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RESEARCH ARTICLE

Taxonomic Characterization and Antagonistic Efficacy of *Streptomyces cavourensis* SKCMM1 Isolated from Sediment of Pichavaram Mangrove Forest

Manikandan Madheslu[1], Ashok Kumar Marimuthu[1], Vaishnavi Jagannathan[1], Duraimurugan Kasiviswanathan[2], Baby Shakila Parimelazhagan[1] and Narendhran Sadasivam[1]*

[1]Department of Biotechnology, Sri Krishna Arts and Science College, Coimbatore - 641 008, Tamil Nadu, India.
[2]School of Community Science and Technology, Indian Institute of Engineering Science and Technology, Shibpur - 711 103, India.

Abstract

Genus Streptomyces under phylum actinobacteria has been recognized as a prolific source for production of bioactive secondary metabolites. Actinobacterial strain designated as SKCMM1 isolated from Pichavaram Mangrove forest sediment was identified as *Streptomyces cavourensis* using polyphasic taxonomic approach. This strain shares 99% sequence similarity with *Streptomyces cavourensis* NBRC 13026^T. Ethyl acetate fraction of the strain SKCMM1 exhibited highest biological activity. FTIR and GCMS analysis of crude compounds isolated from the active ethyl acetate fraction states the presence of several phenolic, hydrocarbon and fatty acid compounds with various bioactivities. This study will be an attempt to understand that the strain holds significant antimicrobial activity against test pathogens and antiproliferative activity against HeLa cell lines (IC_{50} value of 8.9µg/ml).

Keywords: *Streptomyces*, Polyphasic Taxonomy, Liquid – liquid extraction, Spectral analysis, Bioactive compounds.

***Correspondence:** narendhransumathi@gmail.com; +91-9043669722

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INTRODUCTION

Actinobacteria are present in various ecological habitats such as soil, fresh water, backwater, lake, sewage and marine environment[1]. These bacteria are bounteous group of microorganisms that produce a wide array of secondary metabolites such as antibiotics, antitumor agents, immunosuppressive agents, vitamins, enzymes and even cosmetics[2]. On contemplating the microbes of the same region, the marine Actinobacteria are unique for their antibiotic production in fluctuating physical, chemical and biological factors[3]. In recent years the escalating diligence is towards the exploration of novel microbes that manufacture metabolites to combat drug resistant pathogenic microbes[4-6].

Marine ecosystem is a resplendent choice for the isolation of novel microbes that confer splendid outcome in identification, characterization and application of secondary metabolites[7]. Earlier, *Streptomyces sp*. VITMK1 was isolated from Pichavaram region of Tamil Nadu and it yielded a potent antibacterial compound diketopiperazine[5]. Similarly, ethyl acetate crude extract obtained from *Streptomyces* strain CH54-4 was reported for best antimicrobial and anti proliferative activity[8]. In the current study, *Streptomyces* SKCMM1 is isolated from Pichavaram Mangrove forest of Tamil Nadu and identified through polyphasic taxonomic characterization approach. The antagonistic ability of the isolate was assayed through shake flask fermentation and spectral studies (Fig. 1).

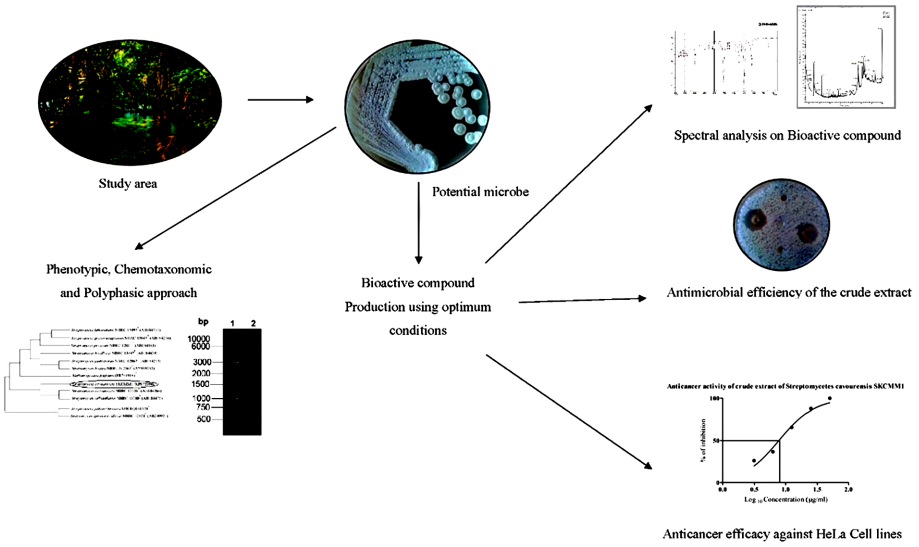

Fig. 1. Schematic representation of the present study

MATERIALS AND METHODS

Isolation and Identification of Potential Actinomycete

Five marine sediment samples were collected from Pichavaram Mangrove forest, Tamil Nadu using a sterile borer at a depth of 10 to 15 cm (Table 1 and Figure 2). The serially diluted (10^{-1} to 10^{-5}) samples were seeded on Starch Casein agar (SCA) (HiMedia, India) plates supplemented with antibiotics (Cycloheximide 50µg/ml and Nystatin 50µg/ml) and incubated at room temperature for 10 days. Characteristic tough leathery colonies of Actinomycetes with a clear zone of inhibition were selected and preserved in SCA slants for further use. Isolates were grown in 50 ml of starch casein broth by submerged culture method (32°C for 7 days), centrifugation was carried out at 8000 rpm for 15 min and the clear supernatant was challenged against selected bacterial pathogens (*E.coli* MTCC 1698*, E.coli* ESBL, *Bacillus pumilis* NCIM2327*, Proteus vulgaris, Staphylococcus aureus* MTCC 3160*, Staphylococcus aureus* (methicillin resistant), *Shigella flexneri* MTCC

1457) by well diffusion method. The potent Actinobacteria was selected based on the vast zone of inhibition and it was taken for further studies.

Table 1. Characteristics of samples collected from Pichavaram Mangrove forest, India.

Sample no	Latitude (N)	Longitude(E) (E)	Temp (°C)	pH
MS1	11° 30' 11''	79° 46' 37''	24	7.9
MS2	11° 28' 37''	79° 47' 15''	28	7.5
MS3	11° 27' 51''	79° 47' 24''	28	8.2
MS4	11° 27' 22''	79° 47' 17''	29	7.7
MS5	11° 27' 24''	79° 46' 46''	26	8.1

MS = Marine Sediment

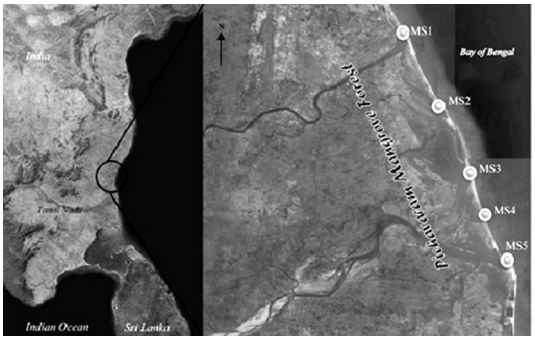

Fig. 2. Schematic representation map showing sampling area

Phenotypic Characterization of Strain SKCMM1

Potential Actinobacterial strain SKCMM1 was characterized morphologically and physiologically as prescribed in International *Streptomyces* Project (ISP)[9]. The micro-morphology of strain was observed under light microscopy and Scanning Electron Microscopy (JEOL JSM-5610) after incubation at 28°C for 7 days. The pigmentation of aerial mycelium and structure of sporophores were observed on different ISP media 1 – 7, which are an ideal aspect in its classification. Colony morphology was recorded with respect to size, colour, aerial mycelium, substrate mycelium and pigmentation. Addition to that, biochemical and physiological examinations were performed according to standard methodology[5, 10]. Standard procedures were used to determine the cell wall amino acids[11] and cell wall sugars[12] of the potent Actinobacterial strain. Fatty acids extracted from isolate SKCMM1 were methylated and analyzed by GC-MS, using the standard sherlock microbial identification system[13].

16s rRNA Gene Sequencing

Genomic DNA was extracted and subjected to polymerase chain reaction[14] targeting 16S rRNA gene using the 5'-AGAGTTTGATCCTGGCTCAG-3'– forward primer and 5'TACGGCTACCTTGTTACGACT-3' – reverse primer[15]. The reaction mixture contained 10X Taq buffer, 25 mM $MgCl_2$, 4 mM dNTPs, Taq polymerase 0.5U, template DNA 5-10 ng and primers at 10 pmol concentration (Fermentas, Thermo Scientific, Vilnius, Lithuania). The PCR conditions comprised of initial denaturation at 94°C for 10 min followed by 23 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 15 sec, extension at 72°C for 1 min and a final extension of 72°C for 10 min carried out in Master thermal cycler (Eppendorf, Germany). Later, amplicon sequencing was done in ABI 3730xl cycle sequencer (Facility from Sciegenom, Cochin, India) and sequences were deposited in GenBank (NCBI). The dendrogram analysis was done in MEGA 4.0 to study the evolutionary similarity of SKCMM1 strain.

Production and Extraction of Bioactive Compound using *Streptomyces cavourensis* SKCMM1

For production, 125 ml of Starch casein broth SKCMM1 (500 ml Erlenmeyer flask) was inoculated with 1 ml *Streptomyces cavourensis* and incubated at 28°C in a rotary shaker (180 rpm) for 7 days. Then, broth was filtered using Whatmann No 42 aseptically and stored at 4°C. In order to select the best choice of solvent for active compound

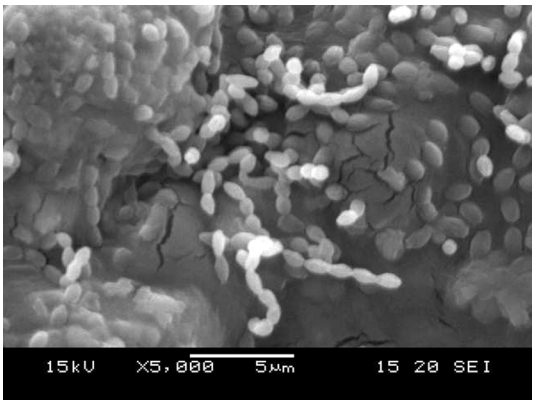

Fig. 3. Electron micrograph image of isolate SKCMM1

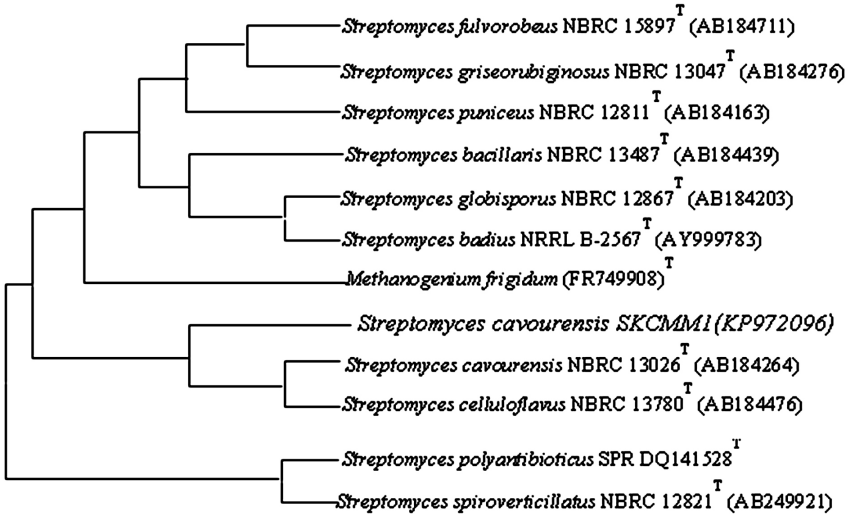

Fig. 4. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain SKCMM1 with related species

extraction, an equal volume of various solvents (hexane, chloroform, ethyl acetate, n-butanol and propanol) along with filtrate were shaken well for 60-90 mins. The collected solvent fractions were concentrated using distillation column and the crude obtained was challenged against test bacterial strains. Based on the zone of inhibition results, ethyl acetate was selected as the best solvent for compound extraction (Figure 5). The fermented broth and ethyl acetate was treated at a ratio of 1:2 to extract bioactive molecules.

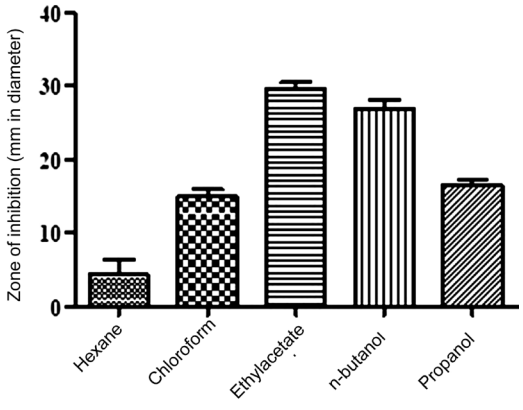

Fig. 5. Results of solvent extraction showing ethyl acetate as best choice of solvent for active compound extraction.

Spectral analysis of ethyl acetate extract

The active extract dissolved in methanol was taken for UV Vis and FTIR analysis using Shimadzu – 1601 UV VIS and Shimadzu IR 8000 respectively. The functional groups present in the sample were detected in the spectral range of 400 to 4000 cm^{-1}. The ethyl acetate sample extract was analyzed by GC-MS [Thermo GC – Trace Ultra Version 5.0]. In this method, a 30m×0.25 mm ZB 5 - MS capillary Standard Non-Polar column with a film thickness of 0.25µm was used. The carrier gas was helium which had a column flow rate of 1 mL/min (at a pressure of 105 kPa). Then, 1.0 µL of ethyl acetate sample was injected with the following column program 50°C@7°C/min to 200°C (3 min) @7°C/min to 280°C (15 min) [Scan range: 40 – 1000 m/z]. Every discrete constituent showed by GC MS tandem analysis was used to compare the compounds and functional groups identified with a standard compound of NIST library (Version 2005).

Anticancer activity of crude extract against HeLa cell lines

The HeLa cell line obtained from National Centre for Cell Science (NCCS), Pune was grown in RPMI-1640 containing 10% fetal bovine serum (FBS). Single cell suspensions were disintegrated from monolayer cells using trypsin-ethylene diaminetetraacetic acid (EDTA). By subsequent medium dilution, the viable cells with a final

Table 2. Antimicrobial activity of the isolates using well diffusion assay

	SKC MM 1	SKC MM 2	SKC MM 3	SKC MM 4	SKC MM 5	SKC MM 6	SKC MM 7	SKC MM 8	SKC MM 9	SKC MM 10	SKC MM 11	SKC MM 12
1	12.3 ± 1.5	0	11.7 ± 1.2	0	13.3 ± 1.5	0	0	0	12.3 ± 0.6	8.7 ± 1.2	10.7 ± 1.2	12.7 ± 1.5
2	17.7 ± 1.2	7 ± 1.0	0	0	11.0 ± 1.7	0	0	11.3 ± 1.5	0	21.0 ± 1.0	0	8.3 ± 1.2
3	27.0 ± 1.0	9.3 ± 1.2	0	0	0.0	0	15.3 ± 1.2	0	6.0 ± 1.7	0.0	18.0 ± 0.0	12.0 ± 1.7
4	12.7 ± 1.5	0	0	7.7 ± 1.2	19.0 ± 1.0	24.0 ± 0.0	9.7 ± 1.2	0	0	23.0 ± 1.7	0	0
5	9.3 ± 1.2	0	0	0	0	0	0	0	7.3 ± 1.5	0	0	0
6	14.0 ± 0.0	0	0	0	0	16.0 ± 1.7	9.3 ± 2.5	0	0	0	0	0
7	11.7 ± 1.2	0	0	0	0	11.0 ± 1.7	0	0	0	0	0	0

1. Staphylococcus aureus MTCC 3160*, 2. Bacillus pumilis* NCIM 2327*, 3. Staphylococcus aureus* (methicillin resistant) *4. Escherichia coli* MTCC 1698 *5. Escherichia coli* (ESBL)*, 6. Shigella flexneri* MTCC 1457, *7. Proteus vulgaris*

density of $1x10^5$ cells/mL were acquired. The cell suspension (100µl) was incubated in 96 well plate (103 cells/well) for 24 hrs to achieve cellular adherence. Later, test samples were added in varying concentrations of 0 – 50 µg/ml dimethylsulfoxide (DMSO) to cells and kept for 48 hr incubation. Afterwards, 15 µl of MTT (5 mg/mL) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4 hrs. Finally, the medium with MTT was then flicked off and the formazan crystals were solubilized in 100 µl of DMSO to measure the absorbance at 570 nm using micro plate reader. The mean value IC_{50} was calculated by nonlinear regression analysis and graph was plotted between percentage of cell inhibition and Log_{10} concentration using GraphPad Prism software.

RESULTS AND DISCUSSION

In the current investigation, five sediment samples collected from mangrove region were had slight variation in their physical parameters. Notably, the temperature of sediments ranged between 24°C to 29°C, while the pH varied from 7.5 to 8.2 which are considered to be optimum for Actinobacterial growth and endurance. Totally twelve actinobacterial isolates obtained from sediment samples were sorted out for superlative samples depending on temperature and pH conditions having potential microbial diversity (Table 1 and Figure 2). Generally, Actinobacteria living in these conditions are capable of growing in extreme and incessantly changing environment which is a quintessential representation for survival in microbial diversity. Also, studies conducted on *Actinobacteria* in mangrove soil by many eminent researchers suggest that this may actually be due to the better quality of the sediments, described in terms of structure, pH and humic substance[8,16].

In the well diffusion assay of current investigation, all the twelve isolates were active against selected clinically pathogenic bacterial strains. Especially the isolate named SKCMM1 showed an extraordinary activity against all the Gram positive and Gram negative reference bacterial strains used in this investigation. The isolate SKCMM1 showed conspicuous

Table 3. Phenotypic characterization of the isolate SKCMM1 on Starch Casein Agar

Phenotypic characteristics	SKCMM1
Colony morphology	Circular, umbonate, entire
Sporophore morphology	Straight or Rectiflexibiles
Spore surface	Smooth
Colour of aerial mycelium	Pale green
Colour of substrate mycelium	Brown
Spore mass	Pale green
Pigment	Brown colour

antimicrobial activity in terms of zone of inhibition (p value = 0.0005) in mm ranging from 8 to 27 against test pathogens (Table 2). For this reason, isolate *Streptomyces* SKCMM1 is focused for centralized assiduity. Generally, actinobacterial populations of mangrove forest are capable of tolerating a wide span of catastrophes, they have not been extensively explored for the registration of novel bioactive compounds. Thus, it is crucial to note that broad spectrum activity of marine microorganisms from hitherto unexplored habitats should be considered novel source for bioactive secondary metabolites.

In the present investigation, morphology of potential isolate SKCMM1 was tested on various media such as Starch casein agar, Actinomycetes isolation agar, Nutrient agar and ISP (International *Streptomyces* Project) media (ISP 1 to 7). The isolate produced circular, pale green colour colonies on Starch casein agar media. The size of the colony measured from 0.5 to 0.75 mm in dm. Further morphological details are given in Table 3. The isolate SKCMM1 showed substantial growth on all the media used for the growth characterization (Table 4). SEM studies revealed well developed and unfragmented aerial and substrate mycelia of the strain SKCMM1 (Figure 3). The spore chain was in straight, rectiflexibiles and spore surface was very smooth, oval shaped and arranged in a long chain.

In chemotaxonomic characterization, no cell sugars were detected on chromatogram comparing with the standard lanes loaded with sugars such as galactose, glucose, arabinose, mannose, ribose and rhamnose. Also in cell wall amino acid analysis, two amino acids spots were detected in sample lane against the standards LL-2,6 diaminopimelic acid and glycine. Presence of LL-2,6 diaminopimelic acid and absence of standard cell wall sugars reveals the cell wall of strain SKCMM1 confirmed that cell wall belongs to type I category. The results were in high agreement with previous studies on *Streptomyces* sp identification[8, 21]. The cell wall fatty acids were extracted from isolate SKCMM1 and analyzed in GC MS (Table 5). Presence of Tetradecanoic acid, Pentadecanoic acid, Hexadecanoic acid and Heptadecanoic acid in the form of methyl

Table 4. Cultural characteristics of isolate SKCMM1 on International Streptomyces Project media.

Media	Growth	Aerial Mycelium	Substrate Mycelium
ISP1	++++	Creamy White	Brown
ISP2	+++	Creamy White	Mild Brown
ISP3	++++	Pale Green	Pale Yellow
ISP4	++++	Pale Green	White
ISP5	++++	Pale Green	Mild Brown
ISP6	++++	Pale Green	Dark Brown (Soluble Pigment Detected)
ISP7	++++	Pale Green	Blackish Brown
NA	++++	Creamy White	Brown
SCA	++++	Pale Green	Brown (Soluble Pigment Detected)
AIA	++++	Creamy White	Brown

Growth as per article; ++++ Excellent; ++ Moderate; +++ Good; + Poor; ISP- International Streptomyces Project; AIA- Actinomycete isolation agar; NA- Nutrient agar.

Table 5. GC-MS analysis of Fatty acid methyl esters in cell wall of isolate SKCMM1

Retention time	Compound name	Mol. Formula	Mol. wt	Area %
19.01	12-Methyltetradecanoic acid	$C_{16}H_{32}O_2$	256	56.60
19.62	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	256	7.10
21.64	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	21.30
23.03	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	284	14.99

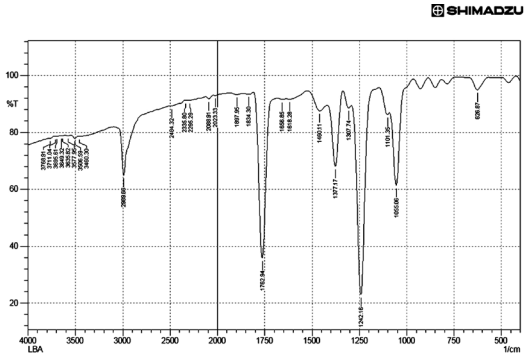

Fig. 6. FT IR Spectrum of active ethyl acetate extract of *Streptomycetes cavourensis* SKCMM1 fermented broth.

esters confirming the cell wall belonged under *Streptomyces*[13].

The isolate SKCMM1 was sequenced using primer specific for *Streptomyces sp* using 16s rRNA gene sequencing and deposited in GenBank (NCBI) as a strain named *Streptomyces cavourensis* SKCMM1 with the following accession number of KP972096. *Streptomyces cavourensis* SKCMM1 showed 99% homology with *Streptomyces cavourensis* strain NBRC 13026^{T} on blast analysis. The GC content of the isolate was about 58.2% which is a typical characteristic of the *Streptomyces*. For dendrogram construction (using MEGA 4.0), 16s rRNA gene sequence of the isolate SKCMM1 and close neighbor of isolate sequence was included in the sequence – based comparative analysis that revealed high similarity with *Streptomyces cavourensis* NBRL 13026 (AB184264) and *Streptomyces cavourensis* NBRL 13780 (AB184476) (Figure 4).

Active compound separation from the fermented broth showed that ethyl acetate is a best choice of solvent (p value = 0.0009) (Figure 5). Similar findings have been reported by various investigators upon using ethyl acetate as a best choice of solvent for extraction of bioactive compounds[5,22]. The active fraction of ethyl acetate fraction was significantly activity against reference bacterial strains used in this study (Table 6). The condensed crude is considered as partially purified bioactive compound and taken for spectral studies.

FT IR spectral studies play a major role in the identification of active compounds. The data obtained through this study will disclose the nature of compounds and functional groups present in the sample. The FT IR results showed 26 elevated peaks in the range between 3800 cm^{-1} and 600 cm^{-1} (Figure 6). The peak values indicated the presence of functional groups such as C=C alkenes, C=O group of acids, C=C alkynes, -C-H stretch (alkane H) and O-H (hydrogen bond, intermolecular, polymeric association). The spectral data of FT-IR reveals the presence of large broader where peaks were observed at 1241cm^{-1} and 1762cm^{-1} indicating the presence of alcohols, ester, ethers and carboxylic acid groups. The two peaks between 1055cm^{-1} and 1377cm^{-1} indicated the presence of primary alcohols and nitro compounds respectively in the sample. The presence of a peak at 2989cm^{-1} indicated alkanes. The presence of 4 peaks between 3200cm^{-1} to 3600cm^{-1} range indicated the presence of monomeric alcohols and phenols.

Table 6. Antimicrobial activity of ethyl acetate crude of SKCMM1 against reference bacterial strains.

Name of the Organism	Zone of Inhibition(mm)
Gram Positive	
Staphylococcus aureus MTCC 3160	34.3 ± 1.52[a]
Bacillus pumilis NCIM 2327	25.0 ± 1.73[a]
Staphylococcus aureus (methicillin resistant)	32.0 ± 1.73[a]
Gram Negative	
Escherichia coli MTCC 1698	24.3 ± 3.1[a]
Escherichia coli (ESBL)	18.3 ± 1.2[a]
Shigella flexneri MTCC 1457	18.6 ± 1.5[a]
Proteus vulgaris	19.0 ± 2.0[a]

a = p< 0.05

Table 7. GC-MS analysis of the ethyl acetate extract of *Streptomycetes cavourensis* SKCMM1 fermented broth

Classification	Compound	%
Aniline Compound	2,4-Dimethoxy- tri phenylamine	0.52
Fatty acid	Pentanoic acid	1.76
	2,4,6-Octatrienoic acid	2.74
Hydroxy Compound	1,4-Dioxane-2,6-dimethanol	13.06
	Caryophyllenyl alcohol	0.67
	3-Cyclohexen-1-ol	0.67
	Geosmin	2.48
Hydrocarbons	1,5-Bis pentane	0.47
	Docosane	0.50
	Cycloheptanone	1.12
	Benzene	2.12
Cycloalkane compound	Cyclohexane	7.58
	Cyclopropane	1.11
Aromatic compound	1,3-Bisbenzo thio phene	8.66
Carboxamide	Cyclopropane carboxamide	0.8
Unknown compounds		55.74

The ethyl acetate extract of *Streptomycetes cavourensis* SKCMM1 was subjected to gas chromatography-mass spectrometry analysis. The identification of compound is based on the peak area and mass by charge ratio. This peak area is directly proportional to the quantity of the compound present in the extract. The volatile metabolic profiling is expressed as chromatogram and the results were tabulated (Table 7). The GC MS results indicated the presence of more than 27 different compounds notably 1, 4-Dioxane-dimethanol (13.06%), 1, 3-Bisbenzo thiophene (8.66%), 1,3,5-trimethyl-2-octadecyl cyclohexane (7.58%), 2,4,6-Decatrienoic acid (2.74%), a-Amyrin (2.24%), Cyclopropane carboxamide (0.8%).

1, 4-Dioxane-dimethanol compound contain two -OH stretch at 2nd and 6th position and FTIR investigation (two larger boarder peaks observed at 1241cm^{-1} and 1762cm^{-1}) also supports the presence of alcohol groups in the partially crude extract. Recently, 1, 4-Dioxane-dimethanol has been filed for its anti-diabetic activity[23]. The second major compound resulted during mass spectroscopic study is 1, 3-Bisbenzo thiophene. Thiophene is a heterocyclic compound containing of planar five membered ring structure. Several, thiophene derivative compounds have been reported for biological activities such as antibacterial activity[24] and antiviral efficiency[25].

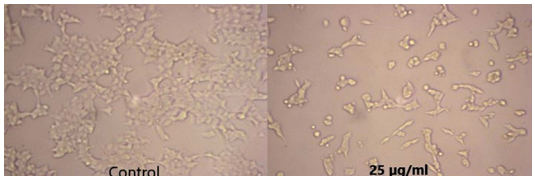

Fig. 7. Anticancer activity of the crude extract of *Streptomyces cavourensis* SKCMM1 against cancer cell lines

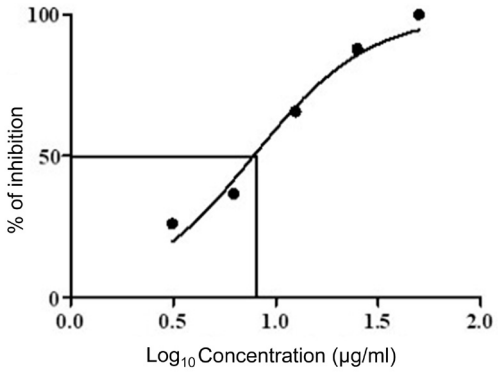

Fig. 8. Dose response curves (GraphPad Prism) of crude extract of *Streptomyces cavourensis* SKCMM1 against cancer cell lines

Thus 1, 3-Bisbenzo thiophene could also be an bioactive thiophene compound against the test pathogens.

The antiproliferative efficiency of SKCMM1 strain against HeLa cell lines has an enormous IC_{50} value of 8.9 µg/ml (R^2 = 0.9732) (Figure 7 and 8). ANCI (American National Cancer Institute) endorses that IC_{50} value of any crude compound falling down to 30 µg/ml as a potent drug[28]. Some of the most dynamic bioactive compound observed during spectral studies were a-Amyrin and Cyclopropane carboxamide despite its small proportion in the crude extract. a-Amyrin belongs to the triterpene class of compounds and has been reported for antimicrobial, antifungal and anti-inflammatory[26]. The presence of a-Amyrin in the crude could be the reason for the anti-inflammatory activity expressed against HeLa cell lines. Similarly, some of the structural derivatives of cyclopropane carboxamide has been reported for antifungal activity[27]. In this, most of them were previously reported for various biological activities such as antimicrobial, anti-inflammatory and cytotoxic activities. Therefore, it is suggested that further investigation may explore the significance of these unnoticed compounds.

In the present investigation, it is discernible that marine Actinobacterial populations from Pichavaram Mangrove region harbor plentiful biological activities particularly antimicrobial compounds production. These regions have magnificently concealed therapeutic and economic biological potential which is highly praised and agreed with the perception of other investigators[4,16-20].

CONCLUSION

As myriad of studies have been conducted on bacteria to look out for novel bioactive compounds, geographical locations such as mangrove forest may offer a potent microbial diversity for scrutinization. As an attempt to identify an effective microbe, here a study was conducted at Pichavaram mangrove forest, Tamil Nadu which culminated intensely propitious *Streptomyces cavourensis* SKCMM1 strain which was isolated and characterized through polyphasic taxonomy. Secondary metabolites proved to have broad spectrum antibacterial and antiproliferative efficiency against HeLa cell lines. Spectroscopic studies of partially purified extract enunciated several activities antimicrobial, antioxidant and cytotoxic compounds. A kindling approach for assessing purification of such compounds may exhibit their connotation in pharmaceutical industries.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest .

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