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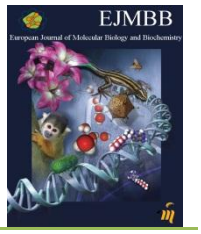

ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES OF MARINE *STREPTOMYCES* SP. PA-32

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ABSTRACT

Marine actinomycetes are potential provider of novel bioactive metabolites and currently emerged as an important source for natural products with unique chemical diversity. The main aim of the study was to evaluate the antimicrobial and cytotoxic potential of crude extract of Marine Streptomyces species isolated from mangrove sediment, Nagapattinum, Tamilnadu, India. Primary and Secondary screening for antimicrobial activity was determined by Cross streak method and Agar disc diffusion method respectively. Among the strains, *Streptomyces* sp. PA32 extract shows anti bacterial, anti fungal and Cytotoxic activities. The results of this study indicate that Marine actinomycetes *Streptomyces* sp. PA32 can able to produce new drug molecule against pathogenic microorganisms.

INTRODUCTION

Actinomycetes are the most economical and biotechnologically valuable class of prokaryotes producing bioactive secondary metabolites notably antibiotics **[**1], anti-tumor agents, immunosuppressive agents [2] and enzymes. A number of structurally unique natural products with antitumor anti-infective **[**and antimalarial bioactivities have been discovered from marine-derived actinomycetes [3]. Multi drug resistance in microorganism is an emerging serious problem in health care sector [4]. The improper usage of antibiotics contributes a major role for drug resistance in pathogenic microbes [5]. Microorganisms acquire resistance towards common antibiotics by altering their metabolism and genetic structure .There is an incessant need to find novel efficient drug molecules against multi drug [6]. Actinobacteria from terrestrial origin produce hundreds of antibiotics which are widely used at present. Some differences could be expected among organisms existing in marine and terrestrial environments due to variation in the physical, chemical and biological factors [7-9].

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It is apparent that the marine environment is a potent source for finding new antibiotics. The species belonging to the genus *Streptomyces* constitute 50% of the total population of soil actinomycetes and are well known for producing a variety of bioactive secondary metabolites including antibiotics, immunomodulators, anticancer & antiviral drugs, herbicides, and insecticides. Although thousands of antibiotics have been isolated from *Streptomyces*, these represent only a small fraction of the repertoire of bioactive compounds produced. So, still there is a chance of discovery of new *Streptomyces* species producing novel compounds from this genus.[10] However, the frequency of rediscovery of known compounds by *Streptomyces* strains was fairly high .Hence, the aim of this study was to investigate the bioactive potential of crude extracts of Marine Streptomyces PA-32 from for its antibacterial, and cytotoxic activities.

MATERIALS AND METHODS

Collection of Soil Sample

Soil samples were collected from Nagapattinum, mangrove (Lat 11°22¢N to 11°30¢N and long 79°45¢E to 79°52¢E), Tamilnadu, India. The soil samples were taken from the 20 cm depth after removing approximately 3 cm of upper soil surface. The samples were placed in the

polythene bags, closed tightly and stored in a refrigerator. The processed samples were given proper identification code.

Screening of potential marine actinomycetes strain

The potential strain was screened by antagonistic activity and potential strain grown at 28°C in starch-casein agar (pH 7.5) for one week. Spores were collected from the slant culture with 10 ml of the same medium broth. Cultivation of the strain was made by transferring 1 ml (ca.10^8 cells/ml) of the spore suspension and incubated at 28°C and 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 100% aged seawater (Plate 1 a, b). Crude culture broth of PA-32 was centrifuged at 10,000 rpm for 30 min at 4°C by maintaining all the physicochemical factors at optimum levels for the culture.

Determination of antimicrobial activity

Culture supernatants, extracts and fractions were used in the disc-diffusion method [11]. A total of five gram positive (*Bacillus subtilis, B. Megaterium, B. Cereus, Staphylococcus aureus* and *Enterococcus faecalis*) and five gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Shigella shiga, S. dysenteriae* and *S. boydii*) and five pathogenic fungi (*Aspergillus niger, A. flavus, A. fumigates, Candida albicans* and *Pencillium* sp.) were used in the antimicrobial screening. The purified antibiotic (30 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 was used. The media used were Mueller-Hint 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 purified antibiotic compounds 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 *B. subtilis, B. cereus, S. aureus, E. coli, S. shiga, S. dysenteriae, A. niger, A. flavus, C. albicans* and *Penicillium* sp. were determined by serial dilution technique [12,13]in the presence of 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. Eggs of the brine shrimp, *Artemia salina*, were hatched in sea water. Ten mature larvae (nauplii) were kept in glass vials containing 10 ml 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 these data, percentage of lethality of the brine shrimp was calculated. From this value, the LC_{50} of the sample was determined [17].

RESULTS

Out of the 125 actinobacterial strains subjected to the preliminary screening process, only 29 isolates showed antagonistic activity against the test organisms. Of the 29 isolates, 5 strains (PA-8, PA-20, PA-32, PA-75 and PA-122) showed good inhibition potential against gram positive and gram negative bacteria. Of these 5 strains, one strain (PA-32) showed higher inhibition potential against gram positive and gram negative bacteria (Table 1).

Antagonistic activity

Antagonistic activity of actinobacterial strains was tested against gram positive and gram negative bacteria. The inhibition zones of strains against the specific test organisms were measured. Maximum inhibition was caused against *B. subtilis* (38 mm) by PA-32 and the minimum inhibition (18 mm) was caused against *E. coli* by PA-20 (Table 1).

Determination of antibiotic compound

The purified antibiotic with R_f value 0.74 was identified from ethyl acetate extract of the metabolites of PA-32 as reddish crystals with a melting point of 110-120°C. The purified antibiotic was odourless and appeared as a pinkish brown spot on the TLC plate. The purified antibiotic showed the maximum absorption peak at the wavelength, 249 nm.

Determination of antibacterial activity

The purified antibiotic 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 and the results were compared with respect to the standard 30 µg/disc ampicillin (Table 2).

Determination of antifungal activity

The purified antibiotic showed antifungal activity against the pathogenic fungi *viz. A. niger, A. flavus, A. fumigatus, C. albicans* and *Penicillium* sp. at a

concentration of 10 µg/disc and 30 µg/disc for each and the results were compared with standard nystatin 20 µg/disc (Table 3).

Determination of minimum inhibitory concentration

Minimum inhibitory concentration of the extract is shown in Table 4. It was found that the purified antibiotic showed good activity against *B. subtilis*, *B. cereus*, *S. aureus*, *E. coli*, *S. shiga*, *S. dysenteriae*, *A. niger*, *A. flavus*, *C. albicans* and *Penicillium* sp. (Table 4).

Cytotoxicity bioassay

Mortality rate of the brine shrimp napulii was found to increase with the increasing concentration of the sample and a plot concentration versus percent morality on graph paper showed an almost linear correlation. The purified antibiotic was also subjected to the brine shrimp lethality bioassay for probable cytotoxic activity. The purified antibiotic demonstrated a strong cytotoxic activity with a LC_{50} value of 0.15 µg/ml (Table 5).

Table 1. Antagonistic activity of actinobacterial strains against human pathogenic bacteria

Antagonistic activity (inhibition zone in mm)										
Strain no.	***B. subtilis***	***B. megaterium***	***B. cereus***	***S. aureus***	***E. faecalis***	***E. coli***	***P. aeruginosa***	***S. shiga***	***S. dysenteriae***	***S. boydii***
PA-8	36	30	21	30	25	21	23	25	31	24
PA-20	30	28	28	23	20	18	33	21	32	21
PA-32	38	34	31	33	29	25	34	28	36	26
PA-75	34	28	24	30	28	21	31	23	31	23
PA-122	28	25	20	25	27	25	21	18	28	21

Table 2. Antibacterial activity of the purified antibiotic and ampicillin.

Test organism	Diameter of zone of inhibition (in mm)		
	Purified antibiotic (30µg/disc)	Purified antibiotic (100µg/disc)	Ampicillin (30µg/disc)
Gram positive			
B. subtilis	20	31	24
B. megaterium	16	22	26
B. cereus	12	20	23
S. aureus	18	25	28
E. faecalis	14	22	24
Gram negative			
E. coli	16	25	21
P. aeruginosa	13	19	24
S. shiga	15	24	20
S. dysenteriae	18	27	23
S. boydii	12	23	26

Table 3. Antifungal activity of the purified antibiotic and nystatin.

Test organism	Diameter of zone of inhibition (in mm)		
	Purified antibiotic (10µg/disc)	Purified antibiotic (30µg/disc)	Nystatin (20µg/disc)
A. niger	10	17	22
A. flavus	11	19	25
A. fumigatus	8	16	23
C. albicans	10	18	28
Penicillium sp.	9	17	26

Table 4. Minimum inhibitory concentration of the purified antibiotic against selected pathogenic bacteria and fungi and standards (Ampicillin and Nystatin).

Test organism	Purified antibiotic (µg/ml)	Ampicillin (µg/ml)	Nystatin (µg/ml)
B. subtilis	23	7	-
B. cereus	18	3	-
S. aureus	28	10	-
E. coli	16	5	-
S. shiga	22	9	-

S. dysenteriae	30	12	-
A. niger	60	-	10
A. flavus	74	-	15
C. albicans	58	-	8
Penicillium sp.	66	-	12

Table 5. Cytotoxic effect of the purified antibiotic and brine shrimp

Concentration of samples (µg/ml)	Log concentration	% Mortality	LC_{50} value (µg/ml)
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	

DISCUSSION

In the present study, a total of 125 actinobacterial strains were isolated from the marine sediments of 6 stations. In the preliminary screening process, only 29 isolates showed antagonistic activity against the test organisms. Of the 29 isolates, four strains (PA-8, PA-20, PA-75 and PA-122) showed good inhibition against gram positive and gram negative bacteria and the strain PA-32 was more potential than other strains.

When the antagonistic activity of actinobacterial strains was tested against gram positive and gram negative bacteria, maximum inhibition was observed against *B. subtilis* (38 mm) in PA-32 and the minimum inhibition (18 mm) was noticed against *E. coli* in PA-20. However, [18] have recorded higher antagonistic activity of actinobacteria against fish disease pathogens (*Aeromonas hydrophila, A. sobria* and *Edwardsiella tarda*). In an earlier study, El-sersy and [19] have reported antagonistic effect of marine *Nocardia brasiliensis* against the fish pathogen *Vibrio damsel*. Absorbance in the UV-visible spectrum revealed a single peak of purified antibiotic compound at 249 nm. In the TLC separation, the purified antibiotic yielded a single band with R_f value of 0.74, similar to the antimicrobial compounds. This would mean that the same purified antibiotic would have been responsible for the antimicrobial activity of the actinobacterial strain, PA-32.

Antibacterial screenings revealed that purified antibiotic has antibiotic activity against all the gram positive and gram negative bacteria tested. The maximum zone of inhibition was found to be 20 mm (30 µg/disc) and 31 mm (100 µg/disc) for *B. subtilis* and 18 mm (30 µg/disc) and 27 mm (100 µg/disc) for *S. dysenteriae*. Whereas, 24 mm and 23 mm for the standard ampicillin. In an earlier study, [20] reported 32 mm (50 µg/disc) inhibition zone against *Klebsiella pneumoniae.* [21] reported that the extract of *Streptomyces tanashiensis* strain A2D showed antibiotic activity against *Bacillus subtilis* (15 mm), *Staphylococcus aureus* (25 mm), *Escherichia coli* (21 mm) and *Klebsiella pneumonia* (23 mm). Purified antibiotic also inhibited all the pathogenic fungi and the maximum zone of inhibition against *A. flavus* was 11 mm (10 µg/ disc) and 19 (30 µg/disc) mm respectively; whereas it was 25 mm for the standard nystatin. In an earlier study, [22] reported 13 mm (20 µg/disc) inhibition zone against *A. flavus.* [23] also reported that antifungal effect of *Nocardia levis* MK-VL_113 against *Fusarium oxysporum* (20 mm).

The minimum inhibitory concentration (MIC) values of purified antibiotic against the test organisms have indicated that the purified compound has shown less antibacterial and antifungal potencies, when compared to the standard antibiotics tested *viz.* ampicillin and nystatin, as revealed by minimum inhibitory concentrations. For purified antibiotic compound, 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. However, performance of the present purified compound is better than that of some other sources: 0.375-3 mg/ml in *Streptomyces* sp. [24] 64-256 µg/ml in *Penicillium* sp. [25] and 16-64 mg/ml of chromium based chemical complex.

Brine shrimp lethality bioassay for cytotoxicity revealed that the purified antibiotic of the actinobacterial strain PA-32 was biologically active. In the experiment, mortality rate of the brine shrimp nauplii increased with the increasing concentrations of the sample and the median lethal concentration (LC_{50}) was 0.15 µg/ ml. But, [25] have reported 17.78 µg/ml as LC_{50} value of penicillin extract, against the brine shrimp mortality, which is quite higher.

Form the present study, it can be concluded that the purified antibiotic has potential antibiotic activity as it showed greater antagonistic and cytotoxic activity; however, further investigations are essential to establish it as an antimicrobial compound.

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