Streptomyces are rich source of bioactive compounds, notably antibiotics, enzyme inhibitors and pharmacologically active agents. Secondary metabolites produced by Streptomyces have been the primary source of antibiotics and more recently, are used as herbicides, anticancer drugs, immunoregulators and antiparasitic compounds. One study estimates that the number of antibiotics produced by the genus Streptomyces is of the order of 100,000, thus many more antibiotics remain to be identified.

Cancer still represents one of the most serious human health problems despite the great progress in understanding its biology and pharmacology. The usual therapeutic methods for cancer treatment are surgery, radiotherapy, immunotherapy and chemotherapy. These techniques are individually useful in particular situations and when combined, they offer a more efficient treatment for tumors. An analysis of the number of chemotherapeutic drugs and their...
sources indicates that over 60% of approved drugs are derived from natural compounds and many have been extracted from actinomycetes.

Lost of the antibiotics are extracellular-secondary metabolites which are normally secreted in culture media and serve as intermediates from primary metabolisms as precursors for their biosynthetic process. As part of our ongoing research of microbial metabolite, we isolated a new strain of *Streptomyces* species from Garden rhizosphere. We, herein, report the isolation of compound 1 from this new species as well as the cytotoxic activities of this compound and crude ethyl acetate extract of ISP-2 broth of this new species.

**MATERIALS AND METHODS**

**Sample collection and isolation of actinomycetes**

The soil samples were collected from the rhizosphere of Sathyabama University garden were carefully transferred to the lab using sterile polythene bags. Actinomycetes were isolated using Starch Casein Agar medium by following the method of. Plates were incubated up to 30 days at 28°C. Distinct actinomycetes colonies were purified by streak plate technique on International Streptomyces Project Number two (ISP-2) medium.

**Fermentation of Selected strain**

The selected actinomycete isolate was inoculated into ISP-2 broth, and incubated at 28°C in a shaker at (200 rpm) for seven to ten days. After incubation the culture broth was filtered through Whatman No. 1 filter paper to get cell free extract. After that, cell free broth was adjusted to pH 5.0 with 1 N hydrochloric acid and equal volume (1:1) of ethyl acetate was added and mixed by vigorous shaking and kept without disturbance. The organic phase was collected and evaporated in incubator at 60-70°C and the residue was stored at-20°C for further use.

**Cytotoxicity Screening**

**Cell line and cell cultures**

Human larynx carcinoma cell line (Hep-2) and Porcine Kidney cell lines (PK-15) were obtained from Cancer institute of Chennai, India. Cells were grown as monolayer culture in Minimal Essential Medium (MEM) medium and incubated at 37°C in a 5% of CO₂ atmosphere.

**Methyl Thiazole Tetrazolium Cell Viability Test (MTT)**

The human laryngeal cancer cell line (Hep-2) and BK-15 cell line were obtained from Cancer institute of Chennai, India. Cells were grown as monolayer culture in MEM medium and incubated at 37°C in a 5% of CO₂ atmosphere. Hep-2 and BK-15 cells (100µl) were seeded in 96 well plates at a concentration of 5X10⁵ cells/ml for 24 hrs. After the incubation the culture medium was replaced with 100ml serum free medium containing various concentrations (2.5, 1.25, 0.625, 0.312, 0.156, 0.078 mg/ml) of actinomycete extracts and incubated for 24 hrs. After that, the medium was refreshed with 100µl of serum free medium (MEM) and 20µl of MTT (5 mg/ml of (3, 4, 5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazoliumbromide) was added. The micro-titer plates were incubated for three hours in dark. The developed colour was measured with ELISA reader at 570 nm. Triplicates were maintained for each treatment. Lethal concentration (LC₅₀) were determined by calculating the % of viability:

\[
\text{% of viability} = \frac{\text{Mean Test OD}}{\text{Mean OD of Control}} \times 100
\]

**Purification Of Crude Extract: Thin Layer Chromatography**

The crude extract of extracellular was purified by thin layer chromatography (TLC) using ethyl acetate & hexane (1:9) ratio. The developed plate was visualized under UV at 366 nm. The distinct bands were visualized and the Resolving Factor (Rf) value was determined by

\[
\text{Rf} = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent}}
\]

**Distance traveled by the solvent**

**Purification of the Compounds By High Performance Liquid Chromatography (HPLC)**

The TLC band was eluted with ethyl acetate by repeated steps and allow for dryness. 20 µl of these eluent was purified further using linear gradient solvent system in HPLC (Shimadzu CLASS-VP V6.13 SP2). The C 18 column is used with the solvent system of methanol and water.

**Identification of Active Actinomycete**

The morphological, cultural, physiological, and biochemical characterization of the isolate was also carried out as described in International Streptomyces Project (ISP). The morphological characters of the isolate SS5 was examined by using...
light microscope as well as scanning electron microscope. The cultural characters of the isolate was studied by cultivating it on different media namely ISP1, ISP2, ISP4, ISP5, and ISP7 and incubated for 7-10 days at 28°C. Colony morphology including color of aerial mycelium, substrate mycelium, reverse side color, melanin pigment production and production of diffusible pigments were recorded.

The physiological characters such as, growth at different pH (5, 7, 9, 10 and 11), temperatures (10°C, 20°C, 30°C, 40°C and 50°C) was also recorded. The biochemical characterization of the isolate was also studied by the procedures. \textbf{Molecular Sequencing}

Genomic DNA was isolated from cells as described by \textsuperscript{11}. The 16S rRNA gene of strain SS 5 was amplified by polymerase chain reaction, using two universal bacterial primers, 1492R (5′-GGTTACCTTGTTACGACTT-3′) and Eubac27F (5′-AGAGTTTGA TCCTGGCTC AG-3′) \textsuperscript{12}. The amplified products were purified using TIANgel mini purification kit, ligated to MD18-T simple vector (TaKaRa), and transformed into competent cells of Escherichia coli DH5α. 16S rRNA gene fragment was sequenced using forward primer M13F (-47) and reverse primer M13R (-48). The derived 16S rRNA gene sequence was compared to the GENBANK database (NCBI), to search for similar sequences using the basic local alignment search tool algorithm.

\textbf{RESULTS AND DISCUSSION}

In the course of screening for novel actinomycetes 40 distinct isolates were obtained from five samples collected from the rhizosphere of Sathyabama University, Chennai, Tamilnadu, India. All the isolates were subjected to primary screening, among that 10\% showed antibacterial activity against all the bacteria, 5\% isolates were active against only for five bacteria used, another 5\% isolates were active against two bacteria used. Among the six active isolate one of the most potent isolate SS 5 described in the present study for its cytotoxic activity. Hep-2 cell lines were cultured for 48 h in the presence of increasing concentration of the extract. The cytotoxic activity was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) method. The concentration of the extract leading to 50\% of cytotoxicity was then determined. The results show that the ethyl acetate extract of the isolate SS5 exhibited LC50 in 0.312 mg/ml concentration against Hep-2 tumor cell lines (Fig. 1).

In order to evaluate the biocompatibility on normal cell lines (PK-15), different concentration (2.5, 1.25, 0.625, 0.312, 0.156, 0.078 mg/ml) of the extract was used. The ethyl acetate extract of the isolate SS5 exhibited IC 50 value against PK-15 cell lines as 2.5 mg/ml (Fig. 1).

The TLC analysis of the ethyl acetate extract SS5 revealed the presence of active compounds that were visualized in iodine chamber, Short UV and Long UV. The iodine chamber did not reveal any bands thus there are no fluorescent compounds in the extract. Short UV showed the presence of single band and also the Long UV showed the presence of single fluorescent band with RF value of 0.605. The band was eluted with ethyl acetate and the presence of active compound was detected by using UV spectrophotometer at

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ISP-1</th>
<th>ISP-2</th>
<th>ISP-3</th>
<th>ISP-4</th>
<th>ISP-5</th>
<th>ISP-7</th>
</tr>
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<tbody>
<tr>
<td>Colour of aerial mycelium</td>
<td>White</td>
<td>pink</td>
<td>White</td>
<td>White</td>
<td>White</td>
<td>Pink</td>
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<tr>
<td>Melanoid pigment</td>
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<td>Nil</td>
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<td>Nil</td>
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<tr>
<td>Reverse side pigment</td>
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<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
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<tr>
<td>Soluble pigment</td>
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<td>Nil</td>
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<td>Nil</td>
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</tr>
<tr>
<td>Spore chain</td>
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<td>RA</td>
<td>RA</td>
<td>RA</td>
<td>RA</td>
</tr>
<tr>
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<td>Smooth</td>
<td>Smooth</td>
<td>Smooth</td>
<td>Smooth</td>
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<tr>
<td>Growth</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
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</tr>
</tbody>
</table>

\textit{ISP – International Streptomyces Project, RA- Retinaculum apertum}

\textit{J PURE APPL MICROBIO, 7(3), SEPTEMBER 2013.}
The active compound have UV visible maximum absorption at 260-280 nm in methanol. It is reported that the most of peptide antibiotic exhibit maximum absorbance at 210-230 nm and 270-280 nm.\textsuperscript{[13-15]}

By analytical HPLC on Shimadzu CLASS-VP V6.13 SP2 C 18 column, the purity of the isolated compound was confirmed by a single peak (Fig: 3). The active compound was obtained in sufficient quantity for spectral analysis and biological testing. The active compound was eluted at time 3.767 min with 100% purity.

The purified HPLC fraction was tested against both human laryngeal carcinoma cell line and PK-15 cell line. The compound 1 was found to be cytotoxic toward Hep-2 with \( IC_{50} \) of 1.84 µg/ml and showed moderate in vitro cytotoxicity against Hep-2 and PK-15 cell lines (Fig. 1). The % of viability for the cell free crude extract of isolate SS 5 is shown in the graph below.

### Table 2. Influence of various physico-chemical parameters on the growth of Streptomyces sp. SU

<table>
<thead>
<tr>
<th>Growth Characteristics</th>
<th>Physiological characters</th>
<th>Streptomyces sp. SU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>30-40°C</td>
<td>Good growth</td>
</tr>
<tr>
<td>pH</td>
<td>7-11</td>
<td>Good growth</td>
</tr>
<tr>
<td>Carbon source utilization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>Good growth</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>Good growth</td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>Good growth</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>Good growth</td>
<td></td>
</tr>
<tr>
<td>D-Maltose</td>
<td>Good growth</td>
<td></td>
</tr>
<tr>
<td>D-Galactose</td>
<td>Good growth</td>
<td></td>
</tr>
<tr>
<td>Mannitol</td>
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<td></td>
</tr>
<tr>
<td>Starch</td>
<td>No growth</td>
<td></td>
</tr>
</tbody>
</table>

% of viability for the cell free crude extract of isolate SS 5

![Graph](image1.png)

**Fig. 1.** Effect of different concentrations of cell free crude extracts from actinomycetes SS5 against Hep-2 and PK-15 cell lines

![Graph](image2.png)

**Fig. 2.** UV visible spectrum of active compound of TLC fraction

![Graph](image3.png)

**Fig. 3.** HPLC Chromatogram peaks of SS5 sample

200-800 nm (Fig: 2).
PK-15 with IC₅₀ of 8 µg/ml.

The potent antibiotic producing strain SS5 was characterized following the procedures recommended by International Streptomyces Project (ISP).

The strain grew well on all the media and produced pinkish white aerial mycelium in ISP-2 and white aerial mycelium in all other medium used (Fig: 4).

The isolate is not produced any melanin pigment and soluble pigment. The isolate showed yellow reverse side colour and produces retinaculum apertum (RA) type spores with extended spiral with smooth surface (Fig: 5).

The isolate SS5 grew well at temperature 30-40°C, pH 7-11 and showed a wide range of carbon compound utilization. It used all the carbon sourced except starch (Table 2).

A BLAST search of the 1450 bp 16S-rRNA gene sequence of the isolate showed 98% homology to Streptomyces sp. The phylogenetic tree was constructed based on neighbor joining method shows that the isolate is most closely related to Streptomyces sp which is also supported by the high boot strap value. Based on the molecular taxonomy and phylogeny the isolate was identified as Streptomyces sp and designated as Streptomyces sp SU. The 16S rRNA sequence of
Streptomyces sp SU has been deposited in the GenBank (NCBI, USA) under the accession number HM 231270. A neighbor-joining tree based on 16S rRNA gene sequences showed that the isolate shares a same clade with Streptomyces sp and occupies a distinct phylogenetic position within the radiation including representatives of the family Streptomycetes (Fig. 6).

**CONCLUSION**

In our search for cytotoxic compound producer from Rhizosphere soil, an Actinomycete SS 5 was isolated, which was capable of producing cytotoxic compound. The strain was identified as Streptomyces sp, based on the morphological, biochemical and molecular studies. Biological activities of the crude extract of the culture were found to be anticancer against Hep-2 cell lines and less toxic to PK-15 normal cell line.

Solvent extraction and purification by TLC, HPLC, helped in isolating the active principles in pure state. The supernatant extract contained predominantly fluorescent compounds with active compound having UV absorption maximum at 260-280 nm. Spectral studies are needed to identify the compound.

**ACKNOWLEDGMENTS**

The authors wish to convey their sincere sense of gratitude to the management of Sathyabama University for their constant support and encouragement.

**Conflict of interest statement**

We declare that we have no conflict of interest.

**REFERENCES**