

ANTIMICROBIAL, ANTIOXIDANT AND CYTOTOXIC PROPERTIES OF *STREPTOMYCES* SP. (ERINLG-01) ISOLATED FROM SOUTHERN WESTERN GHATS

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ABSTRACT

Objective: *Streptomyces* sp. is one of the most important antibiotic producing Gram positive bacteria. The aim of this study was to assess the antimicrobial, antioxidant and cytotoxic effects (against A549 lung adenocarcinoma cancer cell line) of ethyl acetate extract of *Streptomyces* sp. from the soil sample of Doddabetta forest, Nilgiris, Western Ghats of Tamil Nadu.

Methods: Isolation of *Streptomyces* was performed by serial dilution plate technique. The strain was grown in MNGA medium to study the morphology and biochemical characteristics. *Streptomyces* sp. (ERINLG-01) was screened for its antimicrobial activity against pathogenic bacteria and fungi. The strain was subjected to 16S rRNA analysis and was identified as *Streptomyces* sp. (ERINLG-01). The nucleotide sequence of the 16S rRNA gene of the isolate exhibited close similarity with other *Streptomyces* sp. and has been submitted to Genbank. The antibacterial substances were extracted using ethyl acetate from MNGA medium. Antioxidant and cytotoxic effect were also studied.

Results: *Streptomyces* sp. ERINLG-01 was isolated from the soil sample of the Doddabetta forest, Nilgiris, Tamil Nadu, India. Seven RAPD primers were tested against the DNA extracted from the *Streptomyces* sp.; four of them (OPA2, OPA9, OPN15 and OPA20) gave clear and scorable profiles. The ethyl acetate extract showed antimicrobial activity against six Gram negative, five Gram positive bacteria and three fungi. The zones of inhibitions were: 16 mm against *B. subtilis*, *S. epidermidis* and *M. pachydermatis*, 15 mm against *E. aerogenes* and *C. albicans*. The minimum inhibitory concentration values of ethyl acetate extract were: 125 µg/mL against *B. subtilis*, 250 µg/mL against *S. epidermidis*, *M. pachydermatis*, *E. aerogenes* and *C. albicans*. The radical scavenging activity was maximum at 1000 µg/mL (76.11%). Cupric Ion Reducing Antioxidant Capacity of ethyl acetate extract was dependent on the concentration. Ferric reducing antioxidant power assay of ethyl acetate extract showed (1.358 ± 0.04 mM Fe (II)/g) two-fold higher value compared to the standard. Ethyl acetate extract showed 82.4% cytotoxic activity *in vitro* against A549 lung adenocarcinoma cancer cell line at a dose of 1000 µg/mL with IC₅₀ value of 600 µg/mL. The results showed that the ethyl acetate extract of *Streptomyces* sp. ERINLG-01 could be probed further in drug discovery programme.

Conclusion: *Streptomyces* sp. ERINLG-01 showed promising antibacterial, antioxidant and cytotoxic activities.

Keywords: Antimicrobial, Cytotoxicity, Antioxidant, *Streptomyces* sp., RAPD, 16srRNA

INTRODUCTION

Antibiotics are among the most prescribed drugs worldwide but their effectiveness is facing serious clinical concerns especially due to the emergence of resistant bacteria [1]. Other strategies include organic synthesis [2], drug pharmacokinetics modification using nanotechnology [3] and search for molecules with unexploited mechanisms of action [4]. Novel methods and technologies for discovering new drugs from microbial sources have been described [5]. Screening of novel strains are bringing out microorganisms, not yet assayed for their antibacterial activity [6], that can produce innovative molecules or useful templates for new antibiotics development [7]. Actinomycetes are widely distributed in nature and are typically useful in the pharmaceutical industry for their seemingly unlimited capacity to produce secondary metabolites with diverse chemical structures and biological activities [8]. It is essential to continue searching for new antibiotics because of the toxicity of some currently used compounds and the emergence of resistant pathogens [9].

Streptomyces produce over 70% of the known antibiotics, and about 70% of all known medicines have been isolated from actinomycetes bacteria of which 75% and 60% have been used in medicine and agriculture, respectively [10, 11]. *Streptomyces* is a genus of Gram-positive bacterium that grows in various environments, with a filamentous form similar to fungi. The morphological differentiation of *Streptomyces* involves the formation of a layer of hyphae that can differentiate into a chain of spores. This process is unique among Gram-positives, requiring a specialized and coordinated metabolism. The most interesting property of *Streptomyces* is the ability to produce bioactive secondary metabolites such as antifungals, antivirals, antitumorals and antihypertensives. Development of multiple drug resistance in microbes and tumor cells has become a

major problem and has revealed the need to search for new and novel anticancer antibiotics [12]. The present study was aimed at isolating *Streptomyces* sp. from the soil sample of Doddabetta forest, Nilgiris, Tamil Nadu, India and assessing its antimicrobial, antioxidant and cytotoxic properties.

MATERIALS AND METHODS

Sample collection

The soil samples were collected from the depth of 5-15 cm at Doddabetta forest, (Southern Western Ghats), Tamil Nadu, India.

Isolation of *Streptomyces* sp.

Isolation of *Streptomyces* sp. was performed by serial dilution using dilution plate technique. One gram of soil was suspended in 9 ml of sterile distilled water. The dilution was carried out up to 10⁻⁶ dilutions. Aliquots (0.1 mL) of 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶ were spread on the isolation plates containing actinomycetes isolation agar (Himedia, Mumbai). To minimize the bacterial and fungal growth, actidione 30mg/L and nalidixic acid 40mg/L were added. The plates were incubated at 28 °C for 7 to 20 days. The pure colonies were transferred to ISP-2 medium and incubated at 27 °C for five days.

Morphological, physiological and biochemical observations

Cultural and morphological features of ERINLG-01 were characterized following the directions given by the International *Streptomyces* Project (ISP) [13] and the Bergey's Manual of Systematic Bacteriology. Cultural characteristics of pure isolates in various media (ISP 1-7) were recorded after incubation at 28 °C for 7 - 14 days. Morphology of spore bearing hyphae with entire spore chain was observed with a light microscope (Model SE; Nikon) using

cover-slip method in ISP medium (ISP 3 - 6). The shape of cell, Gram-stain, color determination, the presence of spores, and colony morphology were assessed on solid ISP agar medium. Biochemical reactions, different temperatures, NaCl concentration, pH level, pigment production, enzyme reaction and acid or gas production were done following the methods of Balachandran et al., (2012) [14] and Valanarasu et al., (2009) [15].

Extraction

Primary antimicrobial activity was evaluated on Modified Nutrient Glucose Agar medium (MNGA) by the cross streak method against various microorganisms [16]. Culture inoculate of the isolate ERINLG-01 was taken in 500 mL Erlenmeyer flasks containing 150 ml of MNGA medium and incubated at 30 °C in a shaker (200 rpm) for 12 days. After 12th day the culture broth was centrifuged at 8000 g for 20 min to remove the biomass. Equal volumes of ethyl acetate (1:1 v/v) were added. The extract was evaporated to dryness at 40 °C under reduced pressure.

Microbial organisms

The following Gram negative, Gram positive bacteria and fungi were used for the experiment. Gram negative bacteria: *Shigella flexneri* MTCC 1457, *Salmonella paratyphi-B*, *Klebsiella pneumoniae* MTCC 109, *Pseudomonas aeruginosa* MTCC 741, *Proteus vulgaris* MTCC 1771 and *Salmonella typhimurium* MTCC 1251; Gram positive bacteria: *Bacillus subtilis* MTCC 441, *Micrococcus luteus* MTCC 106, *Enterobacter aerogenes* MTCC 111, *Staphylococcus aureus* MTCC 96 and *Staphylococcus epidermidis* MTCC 3615. The reference cultures were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India-160 036; fungi: *Candida albicans* MTCC 227, *Malassezia pachydermatis* and *Aspergillus flavus*. All the fungal cultures were obtained from the Department of Microbiology, Christian Medical College, Vellore, Tamil Nadu, India. Bacterial inoculums were prepared by growing cells in Mueller Hinton broth (MHB) (Himedia) for 24 h at 37°C. The filamentous fungi were grown on sabouraud dextrose agar (SDA) slants at 28 °C for 10 days and the spores were collected using sterile doubled distilled water and homogenized. Yeast was grown on sabouraud dextrose broth (SDB) at 28 °C for 48 h.

Antimicrobial assay

Antimicrobial activities were carried out using disc diffusion method [17]. Petri plates were prepared with 20 mL of sterile Mueller Hinton agar (MHA) (Hi-media, Mumbai). The test cultures were swabbed on the top of the solidified media and allowed to dry for 10 min and a specific amount of crude extract was added to each disc separately. The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. Negative control was prepared using respective solvents. Streptomycin (10µg/disc) was used as positive control for bacteria. Ketoconazole was used as positive control for fungi. The plates were incubated for 24 h at 37 °C for bacteria and for 48 h at 28 °C for fungi. Zones of inhibition were recorded in millimeters and the experiment was repeated twice.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration studies of the ethyl acetate extract were performed according to the standard reference methods for bacteria [17], for filamentous fungi [18] and yeasts [19, 20]. The required concentrations (2000, 1000, 500, 250, 125 and 62.5µg/mL) of the ethyl acetate extract were dissolved in DMSO (2%), and diluted to give serial two-fold dilutions that were added to each medium in 96 well plates. An inoculum of 100µL from each well was inoculated. The antifungal agents Ketoconazole for fungi and Streptomycin for bacteria were included in the assays as positive controls. For fungi, the plates were incubated for 48 to 72 hours at 28 °C and for bacteria, the plates were incubated for 24h at 37 °C. The MIC for fungi was defined as the lowest extract concentration showing no visible fungal growth after incubation time. 5µL of tested broth was placed on the sterile MHA plates for bacteria and incubated at respective temperature. The MIC for bacteria was determined as the lowest concentration of the compound inhibiting the visual growth of the test cultures on the agar plate.

Antioxidant activity

Antioxidant activity of ethyl acetate extract was investigated by DPPH radical scavenging assay, Cupric Ion Reducing Antioxidant Capacity (CUPRAC) assay, Ferric Reducing Antioxidant Power (FRAP) assay and Total Antioxidant Capacity (TAC).

DPPH radical scavenging assay

DPPH (2, 2-diphenyl-1-picryl hydrazyl) radical scavenging activity of ethyl acetate extract was determined based on the method described [21]. 40 µL of various concentrations (125-1000 µg/mL) of ethyl acetate extract was added to ethanolic solution of DPPH (0.1 M, 2960µL). The absorbance of reaction mixture was measured at 517 nm after 30 minutes of incubation in the dark at room temperature. AA (Ascorbic acid) was used as the standard control. The free radical scavenging activity was calculated as follows:

$$\text{DPPH}^{\cdot} \text{ scavenging activity} = [(A_c - A_s / A_c) \times 100]$$

Where A_c is the absorbance of the control, A_s is the absorbance of the extract / standard (AA).

Cupric Ion Reducing Antioxidant Capacity assay

The cupric ion reducing capacity was measured according to the method [22]. The standard antioxidant AA and ethyl acetate extract were mixed with CuCl_2 (1 mL, 10 mM), neocuproine (1 mL, 7.5 mM) and Ammonium acetate buffer (pH 7.0, 1mL, 1M), adjusted to total volume of 4mL. After 30 minutes of incubation at room temperature, the absorbance was measured at 450 nm against blank. In the assay, Cu (II) was reduced to Cu (I) through the action of electron donating antioxidant.

Ferric Reducing Antioxidant Power assay

The assay was performed according to the method of Kubola et al., (2011) [23]. FRAP reagent (50 mL of 300 mM acetate buffer (pH 3.6), 5mL of 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl and 5mL 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) was prepared. FRAP reagent (2960 µL) was mixed with 40 µL of ethyl acetate extract. AA was used as standard as in the other methods. The mixtures were incubated at 37 °C for 4 min and the absorbance was measured at 593 nm. Results were expressed as Fe^{2+} equivalents per gram dry mass.

Total Antioxidant Capacity

The total antioxidant capacity of the compound was evaluated using the phosphomolybdenum method [24]. Ethyl acetate extract (1 mg/mL) was dissolved in a mixture of 2.9mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95 °C for 90 min. After the samples were cooled to ambient temperature, the absorbance of the solution was measured at 695 nm against blank. The results were reported as mg Gallic acid equivalents/g of ethyl acetate extract.

Cytotoxic properties

A549 lung adenocarcinoma cancer cell line was obtained from National Institute of Cell Sciences, Pune. A549 cell line was maintained in complete tissue culture medium Dulbecco's Modified Eagle's Medium with 10 % Fetal Bovine Serum and 2mM L-Glutamine, along with antibiotics (about 100 International Unit/mL of penicillin, 100 µg/mL of streptomycin) with the pH adjusted to 7.2.

The cytotoxicity was determined according to the method of Balachandran et al. (2012) [25] with some changes. Cells (5000 cells/well) were seeded in 96 well plates containing medium with different concentrations such as 1000, 800, 600, 400, 200 and 100 µg/mL. The cells were cultivated at 37 °C with 5 % CO_2 and 95 % air in 100 % relative humidity. After various durations of cultivation, the solution in the medium was removed. An aliquot of 100 µL of medium containing 1 mg/mL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide was loaded in the plate. The cells were cultured for 4 h and then the solution in the medium was removed. An aliquot of 100 µL of DMSO was added to the plate, which was shaken until the crystals were dissolved. The cytotoxicity against cancer cells was determined by measuring the absorbance of the converted dye at 540 nm in an Enzyme linked immune sorbant

assay reader. Cytotoxicity of each sample was expressed as the half maximal inhibitory concentration (IC₅₀) value. The IC₅₀ value is the concentration of test sample that causes 50% inhibition of cell growth, averaged from three replicate experiments.

Molecular analysis

16S rRNA gene amplification

Genomic DNA of ERINLG-01 was isolated by the methods of Hipura *Streptomyces* DNA spin kit-MB 527-20pr from Hi-media. The freshly cultured cells were pelleted by centrifuging for 2 min at 12,000 rpm to obtain 10-15mg (wet weight). The cells were resuspended thoroughly in 300 µL of Lysis solution; 20 µL of RNase A solution was added, mixed and incubated for 2 min at room temperature. About 20 µL of the Proteinase K solution (20mg/mL) was added to the sample and mixed; the resuspended cells were transferred to Hibeat Tube and incubated for 30 min at 55 °C. The mixture was vortexed for 5-7 minutes and incubated for 10 min at 95 °C followed by pulse vortexing. Supernatant was collected by centrifuging the tube at 10,000 rpm for 1 min at room temperature. About 200 µL of lysis solution was added, mixed thoroughly by vortexing and incubated at 55 °C for 10min. To the lysate 200 µL of ethanol (96-100%) was added and mixed thoroughly by vortexing for 15 sec. The lysate was transferred to new spin column and 500 µL of prewash solution was added to the spin column and centrifuged at 10,000 rpm for 1 min and the supernatant was discarded. The lysate was then washed in 500 µL of wash solution and centrifuged at 10,000 rpm for 3 min. 200 µL of the Elution Buffer was pipetted out and added directly into the column without spilling and incubated for 1 min at room temperature. Finally the DNA was eluted by centrifuging the column at 10,000 rpm for 1 min. The 16 S ribosomal RNA gene was amplified by PCR method using primers 27f (5'AGTTTGGATCCTGGCTCAG3') and 1492r (5'ACGGCTACCTTGTACACTT3'). Each PCR mixture in a final volume of 20µL contained 10 mM Tris-HCl (pH.8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each dNTP, 10 pmol of each primer, 50 ng of genomic DNA and 1U of Taq DNA Polymerase (New England Biolabs.Inc). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94 °C for four minutes followed by 30 cycles at 94 °C for one minute, primer annealing at 52 °C for one minute and primer extension at 72 °C for one minute. At the end of the cycling, the reaction mixture was held at 72 °C for 10 min and

then cooled to 4 °C. PCR amplification was detected by 1 % agarose gel electrophoresis and was visualized by ultraviolet (UV)

fluorescence after ethidium bromide staining. The PCR product obtained was sequenced by an automated sequencer (Genetic

Analyser 3130, Applied Biosystem, and USA). The same primers as above were used for this purpose. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks using the NCBI BLAST available at <http://www.ncbi.nlm.nih.gov/>. RAPD analysis was carried out for suitable RAPD primers, such as OPA2: 5'-TGCCGAGCTG-3', OPA9: 5'-GGGTAACGCC-3', OPA10: 5'-GTGATCGCAG-3', OPN15:5'-CAGCGACTGT-3', OPA20: 5'-GTTGCGATCC-3', OPA13: 5'CAGCACCCAC-3' and OPF-05:5'GTG ATC GCA G-3'.

Nucleotide sequence accession number

The partial 16S rRNA gene sequences of isolate ERINLG-01 have been deposited in the GenBank database under accession number KC820653. A phylogenetic tree was constructed using the neighbour-joining DNA distance algorithm using software MEGA [26] (version 4.1).

Statistical analysis

Cytotoxic and antioxidant activities of ethyl acetate extract were statistically analyzed by Duncan multiple range test at P =0.05 with the help of SPSS 11.5 version software package.

Results and discussion

The strain ERINLG-01 was isolated from the soil samples collected from Doddabetta forest Nilgiris, (Southern Western Ghats), Tamil Nadu, India. This strain was Gram-positive filamentous bacterium. The colour of the substrate mycelia was white. The spore chains were white. These characteristic morphological properties strongly suggested that the isolate belonged to *Streptomyces* genus. ERINLG-01 showed good growth on medium amended with sodium chloride up to 9 %; no growth was seen at 10 %.

The temperature for growth ranged from 25 to 37 °C with optimum of 30 °C and the pH range was 6-10 with normal pH of 7. Utilization of various carbon sources by ERINLG-01 indicated a wide pattern of carbon source assimilation. Arabinose, Ribose, Lactose, Xylose and rhamnose did not support the growth of the isolate. ERINLG-01 showed sensitivity towards Gentamicin, Ampicillin, Cephaloridine, Vancomycin, Amikacin, Penicillin, Rifamycin and Norfloxacin. The culture, morphological characteristics and antimicrobial activities of different *Streptomyces* isolates have been reported by several investigators [27].

Table 1: Culture characteristics of *Streptomyces* sp. (ERINLG-01) in different media

| Medium | Growth | Substrate mycelium | Aerial mycelium | Spores | Reverse |
|--------|----------|--------------------|-----------------|--------|-------------|
| ISP 1 | Good | Present | White | White | Brown |
| ISP 2 | Good | Present | White | White | Brown |
| ISP 3 | Moderate | poor | Poor | White | Moderate |
| ISP 4 | Good | Present | White | White | Brown |
| ISP 5 | Good | Present | White | White | Brown |
| ISP 6 | Poor | Poor | Poor | Poor | Light Brown |
| ISP 7 | Good | Present | White | White | Moderate |

ISP1-7: International *Streptomyces* Project;

The result of the sequencing of ERINLG-01 was obtained in the form of rough electrophoregrams. The sequences have been chosen as reference sequences in which unidentified and unpublished sequences were not included.

The phylogenetic tree was obtained by applying the neighbor joining method. Culture characteristics and 16S rRNA studies strongly suggested that our isolate ERINLG-01 belonged to the genus *Streptomyces*. Studies on the microbial diversity by 16S rRNA gene analysis showed that a group of high-GC Gram-positive bacteria (actinomycetes) were dominant in the soil [28]. The identification of isolate ERINLG-01 was confirmed as *Streptomyces* sp. with homology of 100%. The universal primers seem to be sufficient for identifying the genus but not the species. A total of seven RAPD primers were tested against the DNA extracted from the studied

Streptomyces sp.; four of them (OPA-2, OPA-9, OPN15 and OPA20) gave clear and scorable profiles. The profiles were reproducible with sufficient polymorphism. The number of bands and the degree of polymorphism revealed by each primer were clear.

The advantage of RAPD analysis in this study is that it covers the entire genome; therefore it provides sufficient information about differences that might be present inside the genome. In this regard, Williams et al., (1990) showed that RAPD markers cover the entire genome, revealing coding or non-coding regions, repeated or single-copy sequences. Micheltore et al., (1991) reported that polymorphism in RAPD profile might have resulted from base changes that alter primer-binding sites. Ethyl acetate extract of *Streptomyces* sp. (ERINLG-01) showed antibacterial and antifungal activities against bacteria and fungi (2 mg/mL). Of the five Gram

positive and six Gram negative bacteria and three fungi strains studied, ethyl acetate extract exhibited activity against most Gram positive bacteria compared to Gram negative bacteria. The zones of inhibition were: 16 mm against *B. subtilis*, *S. epidermidis* and *M. pachydermatis*, 15 mm against *E. aerogenes* and *C. albicans*.

The minimum inhibitory concentration values of ethyl acetate extract were: 125 µg/mL against *B. subtilis*, 250 µg/mL against *S. epidermidis*, *M. pachydermatis*, *E. aerogenes* and *C. albicans*. Primary screening revealed that MNGA medium was a very good base for the production of antibacterial compounds. Growth and pigment production were observed in glucose as the sole source of carbon. The optimum temperature of 30 °C was found to be effective for growth and pigment production. Maximum antimicrobial compound was obtained at pH 7.0. Earlier report showed that twelve actinomycetes strains were isolated from the soil samples of the Himalaya and ERIH-44 showed both antibacterial and antifungal activity [16]. Normally antibiotic production was higher in medium having glucose (1%) as carbon source. The *Streptomyces* sp.

(ERINLG-01) showed good antimicrobial activity in MNGA medium and indicated that the antimicrobial compounds were extracellular. Most of the secondary metabolites and antibiotics were extracellular in nature and extra cellular products of actinomycetes showed potent antimicrobial activities [31, 32]. The study on the influence of different nutritional media and culture conditions on antimicrobial compound production indicated that the highest biological activities were obtained when MNGA medium was used as a base. Our results indicated that the synthesis of antimicrobial metabolites depended on the medium constituents. In fact, it has been shown that the nature of carbon and nitrogen sources strongly affected antibiotic production in different organisms and the antibiotic production was increased by glucose rich medium [33]. It has been reported that the environmental factors like temperature, pH and incubation have profound influence on antibiotic production. This activity might be due to their ability to complex with bacterial cell wall [34], thus inhibiting the microbial growth and the membrane disruption could be suggested as the mechanism of action [35]. Most of the antimicrobial compounds are extracted using ethyl acetate [36]

Table 2: Physiological and biochemical characteristics of *Streptomyces* sp. (ERINLG-01).

| Characteristics | Results |
|----------------------------------|---------------------------|
| Gram staining | Positive |
| Shape and growth | filamentous aerial growth |
| Production of diffusible pigment | + |
| Range of temperature for growth | 25 °C to 37 °C |
| Optimum temperature | 30 °C |
| Range of pH for growth | 6 to 10 |
| Normal pH | 7 |
| Amylase | + |
| Protease | + |
| Gelatinase | - |
| Indole production | - |
| Growth in the presence of NaCl | 1 to 9% |
| Sugar analysis | |
| Mannose | + |
| Maltose | + |
| Lactose | - |
| Sucrose | + |
| Glucose | + |
| Galactose | + |
| Starch | + |
| Mannitol | + |
| Arabinose | - |
| Xylose | - |
| Rhamnose | - |
| Ribose | - |
| Standard antibiotics | Sensitivity |
| Ciprofloxacin | R |
| Gentamicin | S |
| Ampicillin | S |
| Cephaloridine | S |
| Streptomycin | R |
| Erythromycin | R |
| Vencomycin | S |
| Amikacin | S |
| Penicillin | S |
| Rifamycin | S |
| Norfloracin | S |

+: presence; -: absence; S: Sensitive

Antioxidant activity of ethyl acetate extract of *Streptomyces* sp. (ERINLG-01) was assessed and compared with the standard (Ascorbic acid). The radical scavenging activity of ethyl acetate extract at different concentrations was studied. The radical scavenging activity was maximum at 1000µg/mL (76.11 ± 1.31). Cupric Ion Reducing Antioxidant Capacity of ethyl acetate extract was dependent on the concentration. The standard showed more pronounced Cupric Ion Reducing Antioxidant Capacity than ethyl acetate extract at the concentration of 1 mg/mL. The ferric reducing antioxidant power assay measures the reduction of ferric iron (Fe³⁺) to ferrous iron (Fe²⁺) in the presence of antioxidants. The results showed that the activity was

comparable to the standard ascorbic acid at 40 µL. Ethyl acetate extract (1.358 ± 0.04 mM Fe (II)/g) showed approximately two-fold higher ferric reducing capacity compared to the standard reference Ascorbic acid (2.354 ± 0.13mM Fe (II)/g). The total antioxidant capacity of ethyl acetate extract was determined by the phosphomolybdenum method. This method is based on the reduction of molybdenum Mo (VI) to Mo (V) by the antioxidant compounds and the formation of a green Mo (V)-antioxidant complex with maximum absorption at 695 nm. The high absorbance values indicated that the sample possessed significant antioxidant activity of 0.113 ± 0.03mg GAE/g and Ascorbic acid 0.037 ± 0.03 mg GAE/g.

Ethyl acetate extract showed cytotoxic activity in vitro against A549 lung adenocarcinoma cancer cell line. It showed 82.4% activity at the dose of 1000 µg/mL with IC₅₀ (60.1%) value of 600µg/mL. All the concentrations used in the experiment decreased the cell viability significantly (P<0.05) in a concentration-dependent

manner. Ethyl acetate extracts from *Streptomyces* sp. have been shown to possess cytotoxicity and inhibit cancer cells through a variety of mechanisms including induction of apoptosis [37,38], intercalation and binding with cellular DNA [39], redox-cycling radical formation [40, 41], and inhibition of topoisomerase [42].

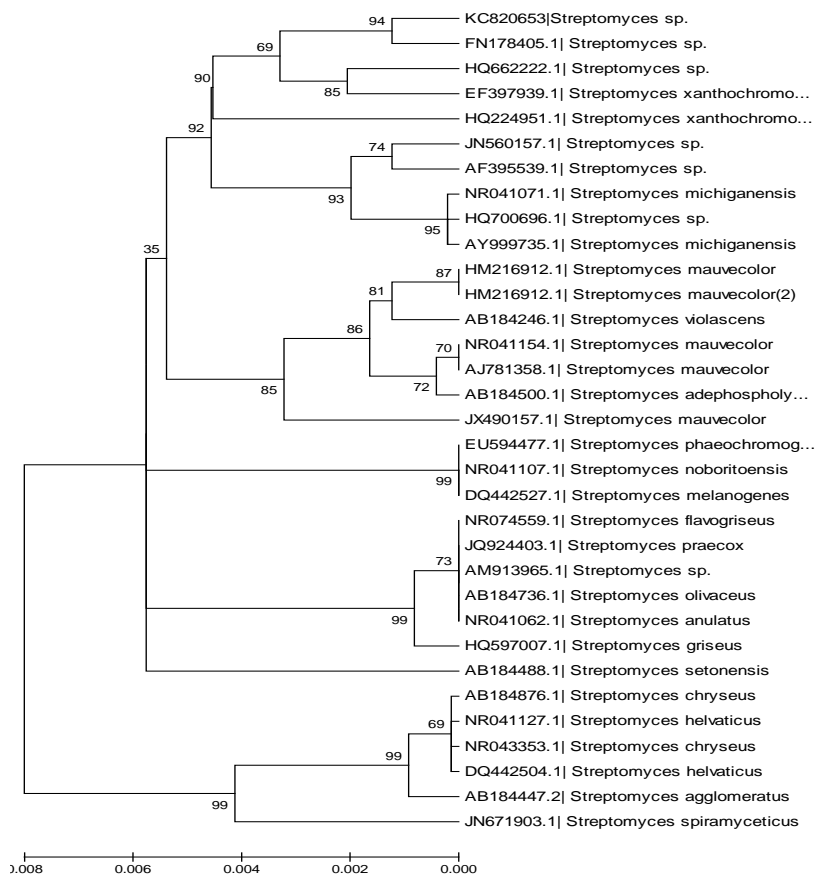


Fig.1: Phylogenetic tree derived from 16S rRNA gene sequences showing the relationship between *Streptomyces* sp. (ERNLG-01) and the other species belonging to the genus *Streptomyces* constructed using the neighbour-joining method. Bootstrap values were expressed as percentages of 1000 replications.

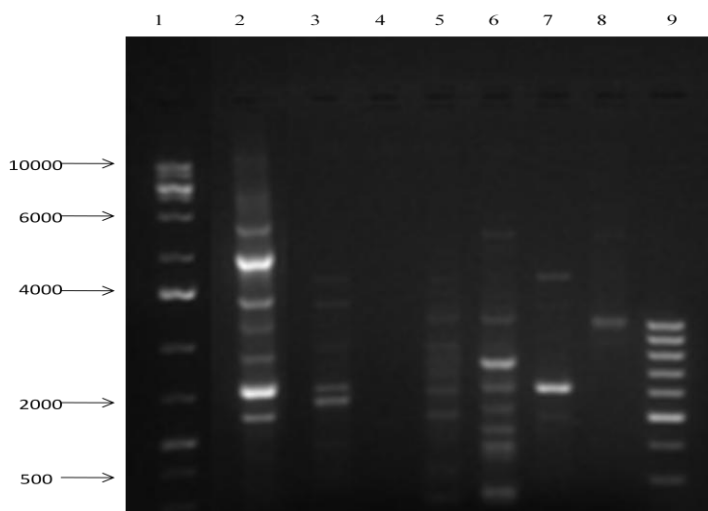


Fig. 2: RAPD analysis carried out with suitable RAPD primers. 1). 1kb ladder, 2). OPA2: 5'-TGCCGAGCTG-3', 3). OPA9: 5'-GGGTAACGCC-3', 4). OPA10: 5'-GTGATCGCAG-3', 5). OPN15: 5'-CAGCGACTGT-3', 6). OPA20: 5'-GTTGCGATCC-3', 7). OPA13: 5'-CAGCACCCAC-3', 8). OPF-05: 5'-GTG ATC GCA G-3' and 9). 100bp ladder

Table 3: Antimicrobial activity of ethyl acetate extract from *Streptomyces* sp. (ERINLG-01) using disc diffusion method (Zone of inhibition in mm) (2mg/disc)

| Organism | Ethylacetate | Streptomycin |
|-----------------------------------|--------------|--------------|
| Gram positive | | |
| <i>Bacillus subtilis</i> | 16 | 22 |
| <i>Micrococcus luteus</i> | 13 | 26 |
| <i>Enterobacter aerogenes</i> | 15 | 22 |
| <i>Staphylococcus aureus</i> | 14 | 14 |
| <i>Staphylococcus epidermidis</i> | 16 | 14 |
| Gram negative | | |
| <i>Shigella flexneri</i> | 9 | 30 |
| <i>Salmonella paratyphi-B</i> | - | 18 |
| <i>Klebsiella pneumonia</i> | 13 | 20 |
| <i>Pseudomonas aeruginosa</i> | 12 | 30 |
| <i>Proteus vulgaris</i> | 10 | 30 |
| <i>Salmonella typhimurium</i> | - | 24 |
| Fungi | | |
| | | Ketoconazole |
| <i>Candida albicans</i> | 15 | 28 |
| <i>Aspergillus flavus</i> | - | 26 |
| <i>Malassesia pachydermatis</i> | 16 | 24 |

'-' no activity; Streptomycin - standard antibacterial agent; Ketoconazole - standard antifungal agent.

Table 4: Minimum inhibitory concentration (2mg/mL) of ethyl acetate extract from *Streptomyces* sp. (ERINLG-01) against tested bacteria and fungi

| Organism | Ethylacetate | Streptomycin |
|-----------------------------------|--------------|--------------|
| Gram positive | | |
| <i>Bacillus subtilis</i> | 125 | 25 |
| <i>Micrococcus luteus</i> | 500 | 6.25 |
| <i>Enterobacter aerogenes</i> | 250 | 25 |
| <i>Staphylococcus aureus</i> | 500 | 6.25 |
| <i>Staphylococcus epidermidis</i> | 250 | 25 |
| Gram negative | | |
| <i>Shigella flexneri</i> | 1000 | 6.25 |
| <i>Klebsiella pneumonia</i> | 500 | 25 |
| <i>Proteus vulgaris</i> | 1000 | 25 |
| <i>Pseudomonas aeruginosa</i> | 500 | 6.25 |
| Fungi | | |
| | | Ketoconazole |
| <i>Candida albicans</i> | 250 | 25 |
| <i>Malassesia pachydermatis</i> | 250 | 15 |

Streptomycin - Standard antibacterial agent; Ketoconazole - Standard antifungal agent.

Table 5: Antioxidant activity of ethyl acetate extract using DPPH and CUPRAC

| Conc. ($\mu\text{g/ml}$) | Ethyl acetate (DPPH) | AA | Ethyl acetate (CUPRAC) | AA |
|----------------------------|----------------------|------------------|------------------------|------------------|
| 100 | 15.78 \pm 0.95 | 90.27 \pm 0.51 | 0.671 \pm 0.01 | 1.642 \pm 0.02 |
| 200 | 25.23 \pm 3.17 | 91.63 \pm 0.76 | 0.784 \pm 0.01 | 1.739 \pm 0.02 |
| 400 | 45.03 \pm 0.69 | 92.53 \pm 0.51 | 0.839 \pm 0.02 | 1.868 \pm 0.01 |
| 500 | 64.12 \pm 1.52 | 93.71 \pm 0.82 | 1.138 \pm 0.02 | 1.853 \pm 0.02 |
| 1000 | 76.11 \pm 1.31 | 95.15 \pm 0.19 | 1.809 \pm 0.01 | 2.081 \pm 0.02 |

AA-Ascorbic acid

Table 6: Antioxidant activity of ethyl acetate extract using FRAP and TAC

| Sample | FRAP mM Fe(II) / g | TAC mg GAE/g |
|------------------------------------|-----------------------|------------------|
| 300 $\mu\text{g/mL}$ Ethyl acetate | 1.358 \pm 0.04 | 0.113 \pm 0.03 |
| AA | 2.378 \pm 0.13 | 0.039 \pm 0.03 |

AA- Ascorbic acid

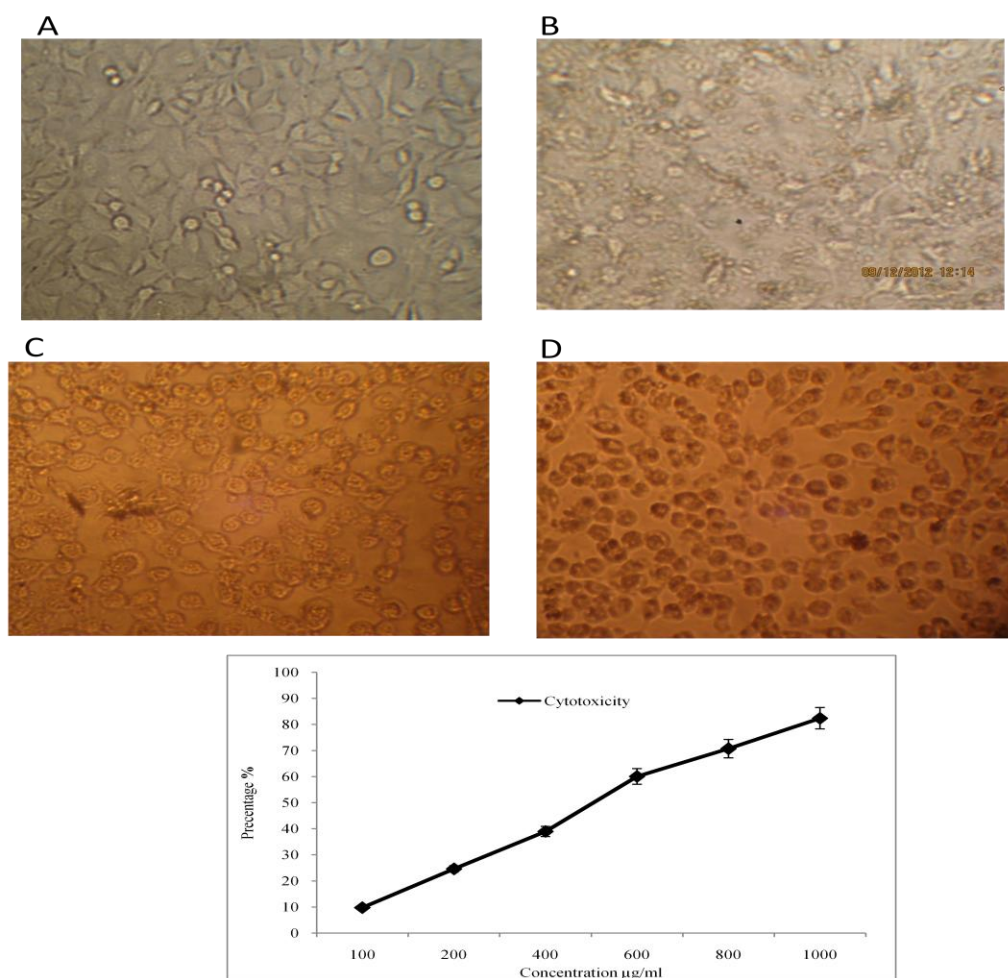


Fig. 3: Cytotoxic effects on cancer cell line (A549) (A) control cell; (B), (C) and (D) treated cells. Data are mean \pm SD of three independent experiments with each experiment conducted in triplicate.

CONCLUSION

Streptomyces sp. (ERINLG- 01) was isolated from the soil samples of the Doddabetta forest, Nilgiris, Tamil Nadu, India. The cell free culture was extracted with ethyl acetate and the extract showed antimicrobial activity against six Gram negative, five Gram positive bacteria and three fungi. Ethyl acetate extract showed prominent antioxidant activity. The ethyl acetate extract was also tested against A549 lung adenocarcinoma cancer cell line. The ethyl acetate extract showed prominent cytotoxic activity in vitro against A549 lung adenocarcinoma cancer cell line. It showed 82.4% activity at the dose of 1000 $\mu\text{g}/\text{mL}$ with IC₅₀ value of 600 $\mu\text{g}/\text{mL}$. The ethyl acetate extract can be probed further in drug discovery programme.

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