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SCREENING, CHARACTERIZATION, IDENTIFICATION AND APPLICATIONS OF BIOACTIVE METABOLITES FROM MARINE ACTINOMYCETES - STREPTOMYCES VARIABILIS

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ABSTRACT

The main aim of the study was to isolate marine actinomycetes which are antagonistically potent to produce the secondary metabolites which have various industrial applications such as antimicrobial, antioxidant (Total antioxidant activity, DPPH assay and $Fe3^{+}$ reducing power assay) and anticancer activity. Primary and Secondary screening for antimicrobial activity was determined by Cross streak method and Agar well diffusion method respectively. In pursuit of this marine sediments were collected from various locations in Chennai and from which five different actinomycetes have been isolated. Among the five isolates (ABTRI 1-5) the strain ABTRI 1 was found to be antagonistically active and its bioactive compounds were produced using ISP-2 medium. Crude bioactive compounds were then extracted and the compounds present within the crude extract of Marine Streptomyces ABTRI 1 were identified by Gas chromatography-Mass spectrometry analysis, which revealed the presence of seven bioactive compounds. The anticancer activity of the secondary metabolite of actinomycetes was evaluated on MCF-7 cell line by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The in vitro cytotoxicity assay on MCF-7 revealed that the secondary metabolite had cytotoxic effect with IC_{50} 292.4µg/ml.

KEYWORDS: Actinomycetes, DPPH, Thin layer chromatography, 16srRNA sequencing and MTT assay.

INTRODUCTION

A filamentous Gram-positive bacterium, characterized by a complex life cycle belonging to the phylum Actinobacteria, which represents one of the largest taxonomic units among the 18 major lineages currently recognized within the Domain Bacteria (Ventura et al. 2007). The bioactive secondary metabolites produced by microorganisms is reported to be around 23,000 of which 10,000 are produced by actinomycetes, thus representing 45% of all bioactive microbial metabolites discovered (Berdy 2005). Among actinomycetes, approximately 7,600 compounds are produced by Streptomyces species (Berdy 2005). Several of these secondary metabolites are potent antibiotics.

Progress has been made recently on drug discovery from actinomycetes by using high-throughput fermentation and screening, combinatorial biosynthesis and mining genomes for cryptic pathways, to generate new secondary metabolites related to existing pharmacophores (Baltz 2008). Marine microorganisms encompass a complex and diverse assemblage of microscopic life forms, of which it is estimated that only 1% has been cultured or identified (Bernan et al. 2004). The actinomycetes (sing. actinomycete) are a large group of aerobic, high G-C percentage gram-positive bacteria that form branching filaments or hyphae and asexual spores. In the Streptomyces species studied, the spores had a two-layered wall and the inner one extended to form the germ-tube wall. Ultra structural changes during the germination of fungal spores have been studied more extensively. When grown on an agar-surface, the actinomycetes branch forming a network of hyphae growing both on the surface and under-surface of the agar. The on-the surface hyphae are called aerial hyphae and the under-surface hyphae are called substrate hyphae.

Actinomycetes have a profound role in the marine environment apart from antibiotic production (Haefner 2003). The degradation and turnover of various materials are a continuous process mediated by the action of a variety of microorganisms (Heald et al. 2001; Bruns et al. 2003). The genus Streptomyces alone produces a large number of bioactive molecules. It has an enormous biosynthetic potential that remains unchallenged without a potential competitor among other microbial groups. A large number of Streptomyces spp. have been isolated and screened from soil in the past several decades (Watve et al.2001).

MATERIALS AND METHODS

Study area and Pre-treatment

Marine sediments were collected in a sterile container from a depth of 20 m from places like Muttukadu and Marina beach in Chennai, Tamilnadu, India. They were brought to the laboratory for further studies. Soil was kept for drying at room temperature for 3 days. Then it was treated with 0.1% Calcium carbonate ($CaCo_3$) and left for 5 days.

Isolation of actinomycetes

About five actinomycete strains were isolated as pure culture by using standard microbiological method such as serial dilution and spread plate method. (Rahman et al 2011).The starch-casein-agar plates were incubated at 30°C for 7 days. After 7 days incubation, the unique whitish pinpoint colonies were observed, selected and purified by subculturing at regular intervals of period.

Primary Antimicrobial screening of Marine Actinomycetes

The isolated marine actinomycetes were screened for their antimicrobial activity by Cross streak method against the human bacterial pathogens and fungal pathogen by antagonistic method. Starch casein agar medium and Nutrient agar medium were prepared separately, sterilized and mixed in laminar chamber and were plated in sterile petriplates. The actinomycetes were streaked (5cm measurement) on one corner of the Starch Casein Nutrient agar (SCNA). After the complete growth of the actinomycetes, the bacterial pathogens were streaked perpendicular (3cm measurement) near to it and incubated at room temperature for 24 hours.

For the antifungal screening, the mycelia agar block (8mm) of the fungal pathogen (Aspergillus niger) were kept in the middle of the respective plate and then the actinomycetes culture were streaked (5cm measurement) on the sides of the Starch Casein Potato Dextrose Agar (SCPDA) and incubated at room temperature for 2 to 3 days. After the preliminary test of the isolates for their antimicrobial activities, the active isolate was subjected to secondary metabolite production.

Production and extraction of Secondary metabolites

The selected potent antagonistic actinomycete was inoculated in ISP-2(International Streptomyces project (or) Yeast Malt broth) medium and kept under shake flask condition at 37°C for 10 days at 150 rpm. After incubation the broth was filtered through Whatman No.1

filter paper, bioactive compound was extracted from the solvent (ethyl acetate) phase. The culture filtrate was centrifuged at 10,000 rpm for 15 min. Equal volume of ethyl acetate was added to the eluted supernatant. The filtrate with bioactive components were concentrated, crude compound was obtained and evaluated for antimicrobial activity, antioxidant activity, etc.

Secondary screening-Determination of the antimicrobial activity

Antimicrobial activity of the crude compound was determined by agar well diffusion method. Wells of 5 mm diameter were bored using sterilized cork borer on Nutrient Agar plates of which the test pathogens *Escherichia coli*, Klebsiella pnuemoniae, Staphylococcus aureus, Bacillus subtilis were swabbed and the crude compound in the concentration of 250-1000 µg/ml were added to the wells. Plates were incubated at 37°C for 24 hrs and the zone of inhibition was measured. Tetracycline was used as positive control.

For fungi spore suspension of the test pathogen Aspergillus niger was prepared and swabbed over the potato dextrose agar plates. Wells of 5mm diameter were bored using sterilized cork borer. Crude compound in the concentration of 250-1000µg/ml were added to the wells. Plates were incubated at 37°C for 48 to 72 hrs and the zone of inhibition was measured. Flucanazole was used as positive control.

Invitro Antioxidant Activity of bioactive compounds from Marine actinomycetes

The bioactive components were concentrated and the crude compound obtained was checked for antioxidant activity.

(a) Phosphomolybdenum assay

Total antioxidant capacity can be calculated by the method described by (Prieto et al., 1999). Various concentrations (200µg-1000µg/ml) from the prepared sample (10mg crude compound/ml DMSO) was been pipetted out and 1ml of the reagent solution was added, followed by incubation in boiling water bath at 95°C for 90mins. After cooling the sample to room temperature, the absorbance of the solution was measured at 695 nm in UV spectrophotometer. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions. Ascorbic acid served as standard. Based on total antioxidant activity, the crude compound was been evaluated for other antioxidant activities (invitro).

(b) Free radical Scavenging Activity

The antioxidant activity was determined by DPPH scavenging assay (Khalaf et al. 2008). Various concentrations (200µg-1000µg/ml) of the crude extract was been pipetted out in clean test tubes. Ascorbic acid was used as reference compound. Freshly prepared DPPH (1,1-Diphenyl-2-picryl hydrazyl) solution (2ml) was added to each tube and the samples were incubated in dark at 37°C for 20 min and read at 517 nm. The data were expressed as the percent decrease in the absorbance compared to the control. The percentage inhibition of radical scavenging activity was calculated.

$$\% \text{ of DPPH Radical Scavenging Activity (\% RSA)} = \frac{\text{Abs. control} - \text{Abs. sample} * 100}{\text{Abs. control}}$$

(c) Ferric (Fe^{3+}) reducing power assay

The concentrated crude bioactive compound was taken in various concentrations (200µg-1000µg/ml) and was mixed with 2.5ml of phosphate buffer (0.2 M, pH 6.6) and 2.5ml of potassium ferricyanide (1%), and incubated at 50°C for 30minutes. Then, 2.5ml of trichloroacetic acid (10% v/v) was added to the mixture and then centrifuged at 3000 rpm for 10 min. Finally, 2.5ml of upper layer solution was mixed with 2.5ml of distilled water and 0.5ml $FeCl_3$ (0.1%) and the absorbance was measured at 700 nm (Makari et al., 2008, Hennebelle et al., 2008). Ascorbic acid served as standard.

Characterization for the selected isolate by morphological and biochemical analysis

Morphological identification - Cover slip culture method

Morphological identification was done by using coverslip culture method, in which spore suspension culture of the actinomycetes was inoculated at the intersection of the SCA medium and cover slip was buried in the solid SCA medium at an angle of 45ºC (Williams and Cross, 1971). The plates were incubated for 5 days. Then the cover slip was removed carefully and kept under glass slide in an inverted position and viewed under microscope.

Various biochemical tests (Indole production, Methyl-red, Voges-Proskauer, Oxidase test, Starch hydrolysis, etc.) were carried out according to the methods of Shirling and Gottlieb (1966), methods outlined in the Bergey's Manual of Systematic Bacteriology (Williams et al., 1989) and in the Laboratory Manual for Identification of Actinomycetes (IMTECH, 1998).

Gram's staining

A loopful of culture was placed in a clean glass slide and it was heat fixed by gently passing over the flame. The smear was flooded with crystal violet and allowed to stand for one minute. The slide was then washed with tap water. Again the smear was flooded with gram's iodine mordant and was allowed to stand for one minute. The slide was washed with tap water. The slide was decolorized using 95% ethyl alcohol. The decolourizer was added drop by drop until crystal violet gets drained out from smear. The slide was washed again with tap water and it was counter stained with safranin for 45seconds.Then, the slide was washed with tap water. The slide was air dried and examined under microscope.

Thin Layer Chromatographic analysis of crude compound

The crude bioactive compound were spotted on the baseline of the silica gel plates (stationary phase) at 1 cm and then allowed to dry at room temperature. The plates were placed in TLC chamber pre-saturated with the mobile phase as Ethyl acetate:Hexane (solvent) in the ratio 1:9.The chromatogram was developed and visualized under UV light and in the iodine chamber, and then the spots were marked. The Rf values were calculated.

R_f value = Distance travelled by the solute / Distance travelled by the solvent.

Identification of bioactive metabolites

The presence of active compounds were been confirmed by thin layer chromatography and the compounds were identified using gas chromatography and mass spectrometry (GC-MS) method, (TSQ QUANTUM XLS). The name of the instrument is Gas Chromatography-Mass Spectrometry and the instrument made is of Thermo scientific. The software required for analytical studies is XCALIBUR (ver-2.2). The column size is of TG-5MS (30mX0.25mmX0.25um). The injector temperature and interface temperature (°C) was at 280°C.

Column Oven Programme

RATE (°C/min)	TEMP(°C)	HOLD (min)
INITIAL	70	1.00
7.5	300	5.00 (TOTAL RUN TIME= 35.67min)

The other Parameters during operation include carrier flow which is of 1.0ml/min, split ratio as 10 and the injection volume is of 1.0 microlitre. The parameters used for mass spectrum includes Scan type (full scan), Scan range (40m/z – 600m/z) and Scan time (0.500s).

Strain identification for the selected isolate by 16srRNA Sequencing

The DNA was isolated by QIAGEN DNA isolation kit and amplified by PCR as per user manual. The PCR products were analyzed on 1% agarose gel for 16S rRNA amplicons. The 16S rRNA amplified fragments were purified using the QIA quick gel extraction kit from the agarose gel and sequenced using automated DNA sequencer (Model 3100). The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) software (http://www.ncbi.nlm.nih.gov/blast) against the 16S ribosomal RNA sequence database. The 16S rRNA partial gene sequence obtained from the isolate was compared with other bacterial sequences by using NCBI BLAST search for their pair wise identities. Multiple alignments of this sequence with the sequences available in the data bank were carried out by Clustal W tool.

Cytotoxicity determination on cancer cell lines

Cell lines and culture conditions

MCF-7 cells obtained from NCCS (National Centre For Cell Science, Pune) were cultured in Rose well Park Memorial Institute medium (RPMI), supplemented with 10% fetal bovine serum, penicillin/streptomycin (250U/ml), gentamycin (100µg/ml) and amphotericin B (1mg/ml) were obtained from Sigma Chemicals, MO, USA. All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were allowed to grow to confluence over 24 hours before use.

Cell growth inhibition studies by MTT assay

Cell viability was measured with the conventional MTT reduction assay, as described previously with slight modification. The MCF-7 cells were seeded at a density of 5×10^3 cells/well in 96-well plates for 24 hours, in 200µl of RPMI with 10% FBS. Then culture supernatant was removed and RPMI containing various concentrations (100µg – 10ng/ml) of test compound was added and incubated for 48 hours. After treatment cells were incubated with MTT (10µl, 5mg/ml) at 37°C for 4 hours and then with DMSO at room temperature for 1 hour. The plates were read at 595nm on a scanning multi-well spectrophotometer. Data represented the mean values for six independent experiments. (Evelyn et al., 2012).

Cell viability (%) = (Average test OD/Control OD) x 100

RESULTS AND DISCUSSION

Isolation of actinomycetes

From the collected marine sediments five different actinomycetes strains have been isolated from Muttukadu regions (Bay of Bengal).Colonies appeared to be powdery white to pale yellow and greyish powder. They are namely ABTRI-1, ABTRI-2, ABTRI-3, ABTRI-4, and ABTRI-5. The above cultures were sub cultured every 15 days and maintained at 4 ºC.

Fig.1 Isolated actinomycetes (ABTRI 1) from marine sediments

Primary Antimicrobial screening of Marine Actinomycetes

The isolates were tested for antibacterial and antifungal activity by cross streak method. For bacterial screening the isolates were streaked in a single line and the pathogens in perpendicular to them. Pathogens include *Escherichia coli* (B), Klebsiella pnuemoniae (C), Bacillus subtilis (D), and Staphylococcus aureus (A). For fungal screening the agar block of fungal strain Aspergillus niger was kept in the centre of the media used and the isolates were streaked by its side. Based on the preliminary screening ABTRI-1 has been selected as an antagonistically active. It showed maximum activity against K.pnuemoniae (3.5cm) and Staphylococcus aureus (1.6cm) as depicted in the fig.2. None of the organisms showed activity against the fungal strain used.

Fig.2 Primary screening of ABTRI 1 against bacterial pathogens

Production and extraction of Secondary metabolites

The selected isolates were cultured in ISP-2 medium and kept in rotary shaker for 7-10 days at room temperature. After the incubation period the broth is filtered with whatman's filter paper and centrifuged at 10000 rpm for 15 minutes. The supernatant has been collected and equal volume of ethyl acetate solvent was added for liquid-liquid extraction.

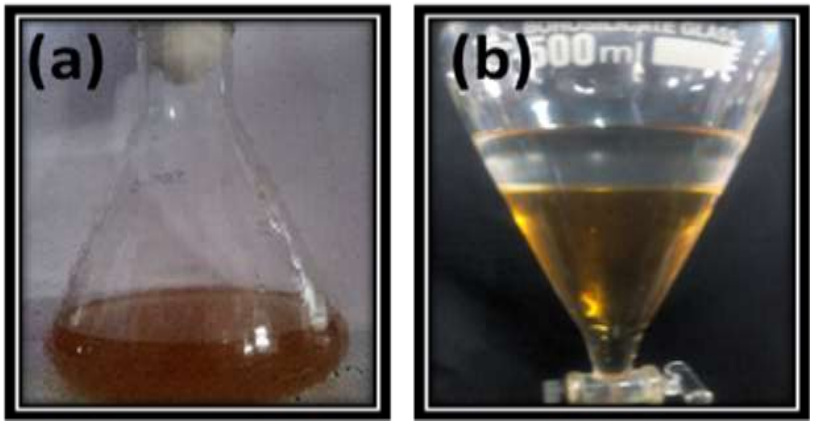

Fig.3 Bioactive compounds - (a) Production and (b) Extraction

Secondary screening-Determination of the antimicrobial activity

Ethyl acetate extract was collected then and used for secondary screening of antimicrobial activity by agar well diffusion method. Nutrient agar for bacteria and potato dextrose agar for fungi was used. The same pathogens (used for primary screening) were tested for secondary screening. Zone of inhibition shows the activity of the compounds against the pathogens. The zones were then measured and recorded. The isolate ABTRI-1 showed activity against

Bacillus subtilis and Klebsiella pneumoniae. The isolate ABTRI 1 did not exhibit antifungal property.

Isolation and screening of marine actinomycetes that can produce bioactive compounds that possess antimicrobial activity has been done by several researchers. C. Sweetline et al., in the year 2012 have done antimicrobial research in the actinomycetes collected from Pichavaram mangroves. In their study thirty eight actinomycetes isolates were screening for the antimicrobial activity against six strains of gram positive and gram negative pathogens namely E.coli, Staphylococcus sp, Salmonella sp, Klebsiella sp, Bacillus sp and Proteus sp.

Table-1: Zone of Inhibition of ABTRI 1 against human bacterial pathogens

S.NO	Name of the organism	Zone of Inhibition (mm)				
		control	250 µg/ml	500 µg/ml	750 µg/ml	1000 µg/ml
1	B.subtilis	22 mm	-	14 mm	15 mm	15 mm
2	S.aureus	22 mm	-	-	-	-
3	E.coli	22 mm	-	-	-	-
4	K.pneumoniae	22 mm	-	14 mm	14 mm	15 mm

Among the 38 isolates tested, seventeen (44.7%) showed antimicrobial activities against more than one genus of test pathogens. Isolate no. KMA02 showed antibacterial activity against all the test pathogens. It showed highest activity against Salmonella sp. Two isolates KMA09 and KMA12 were found to have similar activity against test pathogens but ineffective against Klebsiella sp and Proteus. Isolate KMA04 showed antibacterial activity to three genera of the test pathogens Staphylococcus, Bacillus and E. coli. KMA08 and KMA13 were found to be effective against Staphylococcus, E.coli and Klebsiella (Table 1). By observing the antibacterial activity of the all isolates, KMA02 showed the highest effect on all the pathogens. In the present study, the strains ABTRI 1, ABTRI 2, ABTRI 3, ABTRI 4 and ABTRI 5 were primarily screened. The result of screening was that ABTRI 1 possessed definitive inhibition against Klebsiella pneumoniae and Bacillus subtilis.

Invitro Antioxidant Activity of bioactive compounds from Marine actinomycetes

Phosphomolybdenum assay

Total antioxidant assay has been done for the crude compounds extracted from the five isolates to check the highest antioxidant activity. From the work done ABTRI 1 showed considerable antioxidant activity which is better than other isolates. The gradual increase with

green shades of antioxidant capacity was observed for the compound from ABTRI 1. The results have been given in graphical representation below.

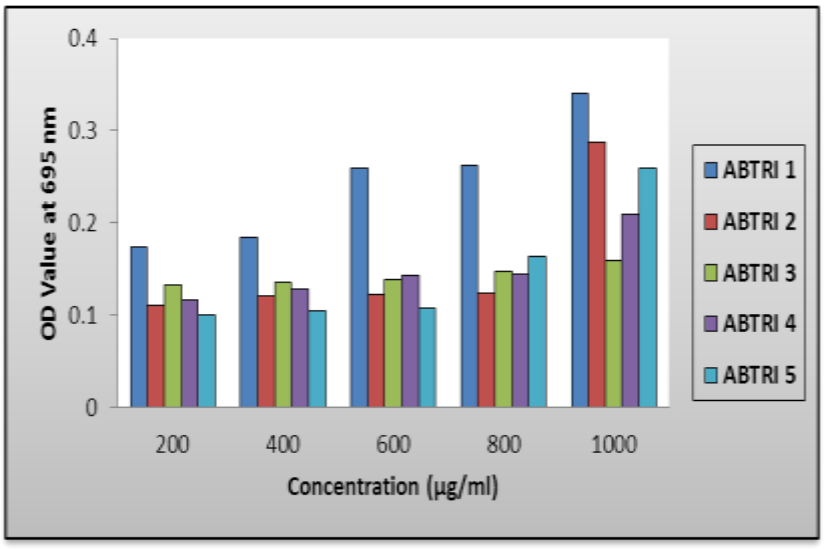

Fig.A Evaluation of Antioxidant activity by Phosphomolybdenum assay

Therefore the total antioxidant activity of the Isolate ABTRI 1 showed maximum activity of 0.340 at the concentration of 1000 µg/ml.

Free radical Scavenging Activity – DPPH assay

Based on the total antioxidant activity results, the strain ABTRI 1 has been chosen for further antioxidant capability by DPPH assay. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as:

(DPPH) + (H-A) ⟹ **DPPH-H + (A)**

(Purple) (Yellow)

Antioxidants react with DPPH, which is a stable free radical and is reduced to the DPPH-H and as consequence the absorbance's decreased from the DPPH radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

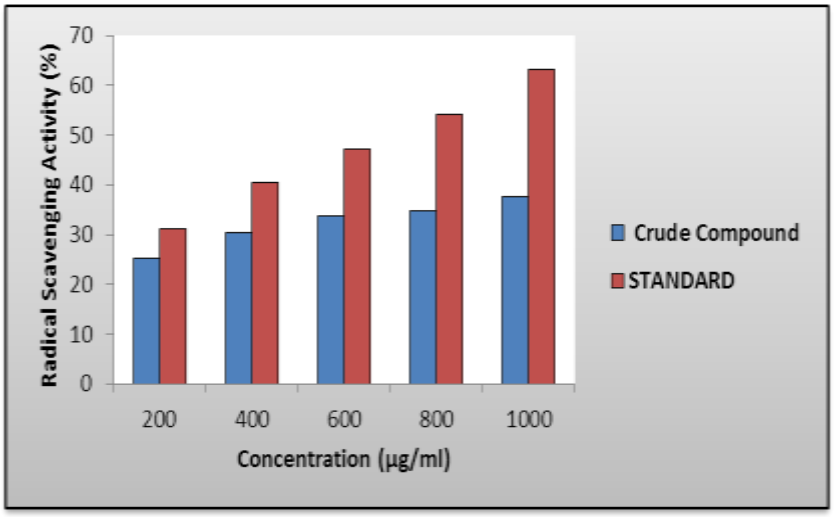

Fig.B Evaluation of Antioxidant activity by DPPH assay

Antioxidant activity of the isolate ABTRI 1 showed better % of RSA 37.73 when compared to the standard value 63.3 % of RSA at the concentration of 1000 µg/ml.

Ferric (Fe^{3+}) reducing power assay

Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm.

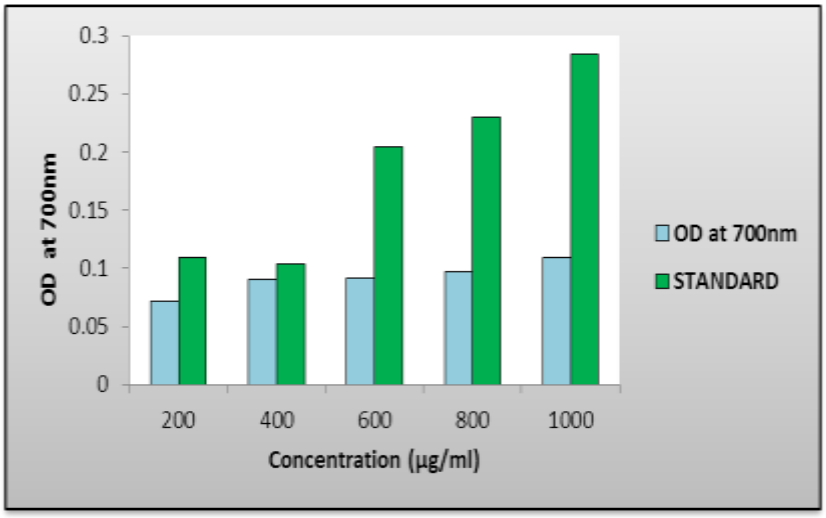

Fig.C Evaluation of Antioxidant activity by Ferric Reducing Power assay

The antioxidant potential of the extract was assessed by DPPH (1,1-Diphenyl-2-picrylhydrazyl) scavenging activity, Fe^{3+} reducing assay, metal chelating assay and DNA inhibition protection assay (Thenmozhi and Kannabiran(2012). The DPPH scavenging

activity was 43.2% at 10 mg/ml of EA extract and compared with ascorbic acid standard. From our study it is shown that DPPH scavenging activity was 37.73% at 10 mg/ml and Fe^{3+} reducing assay was 0.139(OD) at 10 mg/ml. therefore, it is significant that the crude bioactive compounds of the strain ABTRI 1 possess free radical scavenging activity. The findings of the study suggested that the marine isolate ABTRI 1 could be a potential natural source of antioxidants.

Characterization for the selected isolate by morphological and biochemical analysis

Isolate ABTRI 1 showed the highest antimicrobial activity against bacteria and antioxidant activity, so, it was further characterized morphologically and biochemically.

Morphological identification - Cover slip culture method

The microscopic image of the strain ABTRI 1 revealed that the isolate was filamentous in nature with flexible spore chain morphology.

Gram's staining

The strain ABTRI 1 was found to be Gram positive which is evident from the image that it exhibits pink coloured cells.

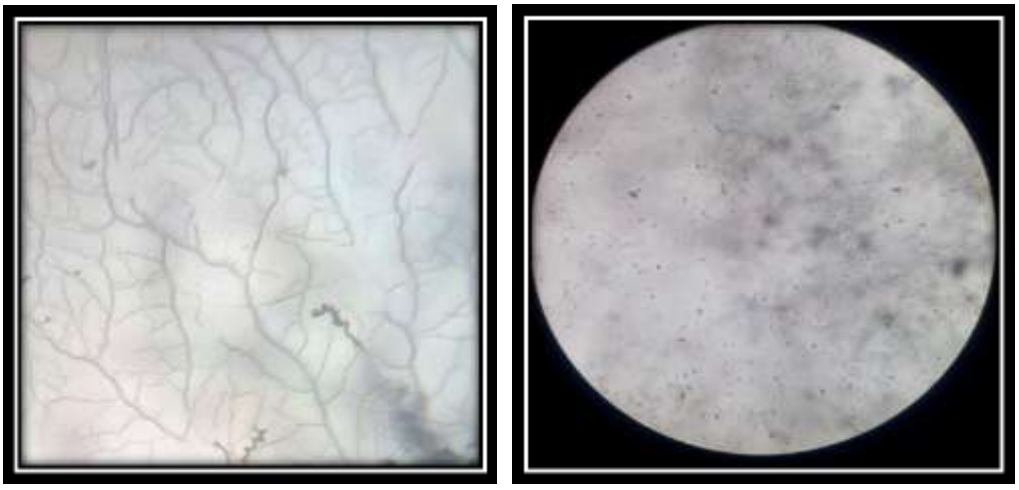

Fig.4 (a) Microscopic view of ABTRI 1 (b) Gram positive-Cocci in shape

The results of biochemical characterization tests revealed that the ABTRI 1 was Gram positive, Indole negative, Methyl red negative, Voges proskauer negative, Oxidase negative, Catalase negative, Carbohydrate utilization:sucrose negative, maltose negative, Galactose positive, fructose positive, Starch hydrolysis positive, and Gelatin liquefaction positive.

Thin Layer Chromatographic Analysis of Crude Compound

Partial purification of secondary metabolites was done by TLC method. Similar to other chromatographic methods during the process, the movement of the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus separation of components in the mixture was achieved. Once separation occurs individual components are visualized as spots at respective level of travel on the plate. Their nature or characters are identified by means of suitable detection techniques. The R_f (Retention factor) values for each spot were measured.

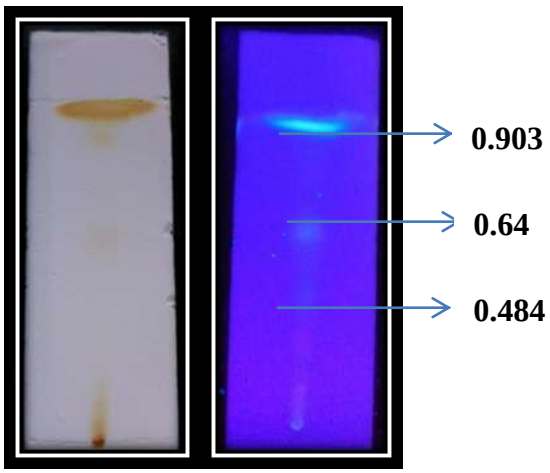

Fig.5 (a) Under iodine (b) Under UV

The partial purification (TLC) with solvent ratio was evaluated to be 0.903, 0.645 and 0.484.The experimental work had been carried out on establishment of antibacterial activity of actinomycetes isolated from salt pan region. Totally 20 actinomycetes was isolated and determined the antibacterial activity against the three human pathogens. Streptoverticillium album showed the high antibacterial activity against the S. aureus. Also, the TLC profile was also studied. To conclude, the study revealed that there was potential activity in sourcing for inhibitory compounds produced from actinomycetes and to identify the antibacterial components for further use. (Gayathri A., 2011). In the present study the strains ABTRI 1, 2, 3, 4 and 5 were tested for antibacterial activity and the ABTRI 1 showed maximum inhibition of 15mm at the concentration of 1000 µg/ml against B.subtilis, K.pneumoniae and the TLC profile revealed the presence of bioactive compounds. The partial purification (TLC) with solvent ratio (Ethyl acetate: hexane) was evaluated to be 0.903, 0.645 and 0.484.

Identification of bioactive metabolites

Interpretation of mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST-011library. The names of the components of the test materials were ascertained.The GCMS analysis for ABTRI 1 indicated the presence of bioactive compounds.

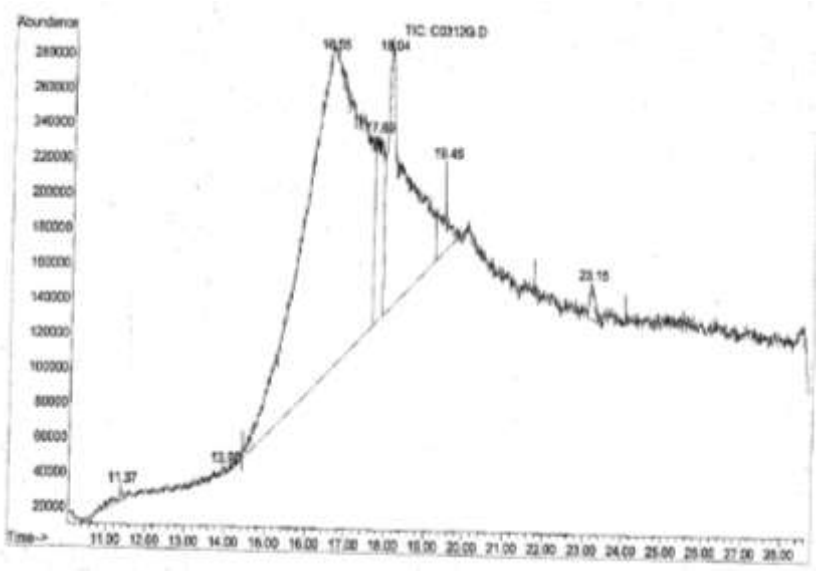

Fig.D GCMS Chromatogram for Bioactive compound (from ABTRI 1)

The active constituents with their retention time (RT), molecular weight (MW) Molecular formula (MF), concentration (peak area %) were determined in fig.D. The GCMS analysis of crude compound showed the presence of seven bioactive compounds with high concentration of Cyclopentaneundecanoic acid having retention time 16.55(RT) and peak area 70.96% , followed by other constituents such as hexadecane having retention time 18.04(RT) and peak area 20.30% ,undecylenic acid having retention time 17.69(RT) and peak area 5.51%, 3-propylglutaric acid having retention time 19.45(RT) and peak area 1.91%, 2-ethylacridine having retention time 23.15(RT) and peak area 0.74%. Other constituents ranged with varying retention time and peak area. The compound Cyclopentaneundecanoic acid has the molecular formula $C_{16}H_{28}O_3$ and molecular weight of 268.

Strain identification for the selected isolate by 16srRNA Sequencing

The 16S rRNA partial gene sequence data was analysed using BLAST search. The selected strain (ABTRI 1) was been identified as Streptomyces sp. and has been designated as Streptomyces variabilis strain NRRL B-3984.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces variabilis strain NRRL B-3984 16S ribosomal RNA gene, partial sequence	2399	2399	99%	0.0	99%	NR_043840.1
Streptomyces griseoincarnatus strain LMG 19316 16S ribosomal RNA gene, complete sequence	2398	2398	99%	0.0	99%	NR_042290.1
Streptomyces griseoincarnatus strain NBRC 12871 16S ribosomal RNA gene, partial sequence	2396	2396	99%	0.0	99%	NR_112312.1
Streptomyces erythrogriseus strain LMG 19406 16S ribosomal RNA gene, complete sequence	2394	2394	99%	0.0	99%	NR_042294.1
Streptomyces labedae strain CSSP735 16S ribosomal RNA gene, partial sequence	2377	2377	98%	0.0	99%	NR_115446.1
Streptomyces aureofaciens strain IMET 43577 16S ribosomal RNA gene, partial sequence	2375	2375	99%	0.0	99%	NR_115193.1
Streptomyces griseorubens strain NBRC 12780 16S ribosomal RNA gene, partial sequence	2372	2372	99%	0.0	99%	NR_041066.1
Streptomyces labedae strain NBRC 15864 16S ribosomal RNA gene, partial sequence	2370	2370	98%	0.0	99%	NR_041192.1
Streptomyces lateritius strain CSSP722 16S ribosomal RNA gene, partial sequence	2368	2368	98%	0.0	99%	NR_115438.1
Streptomyces griseoincarnatus strain CSSP407 16S ribosomal RNA gene, partial sequence	2368	2368	98%	0.0	99%	NR_115369.1
Streptomyces erythrogriseus strain NBRC 14601 16S ribosomal RNA gene, partial sequence	2353	2353	97%	0.0	99%	NR_112438.1
Streptomyces griseoflavus strain LMG 19344 16S ribosomal RNA gene, complete sequence	2351	2351	99%	0.0	99%	NR_042291.1
Streptomyces griseoflavus strain NBRC 13044 16S ribosomal RNA gene, partial sequence	2337	2337	98%	0.0	99%	NR_112349.1
Streptomyces speibonae strain PK-Blue 16S ribosomal RNA gene, partial sequence	2333	2333	99%	0.0	99%	NR_025212.1
Streptomyces althioticus strain CSSP544 16S ribosomal RNA gene, partial sequence	2333	2333	99%	0.0	99%	NR_115392.1
Streptomyces albogriseolus strain NBRC 3413 16S ribosomal RNA gene, partial sequence	2327	2327	99%	0.0	99%	NR_112487.1
Streptomyces viridodiastaticus strain NBRC 13106 16S ribosomal RNA gene, partial sequence	2326	2326	99%	0.0	99%	NR_112371.1
Streptomyces matensis strain NRRL B-2576 16S ribosomal RNA gene, partial sequence	2322	2322	99%	0.0	99%	NR_116076.1
Streptomyces viridochromogenes strain NBRC 3113 16S ribosomal RNA gene, partial sequence	2318	2318	99%	0.0	99%	NR_112482.1
Streptomyces althioticus strain NBRC 12740 16S ribosomal RNA gene, partial sequence	2318	2318	99%	0.0	99%	NR_112254.1

Fig.6 Alignment details for the sequence

Streptomyces variabilis strain NRRL B-3984 16S ribosomal RNA gene, partial sequence
Sequence ID: **ref|NR_043840.1|** Length: 1499 Number of Matches: 1
Range 1: 14 to 1315

Score	Expect	Identities	Gaps	Strand	Frame
2399 bits(1299)	0.0()	1301/1302(99%)	0/1302(0%)	Plus/Plus	

Features:

```
Query  1     GGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCTCCTTCGGGAG  60
Sbjct  14    GGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCTCCTTCGGGAG  73
Query  61    GGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAG  120
Sbjct  74    GGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAG  133
Query  121   CCCTGGAAACGGGGTCTAATACCGGATACTGACCCGCTTGGGCATCCAAGCGGTTCGAAA  180
Sbjct  134   CCCTGGAAACGGGGTCTAATACCGGATACTGACCCGCTTGGGCATCCAAGCGGTTCGAAA  193
Query  181   GCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCAC  240
Sbjct  194   GCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCAC  253
Query  241   CAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACG  300
Sbjct  254   CAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACG  313
Query  301   GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATG  360
Sbjct  314   GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATG  373
Query  361   CAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGA  420
Sbjct  374   CAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGA  433
Query  421   AGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGT  480
Sbjct  434   AGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGT  493
Query  481   AATACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTT  540
Sbjct  494   AATACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTT  553
Query  541   GTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGC  600
Sbjct  554   GTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGC  613
Query  601   TAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGG  660
Sbjct  614   TAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGG  673
Query  661   AGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCG  720
Sbjct  674   AGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCG  733
Query  721   TGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGT  780
Sbjct  734   TGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGT  793
Query  781   GTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGA  840
Sbjct  794   GTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGA  853
Query  841   GTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCA  900
Sbjct  854   GTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCA  913
Query  901   TGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACC  960
Sbjct  914   TGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGC  973
Query  961   ATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTC  1020
Sbjct  974   ATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTC  1033
Query  1021  GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAG  1080
Sbjct  1034  GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAG  1093
Query  1081  CAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTG  1140
Sbjct  1094  CAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTG  1153
Query  1141  GGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCC  1200
Sbjct  1154  GGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCC  1213
Query  1201  GGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCG  1260
Sbjct  1214  GGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCG  1273
Query  1261  GATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTA  1302
Sbjct  1274  GATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTA  1315
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Fig.7 16srRNA Sequence of Streptomyces species

The strain Streptomyces variabilis has 99% identities with E (Expected value) of 0.0. It's score is 2399 bits and it's gap is 0%. The length of the sequence is 1499 and the number of matches is 1.

Cytotoxicity determination on cancer cell lines by MTT Assay

The cytotoxic effect of different concentrations of the crude bioactive compounds of the strain Streptomyces variabilis (ABTRI 1) on MCF-7 cell lines were evaluated by MTT assay in 96-well plates. This assay is often used to measure viable cell where MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole) is reduced to purple formazan in living cell. For determination of IC_{50}, cells were assessed for proliferation inhibition and the IC_{50} value was calculated as 292.4 µg/ml. Less morphological variations were observed. The observed IC_{50} value indicates that the crude bioactive compounds of the strain Streptomyces variabilis was less toxic to normal cells when compared to the malignant cells.

Daryamides, antifungal polyketides isolated from culture broth of a Streptomyces strain, CNQ-085 have been shown to exhibit moderate cytotoxicity against the human colon carcinoma cell line HCT-116 and moderate antifungal activities against Candida albicans (Asolkar et al., 2006). Similarly Chandrananimycins, isolated from marine Actinomadura spp. MO48 have been shown to exhibit antibacterial, antifungal and anticancer activity (Maskey et al., 2003). The assessment of cytotoxicity is very important and a crucial step in the development of new therapeutic drugs for clinical application (Kumar saurav et al., 2011).

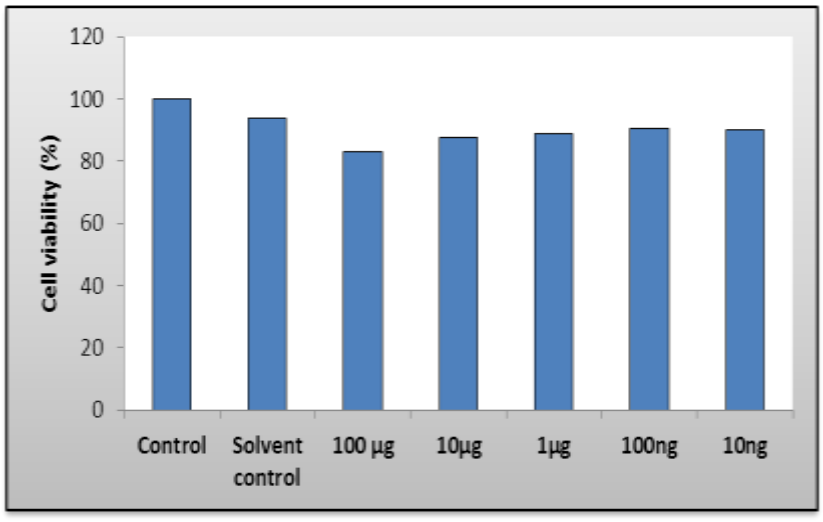

Fig.E Evaluation of Cytotoxicity of Crude compound on MCF-7 Cell lines

The observed IC_{50} value indicates that the crude bioactive compounds of the strain Streptomyces variabilis was less toxic to normal cells when compared to the malignant cells. MTT assay have been conducted to evaluate the cytotoxicity activity of the extract apart from studying their antiproliferative effects towards the MCF-7 cancer cells.

CONCLUSION

Marine environment is a vast diversity where the organisms survive at different environmental conditions. Actinomycetes are considered to be one of the promising tools for drug discovery from 1960's. Marine actinomycetes are more potent since they have developed specific morphological conditions to survive in such a high and varying temperature and pH. Hence they may produce different kinds of secondary metabolites when compared to terrestrial organisms. These marine microbes are also less studied. From the present study we can conclude that the organism Streptomyces variabilis isolated has been found to possess appreciable antimicrobial activity. Antioxidant activity of Streptomyces variabilis is considerable and they are less toxic to normal cells when compared to malignant MCF-7 cell lines. This study suggests that marine-based actinomycetes are the exploiters of new drugs for various diseases, since the actinomycetes are considered to be the "Reservoir of antibiotics".

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