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In vitro Cytotoxic Activity of Bioactive Metabolite and Crude Extract from a New *Streptomyces* sp. SU

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To investigate the cytotoxic activity of actinomycete isolated from Garden rhizosphere. A new strain of *Streptomyces* identified as *Streptomyces sp SU* (Accession no HM 231270) by conventional and molecular method. DNA was isolated and the ITS region of 16s rRNA was amplified by polymerase chain reaction, using two universal bacterial primers, 1492R (52 -GGTTACCTTGTTAC GACTT-32) and Eubac27F (52 -AGAGTTTGATCCTGGCTC AG-32). 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). Blast search sequence similarity was found against the existing non-redundant nucleotide sequence database thus, identified as *Streptomyces sps* SU. Ethyl acetate was the most convenient solvent for the extraction of biologically active metabolites from the culture filtrate of this strain. The active compound 1 isolated from *Streptomyces sp SU* by submerged fermentation and purified by TLC, and HPLC method. The ethyl acetate crude extract exhibited cytotoxic effect in PK-15 and Human laryngeal cell carcinoma cell lines with LC50 values of 2.5 mg/ml and 0.312 mg/ml respectively. The purified compound 1 was eluted by analytical HPLC on Shimadzu CLASS-VP V6.13 SP2 C 18 column, the active compound was eluted at time 3.767 min with 100 % purity. The compound 1 was found to be cytotoxic toward Hep-2 with IC50 of 1.84 μg/ml and showed moderate invitro cytotoxicity against PK-15 with IC50 of 8 μg/ml.

Key words: *Streptomyces sp SU*, HPLC, cytotoxic activity.

Streptomycetes are rich source of bioactive compounds, notably antibiotics, enzyme inhibitors and pharmacologically active agents[1]. 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[2]. 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[3].

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[4]. 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

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sources indicates that over 60% of approved drugs are derived from natural compounds[5] and many have been extracted from actinomycetes[6].

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[7]. 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 [8]. 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 CO_2 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 CO_2 atmosphere. Hep-2 and BK-15 cells (100µl) were seeded in 96 well plates at a concentration of $5X10^3$ 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-2yl)-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 (LC_{50}) 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

Rf = Distance traveled by the solute

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)[9]. 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[10].

Molecular Sequencing

Genomic DNA was isolated from cells as described by [11]. The 16S rRNA gene of strain SS 5 was amplified by polymerase chain reaction, using two universal bacterial primers, 1492R (52 - GGTTACCTTGTTAC GACTT-32) and Eubac27F (52 -AGAGTTTGATCCTGGCTC AG-32)[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 DH5á.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.

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-2yl)-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 1. Growth characters of *Streptomyces* sp. SU in different media

Characteristics	Media used					
	ISP-1	ISP-2	ISP-3	ISP-4	ISP-5	ISP-7
Colour of aerial mycelium	White	pink	White	White	White	Pink
Melanoid pigment	Nil	Nil	Nil	Nil	Nil	Nil
Reverse side pigment	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Soluble pigment	Nil	Nil	Nil	Nil	Nil	Nil
Spore chain	RA	RA	RA	RA	RA	RA
Spore surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Growth	Good	Good	Good	Good	Good	Good

ISP – International Streptomyces Project , RA- Retinaculum apertum

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

Growth Characteristics	Streptomyces sp. SU
Physiological characters	
Temperature	30-40°C
pH	7-11
Carbon source utilization	
Glucose	Good growth
Sucrose	Good growth
Lactose	Good growth
Fructose	Good growth
D-Maltose	Good growth
D-Galactose	Good growth
Mannitol	Good growth
Starch	No growth

200-800 nm (Fig: 2). 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 [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

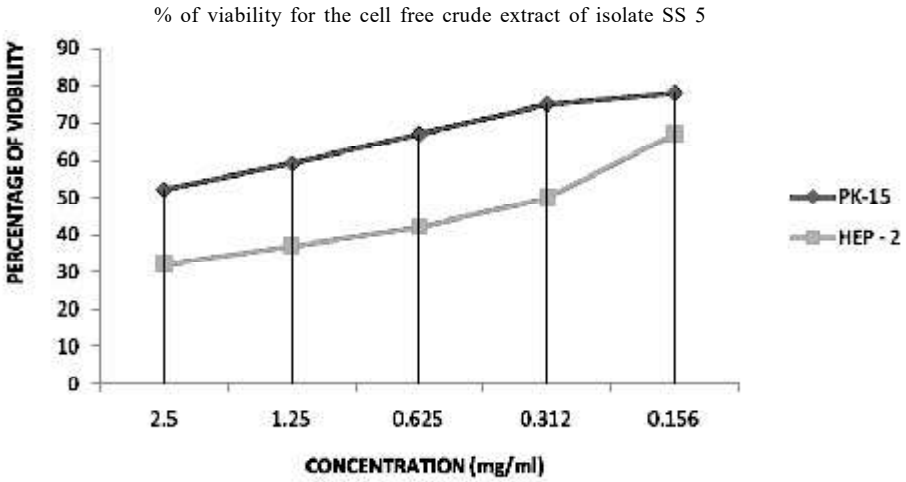

Fig. 1. Effect of different concentrations of cell free crude extracts from actinomycetes SS5 against Hep-2 and PK-15 cell lines

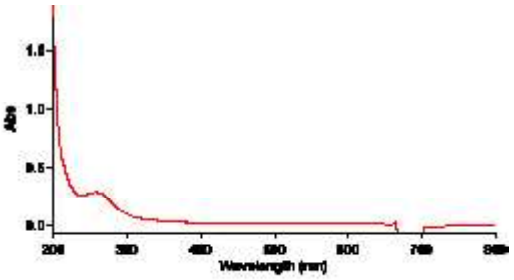

Fig. 2. UV visible spectrum of active compound of TLC fraction

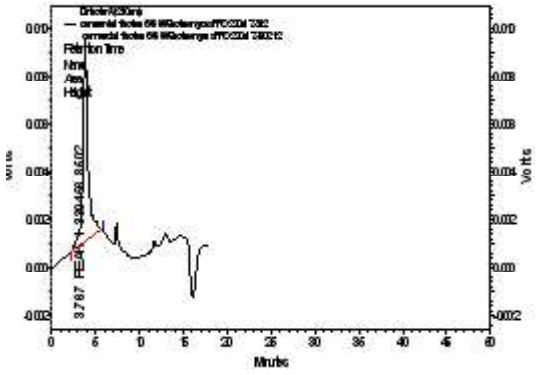

Fig. 3. HPLC Chromatogram peaks of SS5 sample

PK-15 with IC_{50} 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 grow well at temperature 30-40°C, pH 7-11 and showed a wide range of carbon compound utilization. It used all the carbon sourced used 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 r RNA sequence of

Fig. 4. Selected Isolate SS 5 on ISP-2 Medium

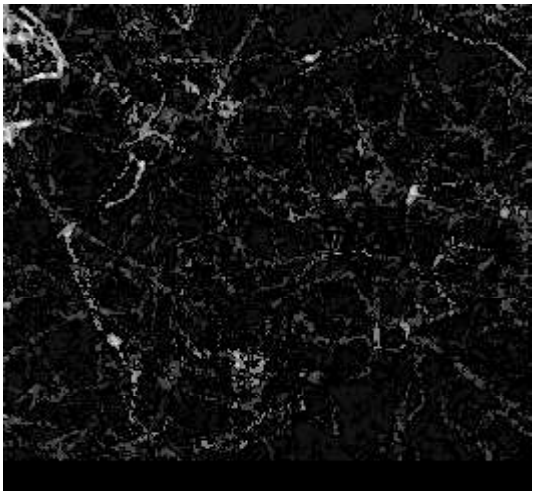

Fig. 5. Scanning electron micrograph of *Streptomyces* sp. SU

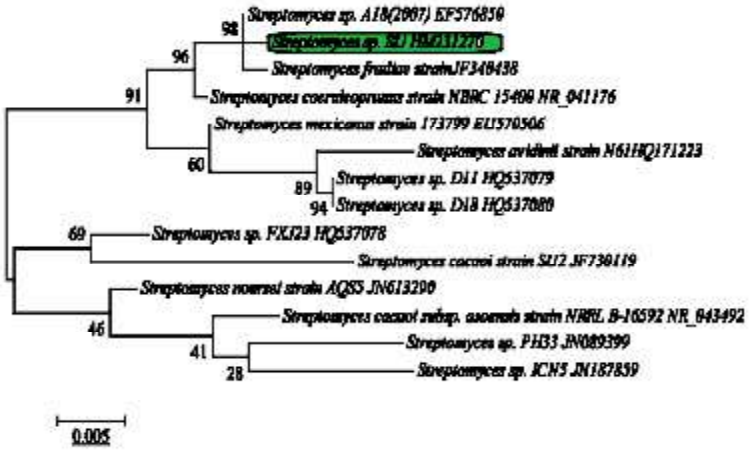

Fig. 6. Phylogenetic tree of *Streptomyces* sp SU based on 16S rRNA gene sequence

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.

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Conflict of interest statement

We declare that we have no conflict of interest.

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