentatively identified as *Streptomyces* (Table 1). The maximum numbers of actinobacterial colonies were observed on Kuster's agar than in starch casein agar. Kuster's agar is the suitable medium for the isolation of mangrove actinobacteria as described in the previous studies[14,15].

The present study shows that out of the 15 actinobacterial strains screened for their anti-microbial activity, only three ethyl acetate extracts of actinobacterial strains showed potent anti-microbial activity (>10 mm each) against seven different pathogens. The actinobacterial strains, namely, SACC 164, SACC 162, and SACC179 showed prominent activity against the pathogens tested (Table 2). Only SACC 96, SACC 161, and SACC 381 were found to be active against *S. aureus*. The strains SACC 96 and SACC 162 showed prominent activity against *K. pneumonia* (19 mm) and the strains SACC 168 and SACC 179 showed maximum activity against *E. coli* (Table 2). Similarly, the *Streptomyces* sp. PM-32 isolated from the sediments collected at the Bay of Bengal coast in a previous study has shown anti-microbial activity against a group of bacterial and fungal pathogens[16]. Our results closely resembled that of previous study with the actinobacteria, mostly *Streptomyces* spp. isolated from the mangrove sediments of Barmanallha and Carbyn's Cove of South Andaman, by showing anti-bacterial activity against the human pathogens[17]. Our results of anti-microbial activity show that the mangrove ecosystems of South Andaman have high potential actinobacteria with bioactivity from which novel compounds can be identified.

The crude extracts that showed more than 50% RLU reduction is considered as the anti-mycobacterial activity. In the present study, the culture filtrates from 15 strains of *Streptomyces* sp. inhibited one or more of the *M. tuberculosis* strains tested. Among the 15 ethyl acetate crude extracts, eight showed more than 50% inhibition against all the three *M. tuberculosis* strains (Table 2). Out of the 15 isolates, SACC 387 showed the maximum percentage of relative inhibition of anti-mycobacterial activity against *Mycobacterium tuberculosis* H37Rv (93.74%) as well as moderate inhibition against *Mycobacterium* all sensitive (85.52%) and MDR (66.72%) (Table 2). Marine-derived antibiotics may be more efficient against pathogens because the terrestrial bacteria have

Table 1 — Cultural and micromorphology of actinobacterial strains

Strains	Cultural characteristics					Micromorphology	
	Growth	Consistency	AMC	RSP	SP	AM	SM
SACC 96	Good	Powdery	Dark gray	-	-	+	+
SACC 161	Good	Powdery	Whitish gray	Golden yellow	-	+	+
SACC 164	Good	Powdery	Dark gray	Orangish yellow	-	+	+
SACC 169	Good	Powdery	Dark gray	Brownish yellow	-	+	+
SACC 162	Good	Powdery	Dark gray	Orangish yellow	-	+	+
SACC 168	Good	Powdery	Dark gray	Brownish yellow	-	+	+
SACC 177	Good	Powdery	Dirty white	Pale brown	-	+	+
SACC 179	Good	Powdery	Gray	Yellow	-	+	+
SACC 186	Good	Rough leathery	Dirty yellow	Brown black	-	+	+
SACC 328	Good	Powdery	Gray	Pale yellow	-`	+	+
SACC 336	Good	Powdery	Cream	Pale yellow	Pale green	+	+
SACC 369	Good	Powdery	Cream	Yellow	-	+	+
SACC 374	Good	Powdery	Creamish white	Pale yellow	Pale yellow	+	+
SACC 381	Good	Powdery	Gray	Brown	Brown	+	+
SACC 387	Good	Powdery	White	Pale brown	-	+	+

+, present; -, absent; AMC, aerial mass color; RSP, reverse side pigment; SP, soluble pigment.

Table 2 — Anti-tubercular and anti-microbial activity of actinobacterial extracts

Strains	Anti-microbial activity (zone of inhibition expressed in millimeter in diameter)									Anti-tubercular activity (% reduction in RLU)		
	M. smegmatis	*S. aureus*	*K. pneumoniae*	*A. hydrophilla*	*P. vermicola*	*S. paratyphi*	*E.coli*	*C. albicans*	*C. neoformans*	MTB H37Rv	SHRE-sensitive MTB	SHRE-resistant MTB
SACC 96	11	10	19	11	0	17	0	0	0	58.44	82.49	85.89
SACC 161	13	7	16	11	0	14	14	0	0	76.85	81.51	29.53
SACC 164	12	0	17	15	14	18	17	0	15	62.87	73.49	57.54
SACC 169	0	0	16	13	14	17	17	0	10	30.35	56.97	50.51
SACC 162	13	0	19	15	15	12	15	0	17	87.85	87.86	54.09
SACC 168	10	0	15	13	14	16	18	0	0	73.38	75.73	70.88
SACC 177	0	0	0	0	0	0	0	0	0	87.94	88.05	23.59
SACC 179	10	0	17	13	12	15	18	0	11	78.5	82.49	7.28
SACC 186	0	0	0	0	0	0	0	0	0	67.37	75.2	64.4
SACC 328	0	0	0	0	0	0	0	0	0	74.56	63.45	72.78
SACC 336	0	0	0	0	0	0	0	0	0	78.64	64.1	48.26
SACC 369	0	0	0	0	0	0	0	0	0	0	0	0
SACC 374	0	0	0	0	0	0	0	0	0	59.29	53.38	81.37
SACC 381	0	11	0	0	0	0	0	0	0	31.31	41.63	75.82
SACC 387	0	0	0	0	0	0	0	0	0	93.74	85.52	66.72

not developed resistance against them[18]. The actinobacterial strains MSU and ANS2 isolated from the marine origin have been reported to exhibit promising anti-mycobacterial activity[4]. The majority of ethyl acetate extracts of actinobacteria tested showed remarkable anti-mycobacterial activity. Our findings clearly demonstrate that marine actinobacteria, especially *Streptomyces* sp., are a good source showing prominent activity against mycobacterial pathogens.

Further investigations are needed in order to determine the active metabolites. Purification of active compounds that selectively act on conserved targets may pave the way for highly effective antibiotics.

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