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Antimicrobial and Cytotoxic Activities of an Actinobacteria (*Streptomyces* Sp. PM-32) Isolated from an Offshore Sediments of the Bay of Bengal in Tamilnadu.

P. Manivasagan, S. Gnanam, K. Sivakumar, T. Thangaradjou, S. Vijayalakshmi and T. Balasubramanian

CAS in Marine Biology, Annamalai University, India

Abstract: A total of one twenty five strains isolated from the offshore sediments of the Bay of Bengal in Tamilnadu were tested for their antimicrobial activity against five Gram-positive, five Gram-negative bacteria and five pathogenic fungi. Of these only 23 strains showed activity against the pathogenic microorganisms and the strain PM-32 was very active. Supernatant was obtained by solvent extraction followed by the chromatographic purification. Spectral data of the purified compound revealed its antimicrobial nature. The fractions showed a steady increase of absorption with the increasing wavelengths of UV-visible light, ranging from 200-500 nm. The BC I fraction showed an additional absorption peak at 249 nm and its minimum inhibitory concentration was between 16 to 74 µg/ml against the tested pathogens. The isolate yielded also showed strong cytotoxicity in brine shrimp lethality assay and the LC_{50} value was 0.15µg/ml.

Key words: Antimicrobial activity · Cytotoxicity · Actinobacteria · Bay of Bengal · Tamilnadu

INTRODUCTION

Actinobacteria are a group of gram-positive bacteria organisms widespread in nature, playing a significant role in the field of biotechnology, because they are the producers of vitamins, enzymes, antitumour agents, immunomodifying agents and, mainly antibiotic compounds [1, 2]. Based on the several studies on microbes, actinomycetes have been adjusted as good sources of antibiotic substances, contributing to three quarters of all known products; especially the *Streptomyces* is prolific [3-7].

For the screening of microorganisms for the production of bioactive compounds, exploration of new soils and habitats has been recommended [8]. In this context, Tamilnadu offshore sediments have been found to be the rich sources of biological compounds [9].

In the present studies, actinobacterial strains, isolated from the Tamilnadu offshore sediments were selected and tested for their antibiotic activity against different types of microorganisms, including human pathogenic bacteria and pathogenic fungi.

MATERIALS AND METHODS

Chemicals: Chemicals used in the experiments were from Merck (Darmstadt, Germany), Rankem (New Delhi), Qualigens (Mumbai) and Sigma (USA). All bacteriological media components were purchased from Hi-Media (Mumbai, India).

Sediment Samples Collection: Sediment samples were collected during February (2008) by Sagar Paschimi coastal research vessel at depths of 15-30m from six stations in Bay of Bengal (Tamilnadu). The sediment samples were stored in the laboratory at 4°C in sterile specimen cups until they were used to isolate the actinobacteria.

Isolation of Actinobacteria: Dilutions (10^{-1}-10^{-4}) of one gram of sediments in sterile 50% aged seawater were prepared and plated on starch-casein agar medium (starch, 10.0g; vitamin free casamino acids, 0.3g; $CaCO_3$, 0.02g; $Fe_3SO_4.7H_2O$, 0.01g; KNO_3, 2.0g; $MgSO_4.7H_2O$, 0.05g; NaCl, 2.0g; agar, 18.0g; pH, 7.2; 50% aged seawater) to isolate the actinobacteria. The media were supplemented with 20 mg/l of nystatin and cycloheximide (100 mg/l) respectively [10] to eliminate bacterial and fungal contaminations respectively. The strains were sub-cultured onto starch casein agar slant (medium with 50% sea water), incubated at 28°C for 28 days to achieve good sporulation and were preserved in 20% glycerol at-80°C.

Corresponding Author: P. Manivasagan CAS in Marine Biology, Annamalai University, India

Fermentation: The strains were grown at 28°C in starch-casein agar (pH 7.5) for one week. Spores were collected from a slant culture with 10 ml of the same medium broth. Cultivation of the strains was made by transferring 1 ml (ca.10^8 cells/ml) of the spore suspension and incubated at 28°C, 250 rpm for seven days in 500 ml Erlenmeyer flasks containing 100 ml of antibiotic production medium, with 2% sucrose, 0.25% yeast extract, 0.1% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.001% $FeSO_4.7H_2O$, 0.002% NaCl, pH 7.5 and 50% aged seawater.

Extraction: Crude culture broth of PM-32 was treated with and centrifuged at 10,000 rpm for 30 min at 4°C by maintaining all the physicochemical factors in optimum levels for the culture. Growth and antibiotic production were estimated at temperature 30°C, pH 7.5 and concentrations of sodium chloride 2.0%. The extraction of the metabolites was carried out, using the ethyl acetate solvent on the basis of best solubility and maximum antimicrobial activities. The solvent ethyl acetate was evaporated and lyophilized after extraction of the antimicrobial agents from the culture medium in a rotary evaporator. A mixture of 28.7 mg of crude antimicrobial agents was obtained from 100 ml of the culture filtrate.

Purification: The ethyl acetate extract (5 mg) was chromatographed on silica gel (Merck, particle size 0.100-0.200 mm) column (22x5 cm) and eluted with chloroform-ethyl acetate (70:30) to give 15 major fractions (Fr M I-XV). The dried residues of all the 15 major fractions were dissolved, each in a specified volume of ethyl acetate, to give 1 mg/ml concentration and tested for their antimicrobial activities against the test organisms by disc-plate method. Among the 15 fractions, fraction no. M-VI exhibited good activity. The active fraction M-VI was purified by chromatography on Sephadex LH20 (Ethyl acetate) to obtain fractions M-VIa to M-VIe. The fraction M-VIc was found to possess good antimicrobial activity. This active fraction M-VIc was further purified by chromatography on Sephadex LH20 (Ethyl acetate) which resulted in one distinct compound good antimicrobial activity. The fractions were scanned in a spectrophotometer (UV-1800-Shimadzu) to determine their absorption spectra of UV-visible light at wavelengths ranging from 200 to 1000 nm.

All the extracts and fractions were subjected to Thin Layer Chromatography (TLC) on silica gel (60 F 254 MERCK; 25 TLC aluminium sheets 20x20cm MERCK) and run in chloroform-ethyl acetate (70:30) to visualize the efficiency of separation of UV fluorescent substances. Each batch was repeated several times to confirm the results.

Determination of Antimicrobial Activity: Culture supernatants, extracts and fractions were used in the disc-diffusion method [11]. A total of five gram positive and five gram negative bacteria and five pathogenic fungi were used in the antimicrobial screening. The BC I (50 µg/disc and 100 µg/disc) was prepared by dissolving it with ethyl acetate. To compare the antibacterial and antifungal activities, Ampicillin (30 µg/disc) and nystatin (20 µg/disc) were used as standard antibiotics respectively. As a negative control, a blank disc impregnated with solvent followed by drying off was used. The media used were Mueller-Hinton on Agar (Difco) for bacteria and Sabouraud-Agar for fungi. The plates (triplicates) were incubated at 37°C for 18 h in the case of bacteria and 28°C for 72 h in the case of fungi. The antimicrobial activities of the compound VII were then determined by measuring the respective zones of inhibition in mm.

Determination of Minimum Inhibitory Concentration: The minimum inhibitory concentrations (MICs) of the compound against *Bacillus subtilis, B. cereus, Staphylococcus aureus, Escherichia coli, Shigella shiga, Shigella dysenteriae, Aspergillus niger, A. flavus, Candida albicans* and *Penicillium* species were determined by serial dilution technique [12, 13] in the presence of a standard Ampicillin (for bacteria) and nysatin (for fungi).

Cytotoxicity Bioassay: Brine shrimp lethality bioassay [14-16] is a recent development in the assay procedure of bioactive compounds which indicates cytotoxicity as well as a wide range of pharmacological activities (e.g. anticancer, antiviral, insecticidal, pesticidal, AIDS, etc.) of the compounds. In brief, the eggs of brine shrimp, *Artemia salina*, were hatched in seawater. Ten mature larvae (nauplii) were kept in glass vials containing 10ml of seawater. The test compound dissolved in DMSO (10 mg/ml) was applied to the nauplii in each vial. However, not more than 50 µl of DMSO was added to the vials containing the shrimps. For each concentration, vials containing the same volume of DMSO plus seawater and shrimps were used as control. After 24 h, the vials were

observed for mortality, if any. The number of survived nauplii in each vial was counted and from this data the percentage of lethality of the brine shrimp nauplii was calculated. From this value the LC_{50} of the sample was determined [17].

RESULTS

Out of the 125 actinomycetes subjected to the primary screening process, only 23 isolates showed activity against the test organisms. Of the 23 isolates, one strain (PM-32) showed higher inhibition potential against gram positive, gram negative bacteria and fungi.

Crude culture broth was centrifuged at 10000 rpm for 30 min at 4°C and lyophilization. The first step in the purification protocol was to concentrate the activity from the growth medium by silica gel column chromatography. The next step in the purification was Sephadex LH20 column chromatography. The overall compound and activity are summarized in Fig. 1.

The fractions showed a steadily increasing absorption at wavelengths ranging approximately from 200 to 500 nm; however, the BC I fraction exhibited an additional absorption peak at wavelength 249 nm compared to those of M-I to MXV fractions.

The BC I with R_f value 0.74 (silica gel GF_{254}, solvent system, chloroform: ethyl acetate = 70:30) was isolated from ethyl acetate extract of the metabolites of an actinobacterial and was obtained as reddish crystals with a melting point of 110-120°C. The BC I was odorless and appeared as a pinkish brown spot on TLC plate.

The BC I at a concentration of 30 µg/disc and 100 µg/disc showed antibacterial activity against five Gram positive *(B. subtilis, B. megaterium, B. cereus, S. aureus* and *E. faecalis*) and five Gram negative (*E. coli, P. aeruginosa, S. shiga, S. dysenteriae* and *S. boydii*) bacteria with respect to the standard 30 µg/disc ampicillin. The results are given in Table 1.

The BC I was tested against the pathogenic fungi *Aspergillus niger, A. flavus, A. fumigatus, Candida albicans* and *Penicillium* species at a concentration of 10 µg/disc and 30 µg/disc for each and the result were compared with standard Nystatin 20 µg/disc. The results are given in Table 2.

The minimum inhibitory concentration of the extract is shown in the Table 3. It was found that the BC I showed good potency against *B. subtilis, B. cereus, S. aureus, E. coli, S. shiga, S. dysenteriae, A. niger, A. flavus, C. albicans* and *Penicillium* species.

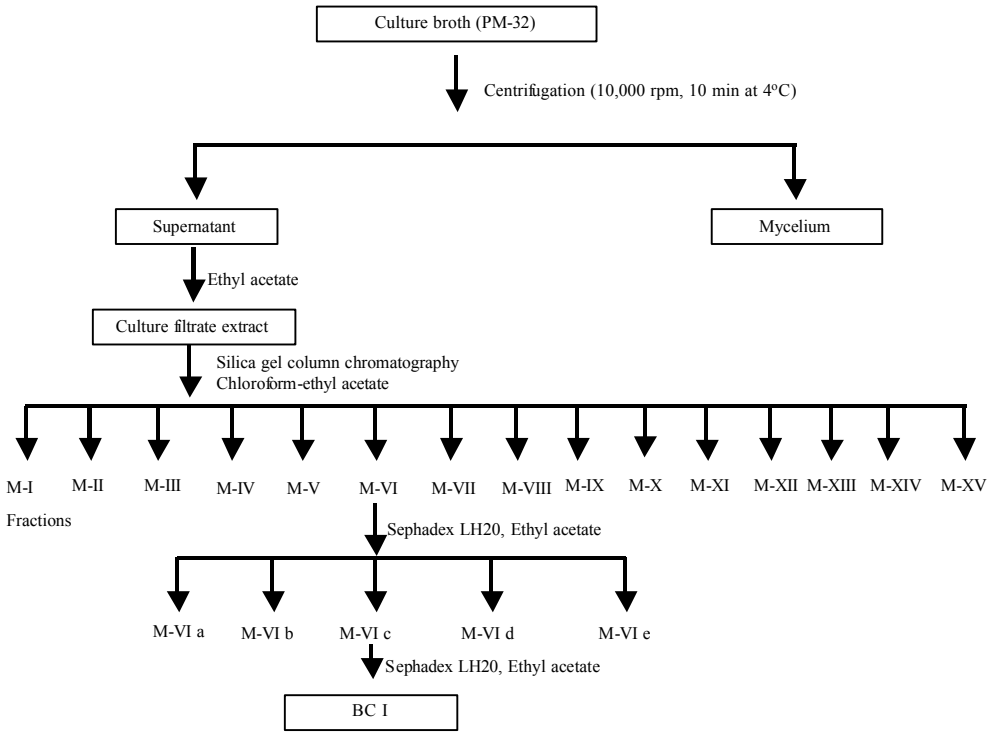

Fig. 1: Scheme for the isolation of pure fractions

Table 1: Antibacterial activity of the BC I and Ampicillin

		Diameter of zone of inhibition (in mm)		
Test organisms	Strain No.	BC I (30µg/disc)	BC I (100µg/disc)	Ampicillin (30µg/disc)
Gram positive				
Bacillus subtilis	QL-166	20	31	24
Bacillus megaterium	QL-38	16	22	26
Bacillus cereus	QL-29	12	20	23
Staphylococcus aureus	ATCC-259233	18	25	28
Enterococcus faecalis	NBIMCC 6783	14	22	24
Gram negative				
Escherichia coli	ATCC 10536	16	25	21
Pseudomonas aeruginosa	CRL	13	19	24
Shigella shiga	ATCC-26107	15	24	20
Shigella dysenteriae	AL-35587	18	27	23
Shigella boydii	AL-17313	12	23	26

Table 2: Antifungal activity of the BC I and Nystatin

	Diameter of zone of inhibition (in mm)		
Test organisms	BC I (10µg/disc)	BC I (30µg/disc)	Nystatin (20µg/disc)
Aspergillus niger	10	17	22
Aspergillus flavus	11	19	25
Aspergillus fumigatus	8	16	23
Candida albicans	10	18	28
Penicillium species	9	17	26

Table 3: Minimum inhibitory concentration of the BC I and standards (Ampiciliin and Nystatin)

Test organisms	BC I (µg/ml)	Ampicillin (µg/ml)	Nystatin (µg/ml)
Bacillus subtilis	23	7	-
Bacillus cereus	18	3	-
Staphylococcus aureus	28	10	-
Escherichia coli	16	5	-
Shigella shiga	22	9	-
Shigella dysenteriae	30	12	-
Aspergillus niger	60	-	10
Aspergillus flavus	74	-	15
Candida albicans	58	-	8
Penicillium species	66	-	12

Table 4: Results of cytotoxic effect of the BC I

Concentration of samples (µg/ml)	Log concentration	% Mortality	LC_{50} value (µg/ml)
0	0.0	0	
10	1.0	39	
25	1.4	57	0.15
50	1.7	91	
100	2.0	100	
200	2.3	100	

Mortality rate of the brine shrimp napulii was found to increase with concentration of the sample and a plot concentration versus percent morality on graph paper showed an almost linear correlation. The BC I was also screened by the brine shrimp lethality bioassay for probable cytotoxic activity. The BC I demonstrated a strong cytotoxic activity with a LC_{50} value (Concentration for 50% lethality) of 0.15 µg/ml (Table 4).

DISCUSSION

A total of 125 actinobacteria strains were isolated from the offshore sediments of Tamilnadu. These isolates were screened for antimicrobial activity using agar-diffusion method. Only 23 strains showed activity against the pathogenic microorganisms and the strain (PM-32) was more active.

In the determination of the UV-visible absorption spectra, the fractions showed a steadily increasing end-absorption with no abrupt peak at the wave lengths of the UV-visible light (200-400 nm), indicating that they might exist in a free form. BC I exhibited a maximum absorption peak at 249 nm.

For a complete characterization of an antibiotic, it should be isolated in pure form as a single component but this is impractical in a screening programme like the present are. However, a little effort was made towards this approach. In the TLC separation, the BC I yielded component with R_f value (0.74) similar to the antimicrobial compounds. This would mean that the same compounds are responsible for the antimicrobial activity of the isolates.

Antibacterial and antifungal screenings revealed the part that BC I has antibiotic activity against all the gram positive and gram negative bacteria tested. The maximum zone of inhibition against *B. subtilis* and *S. dysenteriae* was found to be 20 (30 µg/disc), 31(100 µg/disc) and 18 (30 µg/disc), 27 (100 µg/disc) mm respectively, whereas it was 24 and 23 mm for the standard ampicillin. In earlier studies [18] reported 32 (50 µg/disc) inhibition zone against *Klebsiella pneumoniae.*

All the pathogenic fungi also showed a most activity to the BC I and the maximum zone of inhibition against *A. flavus* was found to be 11 (10 µg/disc) and 19 (30 µg/disc) mm respectively, whereas it was 25 mm for the standard nystatin. In earlier studies [19] reported 13 (20 µg/disc) inhibition zone against *A. flavus.*

The minimum inhibitory concentration values of BC I compound against the tested organisms indicated their noticeable antibacterial and antifungal potencies, in comparison with the standard antibiotics, ampicillin and nystatin. For BC I, the MIC values were 23, 18, 28, 16, 22, 30, 60, 74, 58 and 66 µg/ml, respectively for *B. subtilis, B. cereus, S. aureus, E. coli, S. shiga, S. dysenteriae, A. niger, A. flavus, C. albicans* and *Penicillium* species. Whereas the standard compounds (ampicillin and nystatin) showed MIC values between 3-15 µg/ml for different pathogens tested, which is indicative of their potent antimicrobial properties than the BC I. In previously studies [20] reported 128 (mg/ml) minimum inhibitory concentrations against *A. flavus.* Further work is necessary to establish the antibiotic potential of the isolated compound.

In the brine shrimp lethality bioassay, it was found that the ethyl acetate extracts from the actinobacteria were biologically active. In the experiment, mortality rate of the brine shrimp nauplii increased with the increase in the concentration of the sample and the median lethal concentration (LC_{50}) value was 0.15 µg/ml. In earlier studies [21] reported (LC_{50}) value was 17.78 µg/ml. For the present study, we can conclude that the BC I has potential antimicrobial activity as it showed greater cytotoxic activity; however, further investigations are essential to establish it as an antimicrobial compound.

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