PROSPECTIVE *EFFICACY* OF BIOACTIVE EXTRACT OF NOVEL ACTINOMYCETES AGAINST MALIGNANT CELL AND MULTIDRUG RESISTANT BACTERIA

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Summary

A unique selective enrichment procedure has resulted in the isolation and identification of one new strain of marine-derived Actinomycete. A combination of physiological parameters, chemotaxonomic characteristics, distinguishing 16S rRNA gene sequences, and phylogenetic analysis based on 16S rRNA genes provided strong evidence for the one new strain. This strain can use the various renewal resources, especially agroindustrial wastes, as the potential carbon sources. This leads to the greater possibility for Medicinal Bioactive compound production and reduced pollution caused by those wastes. Biological activity testing of fermentation products from the new marine-derived actinomycetes revealed that several had activities against Multidrug-resistant Gram positive and negative pathogens and malignant cells.

Key words: Actinomycete, 16S rRNA, agroindustrial wastes, Mutlidrug-resistant Bacteria and Malignant cells

Introduction

Cancer still represents one of the most serious human health problems despite the great progress in understanding its biology and pharmacology. An analysis of the number of chemotherapeutic drugs and

their sources indicates that over 60% of approved drugs are derived from natural compounds (1).

The processing of agroindustrial raw materials such as Tapioca produces the large amount of waste, whose accumulation leads to environmental pollution. Due to the high amounts of starch or reducing sugar, those wastes has been recognized as a suitable feedstock for industrial fermentations such as volatile compounds (2).

Actinomycetes naturally produced more than 10,000 bioactive compound which is actively cured many human diseases especially tumor cells. Some novel active compounds from marine actinomycetes are reported at a high frequency (3, 4 & 5) and some of them show strong antitumour activity, which suggests

marine actinomycetes are a promising source of anti-tumour and antibacterial leading drugs(6).

Bacteria will continue to develop resistance to currently available antibacterial drugs by either new mutations or the exchange of genetic information that is, putting old resistance genes into new hosts (7). In many healthcare facilities around the world, bacterial pathogens that express multiple resistance mechanisms are becoming the norm, complicating treatment and increasing both human morbidity and financial costs. New antibacterial agents with different mechanisms of action are also needed.

Diphenyl-picrylhydrazyl radical (DPPH) bleaching is one of the strategies used to evaluate the antioxidant properties of microbial extracts; this method has shown to be rapid and simple and it measures the capacity of microbial extract to bleach the DPPH radical, a nitrogen-centered free radical (8). The structural changes that this radical provokes on compound principles as well as the involved mechanism however, are not clear yet (9)

For *in vivo* screening anticancer compounds expensive cell lines were used leukemic mouse assay and the *in vitro* screening for cancer cells cytotoxicity (10 & 11).Potato disc bioassay is developed based on *Agrobacterium tumefaciens* infection on potato disc is useful for checking anticancer properties of plant extract.Crown gall is a plant neoplastic disease induced by the gram-negative bacterium *A. tumefaciens* (12 & 13). *A. tumefaciens* cause series plant infections with more than 60 dicotyledon families and many gymnosperms lead to great damages(14) .The

A.tumefaciens infection symptoms resemble tumor in mammalian cells(15). The tumor formation starts when bacterial cell transfer part of the Ti (tumor-inducing) plasmid to the infected plant cell genome.

The validity of Crown gall tumor assay predicted on the observation that certain tumorigenic mechanisms are similar in plants and animals like multiplying rapidly without apoptosis (16).

Several modifications on the original Potato disc anticancer protocol(17, 18, 19, 20 & 21) is show that crown gall tumor can be inhibited on potato (*Solanum tuberosum*) tubers discs with apparent correlation with compounds and extracts derived from Actinomycetes known to be active with cancer cell line.

In the present paper studies has identified marine actinomycete as *Streptomyces radiopugnans MS1* and reported antibacterial and anitumor activity, the requirement of natural seawater fermentation optimization of the strain based on further developing a testing method of anticancer activity of fermentation broth.

Materials and Methods

Sample collection

The actinomycete isolate was recovered from marine soil sample collected from Bay of Bengal, Mahabalipuram.It is situated in Chennai, Tamilnadu, India and its geographical coordinates are 12° 37' 0" North, 80° 11' 55" East. 147 soil core samples were collected at a depth of 0-30 cm within a 100 m^2 area in sampling bags in order to avoid external contamination, brought to the laboratory for further analysis and air dried and stored in polythene bag until plating (22 & 23]. The soils from each location were bulked and homogenized to prepare composite samples. Samples were stored at 4 °C in the dark prior to examination and the collected sediment samples were dried at room temperature for 10 days. Air dried soil samples were sieved to exclude large mineral and organic matter particles then ground in a pestle and mortar and kept at 55°C for 10 minutes. (24).

Enrichment and Isolation of Actinomycetes

One gram each of the sieved soil samples was treated with 100ml of tapioca effluent and incubated at an ambient temperature for about a week at 200rpm.

The pretreated samples were diluted 1:10 v/v with sterile distilled water and serial dilutions prepared down to 10^{-4}. One hundred µl of the 10^{-1} to 10^{-4} (25) suspensions were spread, in triplicate, onto Starch casein Nitrate agar (Himedia, Mumbai, India) media (26).The media components included cycloheximide (20 mg/l),pravastatin (10 mg/l), trimethoprim (2 mg/l) and nalidixic acid (10 mg/l) to prevent other non-actinomycete bacteria and fungal growth(27).

After incubation of 7 to 10 days at 28^{o}C, the Actinomycete colonies that developed on the plates were enumerated and were expressed in colony forming units(28).The isolates were identified by various parameters such as Colony morphology, spore arrangement, Staining , physiological, Biochemical reactions (19 & 30) and 16S rRNA gene amplification and DNA sequencing.(31, 32, 33 & 34).

16S rRNA gene Sequencing and Phylogentic analysis

The chromosomal DNA from strain was isolated according to the previously described procedure (35).The 16S rRNA of the strain was amplified using the 16S rRNA preimers, fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and Rp2 (5'-ACGGCTACCTTGTTACGACTT-3'). Cycling conditions were 94ºC for 5 min and then 34 cycles each at 94ºC for 30 sec, 55ºC for 30 sec and 72ºC for 1.5 min.Final extension w as at 72ºC for 5 min(Cfx-96 Real time PCR,Biorad ,USA).The purified DNA fragments were sequenced using sequencer model ABI 3100 sequencer according to manufacturers' instructions (ABI PRISM 3100 Genetic Analyzer User's Manual).Designed according to previous data and a conservative part of 16S rRNA of known actinomycete strains(Accession number : HQ132790& etc) .The purified PCR product was sequenced using an ABI 3730 sequencer. The 16S rRNA sequence of strain *Streptomyces radiopugnans MS1* was aligned manually with available nucleotide sequences retrieved from the GenBank and the RDP using CLUSTAL W (36).Phylogenetic trees were constructed using the Neighbor – joining methods from MEGA version 5 (37).

Fermentation and Preparation of crude extracts

Purified isolates was transferred to one Liter Erlenmeyer flasks which contained 250 ml of the optimized Tapioca effluent medium [Tapioca effluent (75% v/v),Natural sea water(25%v/v),Peptone (0.3% w/v),yeast extract (0.3% w/v),KH2PO4(0.1% w/v),Mgso4(0.5% w/v),CaCO3 (0.4% w/v) pH 7.2~7.4] and cultured at 200 rpm, at an angle of 45º, for 7-10 days at 28 °C. After fermentation the medium was centrifuged with moderate speed approximately 4000 rpm for 10 min at 4^0 C and supernatant was separated. Crude extracts were prepared by adding 60 ml of Ethyl actate to each of the cultures and the extraction allowed proceeding for 2 weeks. The crude solvent extract thus obtained was used to determine antibacterial assay, antioxidant, hemolytic assay, Crown gall tumor assay (anticancer activity) and cytotoxicity assay.

Antimicrobial Screening:

The Antimicrobial assay was done by Bauer *et al*., 1996. Method against Multidrug Resistant Bacteria and Standard bacterial strains

Sources of Multidrug Resistant Microorganism:

S.aureus,B.Subtilis, E.Coli, P.aeruginosa, S.typhi and Candida albicans were obtained from hospitals or pathological labs of chennai. The bacteria were collected on Nutrient agar slants, and were purified twice on different selective medium and enrichment medium. They were later maintained on Nutrient agar agar slants. Most bacteria were already identified at source, however if needed, identification was carried out by cultural characters and biochemical reactions. Susceptibility was confirmed using CLSI broth microdilution panels.

Standard bacterial strains:

The standard bacterial strains used in the study included S.aureus ATCC 25923, B.Subtilis ATCC 6633, E.Coli ATCC 35218, P.aeruginosa ATCC 27853, S.typhi 19430 and Candida albicans 10231from American Type Culture collection (ATCC) from USA.

Disc diffusion method

The paper disc diffusion method was employed [38 & 39). Sterile 6 mm disc filter paper disc (Schleicher & Schul, No. 2668, Dassel, Germany) were impregnated with 20 µl (500µg $disc^-$) of the extract. The bacterial cultures were inoculated on Nutrient Broth (Himedia) and incubated for 24 h at 37±0.1 °C, while the yeast cultures were inoculated on Malt Extract

Broth (Himedia) and incubated for 48 h at 28.0±0.1 °C. Adequate amounts of Mueller Hilton Agar (Himedia) were dispensed into sterile plates and allowed to solidify under aseptic conditions for 5 minutes.. The counts of bacterial and yeast cultures were adjusted to yield $10^7 – 10^8$ ml^{-1} and $10^5 – 10^6$ ml^{-1}, respectively, using the standard McFarland counting method. The test microorganisms (0.1%) were inoculated with a sterile swab on the surface of appropriate solid medium in plates.The agar plates inoculated with the test microorganisms were incubated for 1 h before placing the extract impregnated paper disc on the plates. The bacterial plates were incubated at 37±0.1 °C for 24 h while yeast plates were incubated at 28±0.1 °C for 48 h. After incubation, all plates were observed for zones of growth inhibition and the diameter of these zones was measured in millimeters. All tests were performed under sterile conditions in duplicate and repeated three times. Chloramphenicol (30 µg/disc) and nystatin (30 µg/disc), discs were used as positive controls.

Microdilution method

Minimum inhibition concentration (MIC) assay was performed in Mueller Hinton Broth (Himedia) for bacteria and Malt Extract Broth (Himedia) for fungai, which was included 0.05% phenol red and supplemented with 10% glucose (40). All the test extracts including standard drugs were initially dissolved with in DMSO and the solution obtained was added to Broth to a final concentration of 1000 µg/ml for each crude extract .This was serially diluted by twofold, to attained concentration ranging from 1000 µg/ml to 1.95µg/ml. Each wells of microplate containing 100 µl of each concentration, 95µl of broth and 5 µl of standard inoculums (inoculums size for standard MIC is $2X10^4$ to 10^5 cfu/ml).The negative control well consisted of 195 µl of Broth and 5µl of the standard inoculums. The plates were incubated at 35 °C for 18 – 24 h for bacteria and 30 °C for 48 h for the yeast cultures. All the tests were performed in broth and repeated three times. MIV was determined by observing the change of color in the wells (Red color is no growth where as yellow color is indicate microbial growth).The lowest concentration no color change in the well was considers as the MIC (Xmark ELISA Reader, Bio-Rad, USA).

Antioxidant activity by DPPH radical scavenging assay

Preparation of test solutions:

21mg of each of the Ethyl actate extracts of *Streptomyces radiopugnans MS1* were weighed; Extract was dissolved in distilled Dimethyl sulfoxide (DMSO) separately to obtain a solution of 21mg/ml concentration. Each of these solutions was serially diluted separately to obtain lower concentration ranging from 1,000 µg/ml to 0.9765 µg/ml.

Preparation of standard solution:

10mg of each of Ascorbic acid was weighed separately and dissolved in 0.95ml of DMSO to get 10.5mg/ml concentrations. This solution was serially diluted with Dimethyl sulfoxide to get lower concentrations.

Method:

The assay was carried out in a 96 well microtitre plate. To 200µl of DPPH solution, 10µl of each of the test sample or the standard solution was added separately in wells of the microtitre plate. The final concentration of the test and standard solutions used are 1000 to 1.95 µg/ml. The plates were incubated at 37^{o}C for 20 minutes and the absorbance of each solution was measured at 517nm, using ELISA reader (Xmark ELISA Reader, Bio-Rad, USA) against the corresponding test and standard blanks and the remaining DPPH was calculated (41, 42 &43). EC_{50} (Effective Concentration) is the concentration of the sample required to scavenge 50% of DPPH free radicals. (Masterplex 2010, Hitachi).

$$\text{Radical scavenging (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where A control is the absorbance of the control reaction and A sample is the absorbance in the presence of antioxidant.

Hemolysis assay

Fresh erythrocytes collected from Human healthy person, especially A+ blood sample was used for hemolysis assay for identification of compound activation (44 & 45) .After bleeding and cells were washed three times (46) in 9 volumes of sterile 0.9% sodium chloride solution. After each washing, cells were pelleted by centrifugation at 150x*g* for 5 min and the supernatant was discarded. The final pellet was diluted 1:9 (v/v) in sterile 0.9 % NaCl saline solution than 1:24 (v/v) in sterile Dulbecco's phosphate

buffer saline (D-PBS), pH 7.0 (47) containing 0,5 mM boric acid and 1 mM calcium chloride (48)

In vitro hemolysis studies 96 well microplate was used for component activity identification method descried by Malagoli., 2007. First 100 µL of 0.85 % sodium chloride solution containing 10 mM calcium chloride was placed in every well. For each concentration (100 to 800 µg/mL) to and control, the experiments were set in triplicate. Negative control contained 100 µl of distilled water and positive control containing 20 µL of 0.1% Triton X- 100 in 0.85 % saline.

After 30 min incubation, the cell suspensions were centrifuged at 900x*g* for 10 min (49) and the supernatant was carefully collected, by paying attention not to disturb the pellet. The absorbance at 405/540 nm of supernatant was measured with a UV-Vis Spectrophotometer (SmartSpec ^{TM} Plus spectrophotometer, Bio-Rad, USA). Hemolytic levels were expressed by percentage of hemolysis, calculated with the ratio between the value measured for each sample and that registered for the total hemolysis. (Masterplex 2010,Hitachi).

Crown gall tumor assay (anticancer activity)

Crown gall tumor assay procedure was described by Galsky *et al*., 1980. *Agrobacterium tumefaciens LBA4404* virulent strains were grown for 48 hours in Lauria broth medium included rifampicin (10 µg/ml). Potatoes were surface sterilized in 0.1% mgcl2 solution for 10 minutes and thoroughly washed with autoclaved distilled water. Potato discs (5 mm x 8 mm) were made with cork borer and Transfer the discs into agar plates and keeping them 2/3 submerged in the Agar (1.5%) solution (10 discs per plate).

Prepare 10 ml of the following solutions of anticancer agents in disposable culture tubes (50):10 mg/ml (DMSO) followed by dilution with sterile water to achieve 10ppm, 100ppm and 1000ppm..Similar solutions containing the same concentrations of camptothecin (51) can also be prepared and will serve as positive inhibitory control solutions. Also, prepare a 10 ml control solution containing 5% DMSO in sterile water. Using a sterile micropipette, combine 400 µl bacteria solution with 400 µl of the appropriate test or control solution in Eppendorf tubes. Within 30 minutes after placing the potatoes in the wells, inoculate each potato with 1 drop (50 µl) of the test or control solution, taking care to spread the liquid evenly over the disc surface. Cover the plates, tape the lids using Parafilm

(to minimize moisture loss), and incubate under dry conditions at room temperature for 12 days. Later 12 days, the discs were stained with Lugol's solution (10% KI and 5% I2) for 30 minutes.

Lugol's Reagent stains the starch in the potato tissue a dark blue to dark brown color, but the tumors produced by A.tumefacians will not take up the stain, and appear creamy orange. The stained potato discs were viewed under a dissecting microscope at 10 X magnification and tumors were counted. Twelve replicates were analyzed for the each sample, and all experiments were repeated three times .Data was analyzed using the Analyse-it program**.**

$$\text{Percent inhibition} = 100 - \frac{\text{Average number of tumors of sample}}{\text{Average number of tumors of control}} \times 100$$

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay

A common human solid tumor cell lines, Hela (Human cervical adenocarcinoma) was used for mtt assay. Cell lines were maintained with 10% fetal bovine serum and at 37^{o}C in a humidified atmosphere containing 5% CO_2.Cells were fed with cultured medium 2-3 times per week and sub cultured when confluent.

MTT assay was used to investigate in vitro anti-tumor activity of fermentation broth against several tumor cell lines according to the previously described method (52 & 53). In brief, tumor cells taken from the exponential phase of cultures were seeded into 96 well plates in 180 µl medium at between $2x10^4$ and $3x10^4$ cells /ml and were incubated at 37^{o}C and 5% co_2 for 24 Hrs. The supernatant samples were diluted with RPMI 1640 medium. Sample of the volume 20 µl, along with 20 µl of fermentation media as the negative control, were then added to the plate in triplicate, giving final dilution fold of 5, 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 for the supernatant sample. The cells were incubated for the initial period of 44 hrs, and then with addition of 20 µl of MTT (5mg/ml), for another 4 Hrs in the second period. After pouring out the culture medium, 150 µl of DMSO was added to each well to dissolve the formanan products. The absorbance at 490 nm was record using a micro plate reader (iMark Elisa

reader, Bio-rad, USA). Percentage inhibition rate was measured by the following equation

$$IR(\%) = \frac{OD_{\text{control well}} - OD_{\text{treated well}}}{OD_{\text{control well}}} \times 100$$

In the experiment, anti tumor activity of fermentation broth was evaluated with ID_{50} value .ID_{50} value represented dilution fold of Fermentation broth that caused by using the Masterplex 2010,Hitachi according to the IR value of different dilution sample of fermentation broth. The higher value the stronger is the anti-tumor activity of fermentation broth.

Results

Isolation of actinomycetes

A good number of isolates were obtained from the different soil samples collected.Of the 87 isolates screened so far, 24 of them show antimicrobial activity against test pathogens. Of these, 9 isolates showed good antimicrobial activity against the Gram-positive bacteria, 8 against Gram-negative organisms and 6 against Yeast/Fungi, with one showing broad spectrum of activity.

The isolated strain MS1 is Gram Positive, Substrate mycelium olive gray to dark reddish brown and aerial mycelium white to pinkish, especially on Czapek agars. Spores are moderate gray on most media and reddish gray on Czapek agars. Spore chains are in long, open spirals. The spore surface is spiny. Melanin pigments are not produced on Czapek agar. The Physiological and biochemical properties of strain *MS1* also were very similar to those of *Streptomyces radiopugnans* (Table-1 and Tabel 2).Liquefaction of gelatin, Citrate ,Urea, Arginine ,Lysin, Ornithine decarboxylase ,Galactose orthonitrophenol and VP were all Positive but Indole and H2S was negative .Strain was consumed D-Glucose , L-Arabinose, Galactose,Erythritol,Melibiose, Sucrose,Salicin,Inositol,D-Mannitol,D-Fructose,L-Rhamnose and Raffinose but could not utilize D-xylose and Lactose.

Table:1 ,The morphological characteristics of the Actinomycets isolate *MS1*

Characteristics	**Result**
Gram Staining	Gram Positive

Substrate Mycelium	Olive gray to dark reddish brown
Aerial Mycelium	white to pinkish
Spore color	Gray Color
Spore arrangement	Long and spiral
Spore surface	Spiny

Table:2 Physiological and Biochemical characteristics of the Actinomycets isolate *MS1*

Carbon utilization	**Result**
Gelatin	Positive
Citrate	Positive
Urea	Positive
Arginine	Positive
Lysin	Positive
Ornithine decarboxylase	Positive
Galactose	Positive
VP	Positive
Indole	Negative
H_2S	Negative
Glucose	Positive
L-Arabinose	Positive
Galactose	Positive
Lactose	Negative
Erthritol	Positive
Melibiose	Positive
Sucrose	Positive
Cellulose	Positive
D-Xylose	Negative
Inositol	Negative
D-Mannitol	Negative
D-Fructose	Positive
L-Rhamnose	Positive
Raffinose	Negative
Nacl Tolerance	
4%(w/v) Nacl	Positive

7%(w/v) Nacl	Positive
10%(w/v) Nacl	Positive
13%(w/v) Nacl	Negative
Temperature	
30^{o}C	Strong
40^{o}C	Moderate
50^{o}C	Weak
pH for Growth	
Range	5 to 9
Optimum	7.4
Cell wall aminoacid analysis	L-DAP (L-diaminopimelic acid)

The nearly complete 16sRNA sequence of strain MS1 was generated (Genebank accession No. HQ623051).Phylogentic analysis of 16sRNA sequences of the strain and related taxas showed almost complete 16sRNA sequence of the strain had 99% similarity to that of *Streptomyces radiopugnans* (Genbank accession no. DQ912930) (Figure:1).As a Result ,strain MS1 was identified as *Streptomyces radiopugnans MS1*

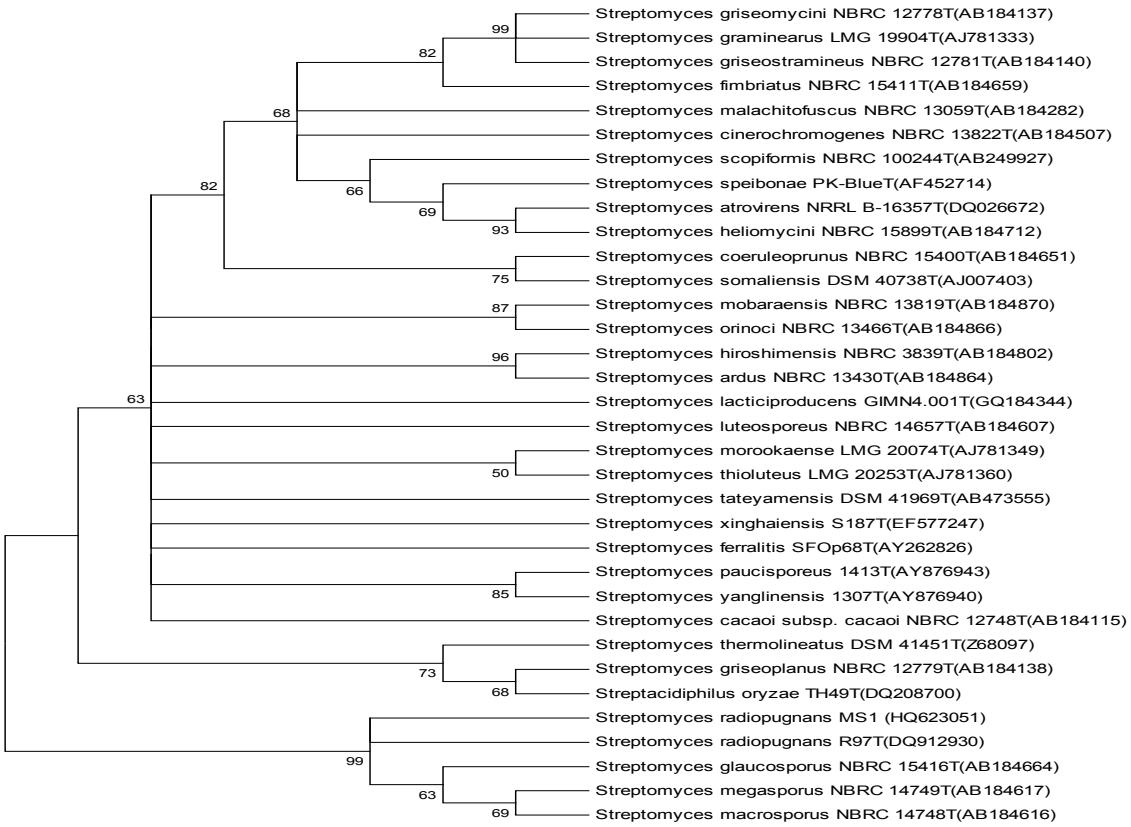


Figure:1

The evolutionary history was inferred using the Neighbor-Joining method (54) .The optimal tree with the sum of branch length = 0.24861088 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches . The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (55) and are in the units of the number of base substitutions per site. The analysis involved 34 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 888 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (56).

Antimicrobial Activity

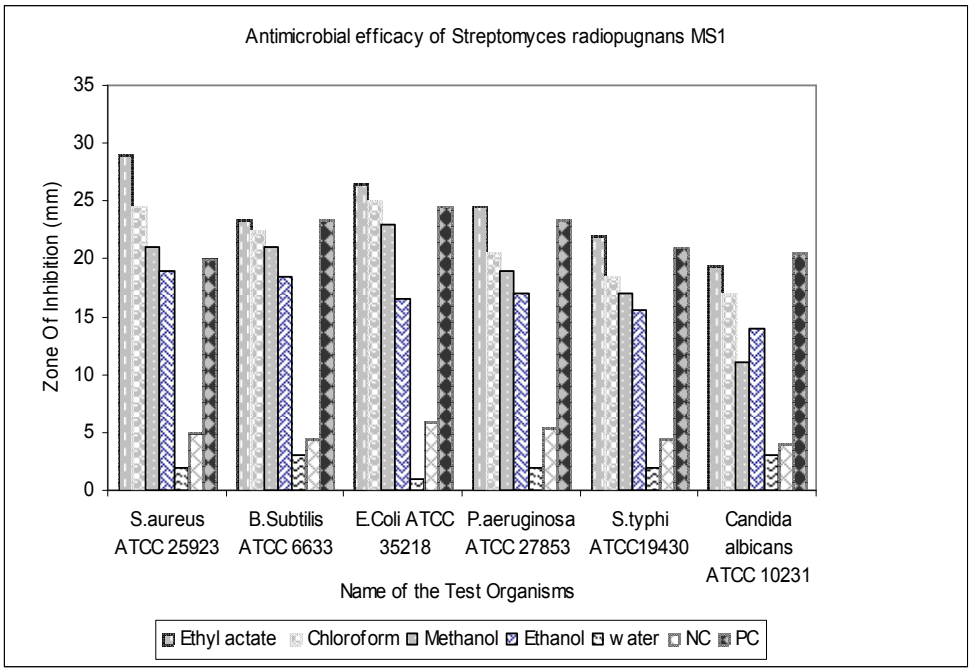
Disc Diffusion Test

Extracts of *Streptomyces radiopugnans MS1* has shown inhibition effects on the growth of all the organisms tested, but their inhibition ability was differ from one organism to another.(Table-3) In almost all, the tested organisms growth was inhibited by Ethyl actate and chloroform. Ethyl actate extract has shown higher range of inhibition diameter from 19.5 to 25.5mm.but ethyl acetate extract has shown inhibition ranges from 17 to 23 mm. Chloramphenicol and nystatin have shown inhibition diameter ranges from 20 to 24.5 mm(Figure:2).

Table :3 Antimicrobial efficacy of *Streptomyces radiopugnans MS1*

	Zone of inhibition(mm)						
Name of the Test organisms	**Ethyl actate**	**Chloroform**	**Methanol**	**Ethanol**	**Water**	**NC**	**PC**
S.aureus ATCC 25923	29.0±0.14	24.5±0.14	21±0.14	19.0±0.07	2±0.22	5.0±0.22	20.0±0.07
B.subtilis ATCC 6633	23.5±0.07	22.5±0.14	21±0.07	18.5±0.14	3±0.01	4.5±0.22	23.5±0.07
E.coli ATCC 35218	26.5±0.14	25.0±0.14	23±0.07	16.5±0.14	1±0.22	6.0±0.07	24.5±0.07
P.aeruginosa ATCC 27853	24.5±0.14	20.5±0.07	19±0.14	17.0±0.14	2±0.22	5.5±0.22	23.5±0.14
S.typhi ATCC19430	22.0±0.07	18.5±0.14	17±0.14	15.5±0.07	2±0.08	4.5±0.22	21.0±0.07
Candida ATCC 10231	19.5±0.07	17.0±0.07	11±0.14	14.0±0.07	3±0.01	4.0±0.22	20.5±0.07
Values are mean ±SD,NC:Negative Control & PC:Positive Control							
Neagtive control :DMSO control,Positive control:Chloramphenicl/nystatin,Methanol extract							

Figure:2



Minimal Inhibitory Concentration Determination (MIC)

The Ethyl actate and chloroform of *Streptomyces radiopugnans MS1* were tested at different concentrations for antimicrobial activity; the extent of their inhibitory activities against the test organism could be well understood only by comparing the MICs values obtained. All the consequent to tested organism are shown in table 4.

Table:4 , Minimum inhibitory concentration of various extract of *Streptomyces radiopugnans MS1*

Name of the Test organisms	Chloroform	Ethyl actate	Standard
S.aureus ATCC 25923	32	63	32
B.Subtilis ATCC 6633	63	125	32
E.Coli ATCC 35218	32	125	32
P.aeruginosa ATCC 27853	32	63	32

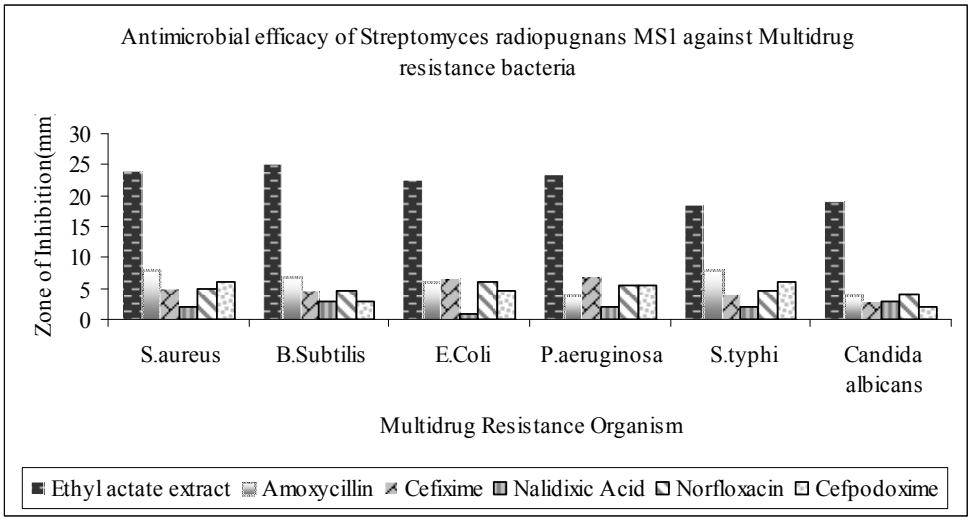
S.typhi ATCC19430	63	125	32
Candida albicans 10231	32	125	32
Standard :Chloramphenicol/Nystatin			

MS1 extracts showed antimicrobial activity against at least six of the types of Multidrug resistant microorganisms tested, as exhibited by an agar diffusion assay (Table 5). Extract of *Streptomyces radiopugnans MS1* showed the most potent activity against all the microorganisms studied and values determined by CLSI for these organism; interpretive categories are those assigned by FDA.

Table:5 Antimicrobial efficacy of Streptomyces radiopugnans MS1 against Multidrug resistance bacteria

	Zone of inhibition(mm)					
Name of the Multidrug Resistant organisms	Ethyl actate extract	Amoxycillin	Cefixime	Nalidixic Acid	Norfloxacin	Cefpodoxime
S.aureus	24.0±0.14	8.0±0.22	5.0±0.01	2.0±0.01	5.0±0.14	6.0±0.22
B.Subtilis	25.0±0.14	7.0±0.14	4.5±0.01	3.0±0.14	4.5±0.01	3.0±0.01
E.Coli	22.5±0.07	6.0±0.01	6.5±0.14	1.0±0.01	6.0±0.22	4.5±0.14
P.aeruginosa	23.5±0.22	4.0±0.14	7.0±0.22	2.0±0.14	5.5±0.14	5.5±0.14
S.typhi	18.5±0.14	8.0±0.14	4.0±0.14	2.0±0.22	4.5±0.01	6.0±0.14
Candida albicans	19.0±0.14	4.0±0.22	3.0±0.22	3.0±0.14	4.0±0.07	2.0±0.07

Figure :3



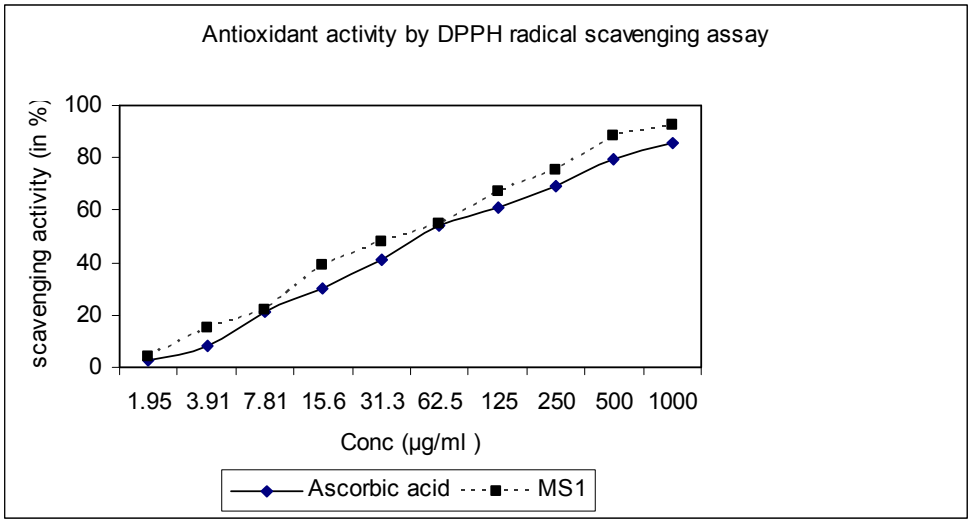
Antioxidant activity by DPPH radical scavenging assay

The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. Figure 2 illustrates a decrease in the concentration of DPPH radical due to the scavenging ability of the soluble constituents in the extract of *Streptomyces radiopugnans MS1* and the standard ascorbic acid, as a reference compound, presented the highest activity at all concentrations(Table-6). The EC_{50} values were found to be 24.59 µg/ml and 18.37 µg/ml for extract of the *Streptomyces radiopugnans MS1* and ascorbic acid respectively See Figure 4.

Table:6 , Antioxidant activity by DPPH radical scavenging assay

Tested Material	**Concentration (µg/ml)**	**% D.P.P.H Radical Scavenging (±SEM)**	**EC 50 (µg/ml)**
Ascorbic acid	1.95	2.9845±0.50	18.37
	3.91	7.923±0.24	
	7.81	20.987±0.49	
	15.63	30.3982±0.19	
	31.25	40.758±1.92	
	62.5	53.982±0.55	
	125	61.23±0.75	
	250	69.29±1.22	
	500	79.326±1.05	
	1000	85.575±0.41	
Extract of Streptomyces Lapedae MS2			
	1.95	3.9842±0.50	24.59
	3.91	15.2567±0.60	
	7.81	21.786±1.00	
	15.63	39.0987±1.13	
	31.25	47.98±0.42	
	62.5	54.872±1.22	
	125	67.23±1.97	
	250	75.567±0.91	
	500	88.2678±1.08	
	1000	92.23±1.05	

Figure:4



Hemolytic activity

The EC_{50} was achieved by non-linear regression analyses and EC_{50} value lower than 194.09 µg/mL and was considered as active for hemolytic activity

Crown gall tumor assay:

Results showed that Ethyl actate extract of *Streptomyces radiopugnans MS1* inhibit tumor growth in highly significant way in a concentration dependent manner across the strains (Table-7). Significant tumor inhibition was observed at 100ppm and 1000ppm concentrations, but not at 10ppm. Maximum 83%,67%, and minimum 14% tumor inhibition was recorded for *Agrobacterium* strains.In the present investigation, Camptothecin served as a positive control and 100% tumor inhibition was observed.These results are significant at $P<0.0001$ for bacterial strain as well as for the concentration.

Table:7 Statistical analysis of tumor inhibition by the extract and tumor induction by strain of *Agrobacterium tumefaciens LBA4404.*

n	12				
Tumor inhibition by extract	**N**	**Mean**	**SE**	**Pooled SE**	**SD**
1000ppm	3	82.4	1.58	2.15	2.7
100ppm	3	74.9	3.85	2.15	6.7
10ppm	3	15.0	0.58	2.15	1.0
Control	3	15.3	0.88	2.15	1.5
Source of variation	**Sum squares**	**DF**	**Mean square**	**F statistic**	**P**
Extract	12179.4	3	4059.8	294.04	<0.0001
Residual	110.5	8	13.8		
Total	12289.8	11			
LSD Contrast	Difference	95% CI			
1000ppm v control	67.0	60.1 to 74.0		(significant)	
100ppm v control	59.6	52.6 to 66.6		(significant)	
10ppm v Control	-0.4	-7.4 to 6.6			

Extracts which show anticancer activity at concentrations proved to have antimicrobial activity against *A.tumefaciens LBA4404* were considered as negative results (Table 8 and Table 9).

Table:8 Effect of the extract of *Streptomyces radiopugnans MS1* on tumor inducing strain of *Agarobacterium tumefacians LBA4404.*

Extract Concentration	Mean number of tumor ±SEM	% inhibition
10ppm	6.4 ±0.15	14
100ppm	2.5 ±0.20	67
1000ppm	1.3 ± 0.20	83
Negative Control	7.5 ± 0.52	

Table:9 Inhibition of *A.tumefaciens LBA4404* exposed to various *Streptomyces radiopugnans MS1* using agar well diffusion technique at different concentration dose.

	ıncentration of MS1 extract (µg/ml)							
							0	
Solvent Name	y	AU	Y	AU	y	AU	y	AU
Ethyl actate	0	0	0	0	0	0	0	0
Methanol	0	0	1.1	1.8	1.2	2	2	3.3
Chloroform	0	0	0	0	0	0	0	0
Ethanol	0	0	0	0	0	0	1	1.7
Water	0	0	0	0	0	0	0	0
Positive Control	0	0	0	0	0	0	0	0
y = diameter of inhibition zone (cm); AU=y/x µl of plant extract where x =diameter of well (6mm)								

Screening of marine *Streptomyces radiopugnans* MS1 having cytotoxic activity by the MTT assay

All the standard anticancer agents, the IC50 value was less than 10µg/ml against at cell lines (Table-10). The sensitivity of the cell lines to five antibiotics without anticancer activity and three organic solvents is summarized in Table 11. Only the IC50 value of chloramphenicol was less than 20 µg/ml against the HELA cell lines, other antibiotics and organic solvents had no effects on the cancer cells tested. These data was useful for MTT assay utilized in our study was appropriate for screening anticancer agents with cytotoxic activity.

Table :10 Antitumour activity of marine *Streptomyces radiopugnans MS1* determined by the MTT assay

Compound and Strain	**IC_{50} (µg/ml)/ID_{50}[a] HELA**
Mitomycin C	0.007
Vincristine sulfate	0.004
Methotreate	0.005
Cisplatin	0.55

Bleomycin	0.950
5-Flurouracil	0.150
MS1	1:320

[a]IC50 for control drugs; ID50 for broth Extract.

Table 11: Effect of non-anticancer agents on tumor cells tested by the MTT assay.

Compound 20 µg /ml	**Growth inhibition rate % HELA**
Water	0
DMSO	0
Methanol	2.5
Chloroform	3.6
Ethyl actate	4.2
Ethanol	4.8
Kanamycin	14.7
Micronomicin	18.3
Linomycin	20.7
Gentamicin	13.5
Chloramphenicol	76.7

The Extract at a dilution of 1:320 displaying activity as a function of the growth inhibition rate is 100% of marine *Streptomyces radiopugnans MS1* and exhibited strong activity against Hela cells with ID_{50} value of 25.33.

Discussion

In the present study, extract of marine Actinomycetes MS1 strain was found to have strong antitumour activity against Crown gall tumor assay and Hela cell lines.

The Biochemical, physiological and morphological properties of the test strain are dependable with its assignment to the genus Streptomyces . It is also clear that the organism forms a distinct phylo genetic line in the 16sRNA tree and belongs to the group of streptomycetes(57 & 58).The strain was further identified as *Streptomyces radiopugnans MS1*.

Study was interesting to note that antibiotic-resistant bacteria showed more sensitivity to the investigated *MS1* extracts. This has clearly indicated that antibiotic resistance does not interfere with the antibacterial action of *Streptomyces radiopugnans MS1* extracts and these extracts might have different modes of action on test organisms.

In latest years much consideration has been dedicated to natural antioxidant and their connection with health benefits (59) There are several ways available to assess antioxidant activity of compounds. An easy, rapid and sensitive method for the antioxidant screening of Actinomycetes extracts is free radical scavenging assay using 1,1, diphenyl-2-picryl hydrazyl (DPPH) stable radical spectrophotometricall. In presence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases (60) In this study, the scavenging activity of Ethyl actate extracts was found to be dose dependent i.e., higher the concentration, more was the scavenging activity. Though the DPPH radical scavenging abilities of the extracts were less than that of ascorbic acid, the study proved that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

The hemolysis assay was used as a test for the interaction of compounds with the erythrocytes. Hemolytic activity can be a result of pore formation in the cell membranes thus changing membrane permeability or it can be due to the alteration of sodium–potassium and calcium–magnesium ATPase activities (61). Measuring hemolytic activity is important as it is an indicator for cytotoxicities. The *in vitro* hemolysis test has also been employed by many different groups for the toxicological evaluation of different streptomyces. Mechanical stability of erythrocytes membrane is good indicator of various *in-vitro* cytotoxicities Performing hemolytic assay is important to determine whether a drug possessing antioxidant and other bioactivities can be used in pharmacological applications (62).

Anticancer Crown gall tumor assay is a precious contrivance that indicates anticancer activity of test compound by their inhibition crown galls formation that was induced in wounded potato tissues by *A. tumefaciens LBA4404*. This bioassay was a perceptive, bench-top anticancer assay for chemicals that disrupt the cell cycle (mitosis, S phase, etc.) despite of their mode of action (63).A number of scientists have been used these process over the past 15 years, and they appear to be malleable to the purpose of standardization or quality control of bioactive compounds in such heterogeneous botanicals.

Streptomyces radiopugnans MS1 plays an important in anticancer potential; the Ethyl actate extract was evaluated for anticancer activity. It was shown that tumor formation was observed when *Agrobacterium* strains alive on living potato disc. The potato discs were often damaged due to the contamination and other physiological factors when there was no tumor formation. Thus successful addition of *Agrobacteirum* on living potato disc is desired for anticancer test of Ethyl actate extracts. (64)

In conclusion, these crude extract with such high potency and minimum hemolysis would be suitable candidates for use against these Crown gall tumor assay and cancerous cell lines as efficient and novel anticancer agents.Optimization of Crown gall tumor assay practically to be more accurate for screening anticancer compounds as well as using MTT assay.

Using sensible methods is more constructive for optimizing any experiment as a substitute of using other kind of optimization especially in biological experiments where case-by-case study is significant. In our case study *Streptomyces radiopugnans MS1* is clear model about the vital of using more than one concentration to explore the activities of the tested crude extracts. Modified Crown gall tumor assay procedures could routinely be employed as comparatively rapid, inexpensive, safe, and statistically reliable prescreen for anticancer activity. We suggest using Crown gall tumor assay examination to study more crude extracts especially in developing countries where the cost of mammalian tissue culture or investigational animals is high and to sustain the research focused on discovery of new natural compounds which could help a lot for investigating or breakthrough new medicinal drugs.

Our study on the *Streptomyces radiopugnans MS1* isolated from the marine demonstrated its higher anticancer properties and a great potential source of anticancer compounds. The metabolites from strain *MS1* will be intensively investigated in our further study.

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