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Antimicrobial, Antioxidant and Anticancer Activity of Actinomycete, Streptomyces rochei isolated from the Marine soil

[1] Pavithra K, 2[*] Gomathi N
1 PG Student, Department of Microbiology, Valliammal College for women
2[*] Corresponding Author, Assistant Professor, Department of Microbiology, Valliammal College for Women, E-9, Anna nagar East, Chennai – 600102, Tamil Nadu, India.

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ABSTRACT-The Marine environment comprises a huge diverse group of microbes and act as a good source of many novel bacteria producing bioactive molecules. Many novel strains can be isolated from marine soil and tested for its biological activity. The present study aimed to isolate Actinomycetes from marine soil sample and screened for the production of secondary metabolite, antibiotic. The screened isolate was subjected for crude extraction of compounds. The crude extract of Actinomycete was tested for Antibacterial, antifungal, Antioxidant and Anticancerous activity.

Keywords: Actinomycetes, Marine soil, Antibacterial, Antifungal, Antioxidant and Anticancerous.

I. INTRODUCTION

Marine environment was recently found to be one of the important sources for the isolation of new strain of Actinomycetes with potentiality to produce chemically diverse compounds with a wide range of biological activities. Actinomycetes are a group of prokaryotic microorganisms which are gram positive bacteria that may look like fungi and grow as filamentous mycelia and form spores. They produce a variety of bioactive compounds such as antibiotics, vitamins and enzymes and the secondary metabolites produced by Actinomycete can be used to treat bacterial and fungal infections and also to kill cancer cells (1).

Representative genera of actinomycetes include Streptomyces, Actinomyces, Arthrobacter, Corynebacterium, Frankia, Micrococcus, Micromonospora and several others. Secondary metabolites produced by marine Actinomycetes may form the basis for the synthesis of novel therapeutic drugs, which may be efficient to combat a range of resistant microbes. Above 500 species of Streptomyces account for 70–80% of relevant secondary metabolites with small contributions from other genera, such as Saccharopolyspora, Amycolatopsis, Micromonospora and Actinoplanes (2).

Some of the most extensively researched Actinomycetales are members of the genus Streptomyces. Streptomyces spp. are saprophytic bacteria found in soil as well as aquatic environments, which possess a variety of morphological forms that often resemble fungi (3). The present study examined the antimicrobial, antioxidant and anticancer activity of crude extracts of Actinomycetes isolated from the marine soil.

II. METHODOLOGY

2.1 Collection of marine soil sample

Marine soil samples were collected from Pattina pakkam beach and Besant nagar beach (wet soil) Chennai, India. The collected soil samples were stored in sterile plastic bags.

2.2 Isolation of Actinomycetes

One gram of soil sample was weighed and suspended in 10 ml of sterile distilled water. The serial dilution technique was performed in the wet soil and the range of dilution is 10^{-1} to10^{-9}. The starch casein agar (SCA) medium was prepared, sterilised and was cooled. The media was supplemented with fluconazole and Gentamycin to avoid fungal and bacterial contamination. The prepared medium was then poured into the petriplates. After the solidification of the medium, the plates were labelled and inoculated with 100 µl suspension of different dilution (10^{-3}, 10^{-4}, 10^{-5}) and was spread on the surface of the agar using L rod and the plates were incubated at 37ºc for 5 days.

2.3 Primary screening of secondary metabolite by Cross streak method(4)

Starch casein agar and Nutrient agar media were prepared in equal volume, sterilized and mixed well. Then the Starch Casein Nutrient agar medium was poured into sterile petriplates and allowed to solidify. The isolated Actinomycetes were inoculated on each plate by single streak method and the plates were incubated for 4-5 days. 24 hours culture (Escherichia coli, Bacillus subtilis,

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Staphylococcus aureus, Pseudomonas aeruginosa) were streaked by perpendicular streaking method, incubated overnight and observed for bacterial growth inhibition.

2.4 Identification of secondary metabolite (antibiotic) producing bacteria

2.4.1 Preliminary Test:

The isolate screened for secondary metabolite was subjected to gram's staining.

2.4.2 Identification of bacteria based on 16s rRNA sequence analysis

16S rDNA partial gene sequencing

Chromosomal DNA was isolated from the isolate by the standard phenol/chloroform method**.** The isolated genomic DNA was used as a template in PCR amplification of 16S rRNA. The Universal primers used for amplification of 16S rRNA are 16S-RS-F (forward) 5' CAGGCCTAACACATGCAAGTC-3' and 16S-RS-R (reverse) 5'-GGGCGGWGTGTACAAGGC-3'. The amplified 16s RdnA gene from the isolate was sequenced.

2.5 Extraction of bioactive compounds from selected Actinomycetes

Starch Casein broth was prepared, sterilized and the isolate of antibiotic producing Actinomycete was inoculated into the broth and incubated in the shaker at 37ºc for 7 to 10 days. Then the broth was centrifuged at 10,000rpm for 10 minutes. The supernatant was collected and added with an equal volume of ethyl acetate and shaken vigorously for 2 hours. The mixture was kept undisturbed overnight for complete extraction of metabolite. After 24 hours of incubation, the upper layer (clear solvent phase) was collected and poured in to the sterile petriplates and allowed to dry for 1 day. The extract was scrapped using 1ml of ethyl acetate and the crude compound was been transferred to sterile tubes.

2.6 Antibacterial activity (Agar well diffusion method)

The antibacterial activity of the crude compound was tested for the bacteria – Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis and Staphylococcus aureus by Agar well diffusion method (5,6). Mueller Hinton agar was prepared, sterilized and the medium was then poured into sterile petridishes and allowed to solidify. The inoculum was prepared for each bacterium by using the direct colony suspension procedure (CLSI). The inoculum density was standardized using a barium sulphate turbidity standard, equivalent to 0.5 McFarland standard. The medium was lawned with the bacterial culture using sterile cotton swabs. Then the five wells of diameter 4mm were cut using cork –borer. To these cut wells, different volumes of the crude compound (25, 50,75 and 100µl) of the prepared crude compound was loaded and 25µl of Gentamycin loaded into the well acts as control. The plates were incubated at 37ºc for 24 hours and the zone of inhibition was measured.

2.7 Antifungal activity

Antifungal activity of the crude extract of Actinomycete was tested for the fungi – Aspergillus niger and Candida albicans. The inoculum was prepared for each fungus using Sabouraud dextrose broth and incubated for 48 hours at room temperature. The assay was performed by agar well diffusion method. Sabouraud dextrose agar (SDA) was prepared, autoclaved and then poured into sterile petridish and allowed to solidify. The medium was lawned with the fungal culture. Then the five wells of diameter 4mm were cut using cork –borer. To these cut wells, different volumes of the crude extract (25, 50,75and 100µl) was loaded into the well. Fluconazole and clotrimazole served as control. The plate was incubated for 48 hrs at 28ºc for Aspergillus niger and for 24 hrs at 37ºc for Candida albicans and zone of inhibition was measured.

2.8 Antioxidant activity by DPPH assay

DPPH assay was performed to determine the antioxidant activity of the crude extract of Actinomycete (7). DPPH (1, 1-diphenyl-2-picrylhydrazyl) is characterized as a stable free radical. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then give rise to reduced form (Blosi,1958) with a loss of this violet colour (although there would be expected to be a residual pale-yellow color from picryl group still present). Various volumes (50,100,150,200 and250µl) of the crude extract were transferred into clean test tubes. Freshly prepared DPPH (1, 1-diphenyl-2-picrylhydrazyl) solution (1ml) was added to each test tubes and incubated in dark at 37ºC for 30 minute and read at 517nm. The percentage of cell viability was calculated using the following formula:

% Cell viability = 100*(Absorbance of control – Absorbance of sample)/Absorbance of control

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2.9 ANTI-CANCER ACTIVITY

2.9.1 Cell culture

The cell lines were obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were cultured in 25 cm^2 culture flasks and incubated at 37 °C in a humidified atmosphere of 5% CO_2. The cells were provided with liquid growth media DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 100 U/ml penicillin G and 100 µg/ml streptomycin (Hi-media). The DMEM consists of glucose (4.5 g/l), L-glutamine (2 mm), sodium bicarbonate (1.5 g/l), HEPES (10 mm) and sodium pyruvate (1.0 mm). The cell confluency was measured using trypan blue dye exclusion method. Cells were passaged (sub-cultured) when 85-90% confluency was obtained.

2.9.2 Anticancer activity on cell lines – MTT assay

The anticancer activity of the crude extract of Actinomycete was tested against lung cancer cell line (A549) by MTT (3-4,5- dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide assay (8). The cells were seeded in 96-well microplates (1 x 10^6 cells/well) and incubated at 37°C for 24 hrs in 5% CO_2 incubator and allowed to grow till 90% confluence. Then the medium was replaced and the cells were treated with crude extracts of different volumes such as 25µl, 50µl, 75µl, 100µl, 125µl / ml and incubated for 24 hrs. The cells were then washed with phosphate-buffer saline (PBS, pH 7.4) and 20 µl of (MTT) solution (5 mg/ml) was added to each well. The plates were then allowed to stand at 37ºC in the dark for additional 2-4 hrs. The formazan crystals were dissolved in 100 µl DMSO and the absorbance was at 570 nm using Spectrophotometer. The morphological changes of untreated (control) and treated cells were observed under inverted microscope after 24 hrs and photographed. The percentage of cell viability was expressed as in formula.

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

III. RESULTS

Fig1: wet soil sample from Pattina pakkam beach

Fig 2: wet soil sample from Thiruvanmiyur beach

Fig 3: wet soil sample from Besant nagar beach

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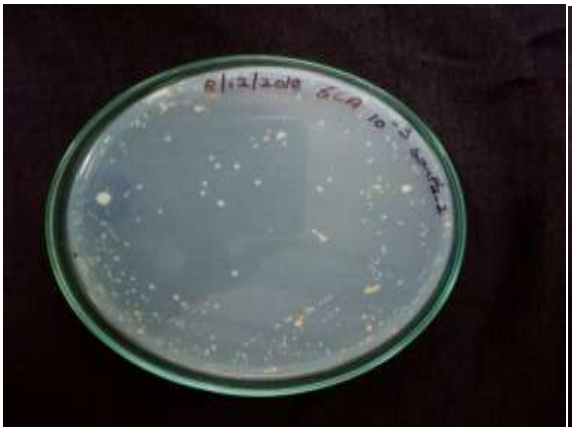

Fig 4: Plate showing Bacterial isolates from soil of Pattina pakkam beach

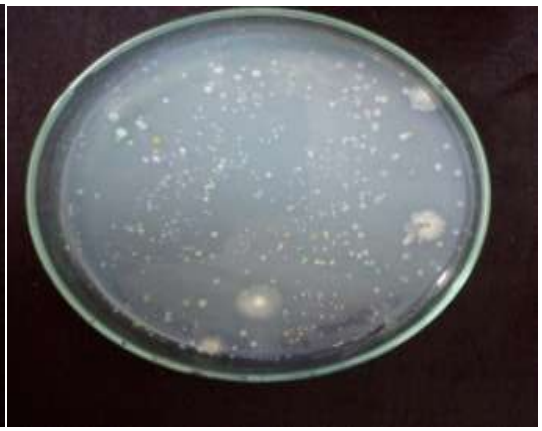

Fig 5: Plate showing bacterial isolates from marine marine soil of Thiruvanmiyur beach

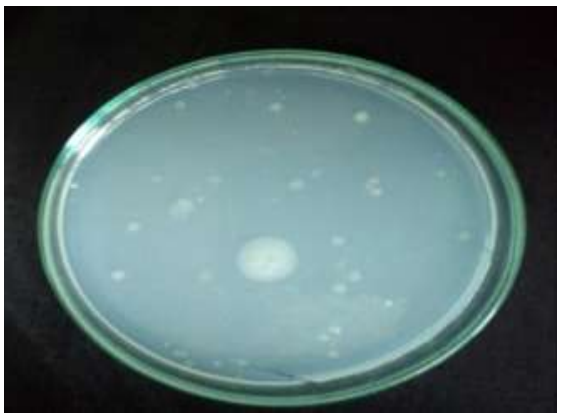

Fig6: plate showing from bacterial isolates from marine soil of Besant nagar beach

Fig 7: Bacterial isolate from marine soil showing inhibition of growth of tested bacteria near the centre streak (Cross streak method)

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Identification of bacteria based on 16srRNAsequence analysis

16S rRNA gene partial sequence (~682bp)

GGGATTATGTGGCCGAGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGA
AACGGGGTCTAATACCGGATACTGATCCTCGCTGGCATCTAGGTTCGAAAGCTCCGGCGGTGCAG
GATGAGCCCGCGGCCTATCAAGCATTTTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGG
TAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGAC
GGCCTTCGGGTTGTAAATTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCC
GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTACCGGTATTATTGGG
CGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGGTTGTGAAAGCCCGGGGCTTAACCCCGGGAGT
CGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCA
GATATCAGGAGGTACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACAGACGCTGAGGAGCGTG
GGAGCGAACAGGATTAGATACCCTGGTAGTCCA

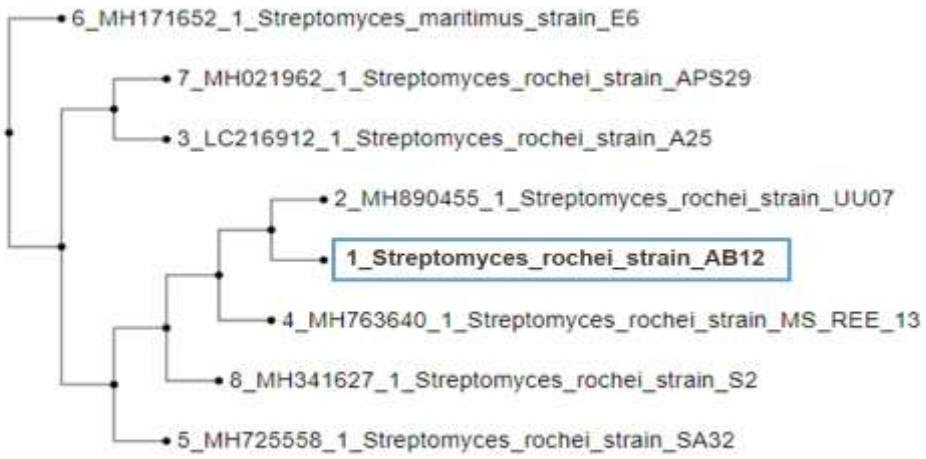

Table 1: Taxa retrieved from the GenBank

Fig 8:Small scale cultivation of Streptomyces rochei

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Fig 9: Crude Extraction of Streptomyces rochei isolated from Marine soil

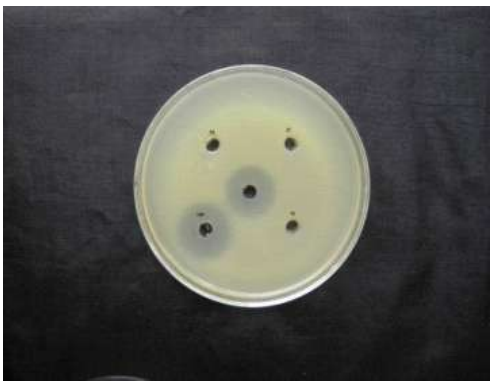

Fig10: Antibacterial activity of crude extract of Streptomyces rochei against Escherichia coli

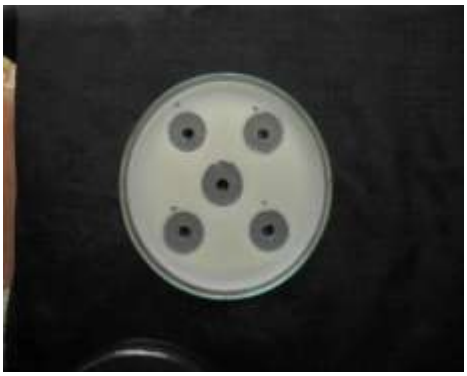

Fig 11: Antibacterial activity of crude extract of Streptomyces rochei against Bacillus subtilis

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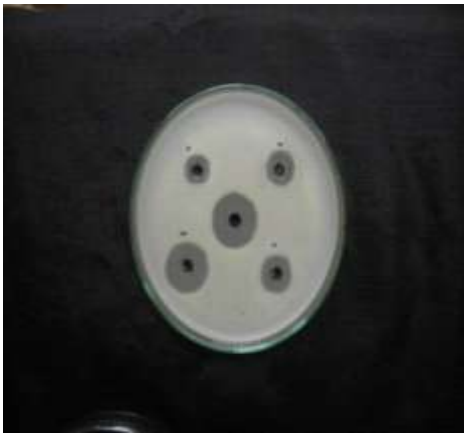

Fig 12: Antibacterial activity of crude extract of Streptomyces rochei against staphylococcus aureus

Fig13: Antifungal activity of crude extract of Streptomyces rochei against Aspergillus niger

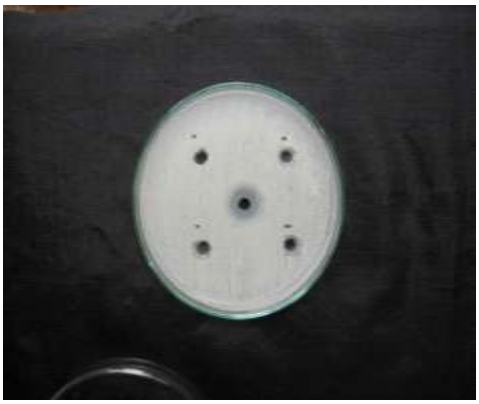

Fig 14: Antifungal activity of crude extract of Streptomyces rochei against Candida albicans

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Organisms	Zone of inhibition(mm) for different volumes of crude extract of Streptomyces rochei				Antibiotics (10µg/ml) Gentamycin
	25µl	50µl	75µl	100µl	
Staphylococcus aureus	9	14	17	21	23
Escherichia coli	Nil	Nil	Nil	21	21
Pseudomonas aeruginosa	Nil	Nil	Nil	Nil	20
Bacillus subtilis	20	21	22	23	22

Table 2: Antibacterial activity of crude extract of Streptomyces species isolated from Marine soil

Organism	Zone of inhibition(mm) for different volumes of crude extract of Streptomyces rochei				Antibiotics(10µg/ml) Clotrimazole and Fluconazole
	25 µl	50 µl	75 µl	100 µl	
Candida albicans	Nil	Nil	Nil	Nil	21
Aspergillus niger	Nil	Nil	6	8	13

Table 3: Antifungal activity of crude extract of Streptomyces rochei isolated from marine soil

S.NO	Sample	Volume crude metabolites (µl/ml)	OD	DPPH activity
1.	S_1	50	0.808	11.88
2.	S2	100	0.744	18.86
3.	S3	150	0.661	27.91
4.	S4	200	0.559	39.04
5.	S5	250	0.477	48.30

Table 4: Antioxidant activity of Crude extract of Streptomyces species by DPPH assay

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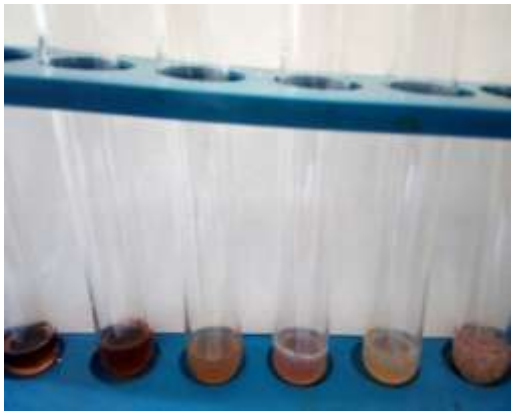

Fig 15 Antioxidant activity of crude extract of Streptomyces rochei by DPPH Assay

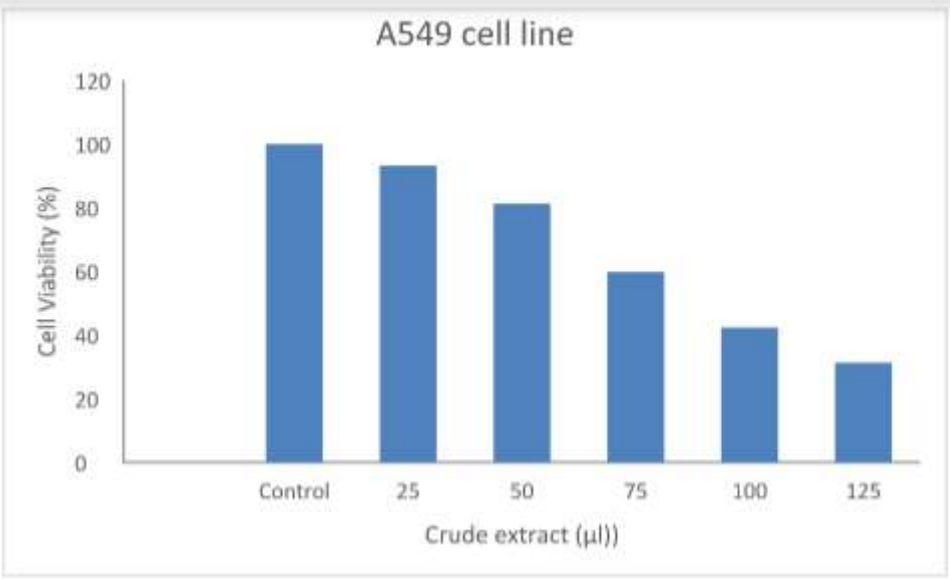

Chart 1: Anticancer activity of crude extract of Streptomyces rochei

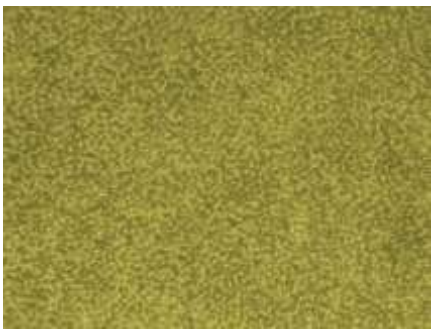

Fig 16: control (lungcarcinoma) A549

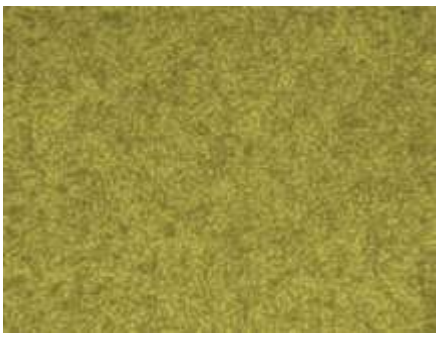

Fig 17: Toxicity of 25µl/ml

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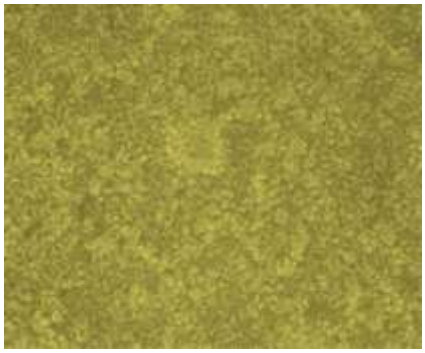

Fig 18: Toxicity of 50µl/ml

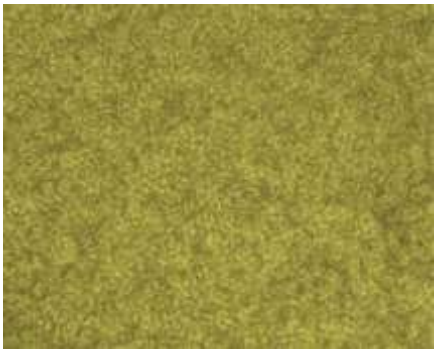

Fig 19: Toxicity of 75µl/ml

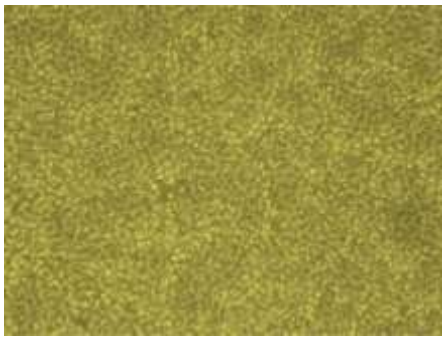

Fig 20: Toxicity of 100µl/ml

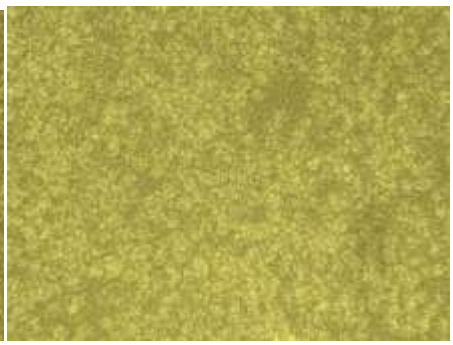

Fig 21: Toxicity of 125µl/ml

The crude metabolites extracted from Streptomyces rochei were tested for anticancer activity using lung carcinoma cell line (A549). The crude metabolites showed anticancer activity and 50% of inhibition (Ic50) was determined as 75µl of crude extract.

(A549) Lung carcinoma cell line				
Volume of crude extract (µl)	**Absorbance**		**Average**	**Cell Viability (%)**
	I	**II**		
Control	0.968	0.964	0.966	100
25	0.903	0.898	0.9005	93
50	0.789	0.782	0.7855	81
75	0.58	0.576	0.578	59
100	0.412	0.407	0.4095	42
125	0.301	0.306	0.3035	31

Table 5: Anticancer activity of crude compound from cell line

IV. DISCUSSION

In the present study, marine soil samples (Fig 1,2&3) were collected from three different beaches in Chennai and was processed for the isolation of Actinomycetes. The three isolates (fig 4,5&6) obtained from three different marine soil

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samples were screened for secondary metabolites production by cross streak method Out of three isolates, one isolate showed the production of metabolite that inhibited the test pathogens - Escherichia coli, Staphylococcus aureus and Bacillus subtilis and Pseudomonas aeruginosa in the cross-streak method (Fig: 7). Actinomycetes isolated from marine soil were screened by cross streak method and out of 52 isolates of marine actinomycetes, about 43 marine actinomycetes showed good antimicrobial activity (9).

The Actinomycete which showed growth inhibitory activity against the test pathogens was subjected to Gram's staining and 16S rRNA gene sequencing. Phylogenetic tree was generated from the 16S rRNA gene sequence data using neighbor-joining tree building algorithm. The bacterial species is identified as Gram-positive rod, Streptomyces rochei. Streptomyces rochei was cultivated in small scale for the extraction of bioactive metabolites.

Ethyl acetate extract of Streptomyces rochei was tested for their antimicrobial activity by agar well diffusion method (Table 2). It had shown inhibitory activity against Escherichia coli with a zone of inhibition 21mm only for 100µl of crude metabolites and the zone inhibition was found to be equal with the antibiotic (Fig 10). Bacillus subtilis was inhibited with a zone of inhibition 20mm, 21mm, 22mm and 23mm for different volumes (25µl,50µl,75µl,100µl) of crude metabolites (Fig 11). Staphylococcus aureus was inhibited with a zone of inhibition 9mm, 14mm, 17mm and 21mm for different volumes(25µl,50µl,75µl,100µl) of crude metabolites (Fig 12) The isolates was not found to have an inhibitory activity against Pseudomonas aeruginosa.

The crude extract of Streptomyces rochei was also tested for antifungal activity and had not inhibited Candida albicans (Fig 14) but inhibited Aspergillus niger with a diameter of zone of inhibition 5mm, 7mm, 8mm and 10mm for different volumes (25µl,50µl,7µl5,100µl) of crude metabolites (Fig13 & Table 3). The crude metabolites extracted from the Actinomycetes inhibited E. coli, Staphylococcus, Bacillus, Klebsiella and Pseudomonas species (10)

The percentage scavenging of DPPH radical for the different volume (50µl, 100µl, 150µl, 200µl, 250µl) of crude metabolites was found to be 11.886, 18.86, 27.917, 39.04 and 48.30 (Table 4, Fig 15). The ethyl acetate extract of Streptomyces species isolated from marine soil showed 43.2% DPPH free radical Scavenging Activity(11).

The crude extract containing metabolites from Streptomyces rochei was also tested for anticancer activity using lung carcinoma cell line - A549 (Fig 16-21). The crude metabolites showed anticancer activity and 50% of inhibition (Ic50) was determined as 75µl of crude extract. (Table5 and Fig: 19). Streptomyces strain, isolated from humus soils in the Western Ghats, has an anticancer activity against HepG2 (hepatic carcinoma) and HeLa (cervical carcinoma) in vitro (12).

V. CONCLUSION

The Streptomyces rochei isolated from the marine soil showed potential antimicrobial activity against the tested bacteria and fungi. The crude bioactive compounds in the extract of Streptomyces rochei showed antibacterial, antifungal, antioxidant and anticancer activity. Streptomyces rochei isolated from marine source act as a good source of extracting potential antimicrobial and anticancer compounds and hence basis for the further identification of novel compounds from Streptomyces rochei. The compounds present in the crude extract of Streptomyces rochei can be identified, purified and tested in future to be used as a potential source of Pharmacological drug.

REFERENCES

[1]. Fenical W and Jensen PR (2006) Developing a new resource for drug discovery: marine actinomycete bacteria. Nat Chem Biol 2:666–673

[2]. Renu Solanki · Monisha Khanna · Rup Lal, Bioactive compounds from marine actinomycetes, Indian J. Microbiol. (December 2008) 48:410–431

[3]. Hodgson, D.A. Primary metabolism and its control in Streptomycetes: A most unusual group of bacteria. Adv. Microb. Physiol. **2000**, 42, 47–238.

[4]. Holt JG.UK:Cambridge University Press;1989.p.4 cross streak

[5]. Magaldi S., Mata-Essayag S., Hartung de Capriles C. Well diffusion for antifungal susceptibility testing. Int. J. Infect. Dis. 2004; 8:39–45.

[6]. Clinical Laboratory Standards Institute, Performance standards for antimicrobial disk susceptibility tests; approved standard - 9th ed. CLSI document M2-A9. Clinical Laboratory Standards Institute, Wayne, PA, 2006; 26: 1.

[7]. Molyneux P. The use of the stable free radical diphenylpicryl-hydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J Sci Technol, 2004; 26: 211–219.
[8]. Tim Mosmann. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. J Immunol Methods, 1983; 65: 55-63.
[9]. Vijayabharathi R, Bruheim P, Andreassen T, et al. Assessment of resistomycin, as an anticancer compound isolated and characterized from Streptomyces aurantiacus AAA5. J Microbiol. 2011;49(6):920 - 926
[10]. Raja Sekar Reddy Athoon, Janardhan A. Evaluation of anti-microbial and anticancer activity of secondary metabolites from marine Actinomycetes. J Pharm Res 2012; 5(1): 391-393.
[11]. Mohankumar Thenmozhi, Krishnan Kannabiran. Antimicrobial and antioxidant properties of marine Actinomycetes, streptomyces sp VITSTK7. Oxid Antioxid Med Sci 2012; 1(1): 51-57
[12]. Gunasekaran Mohanraj, Thangavel sekar. Isolation and screening of Actinomycetes from marine sediments for their potential to produce antimicrobials. Int J Life Sc BT & Pharm Res 2013; 2(3): 115-126.