

IN VITRO CYTOTOXIC ACTIVITY OF BIOACTIVE METABOLITE AND CRUDE EXTRACT FROM A NEW ACTINOMYCETE *Streptomyces avidinii* STRAIN SU4

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ABSTRACT

Objective: To investigate the in-vitro cytotoxic activity of crude extract and bioactive metabolite of actinomycete isolated from marine sediment.

Methods: A new strain of *Streptomyces* identified as *Streptomyces avidinii* strain SU4 (Accession no HM JF730120) by conventional and molecular method. The active compound 1 isolated from *Streptomyces avidinii* strain SU4 by submerged fermentation and purified by TLC, and HPLC method.

Results: The ethyl acetate crude extract exhibited cytotoxic effect in Hep-2 cell line and VERO cell line with LC₅₀ values of 64.5 µg and 250µg 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 4.292 min with 100 % purity. The compound 1 was found to be cytotoxic toward Hep-2 with IC₅₀ of 2.84 µg/ml and showed moderate invitro cytotoxic against VERO with IC₅₀ of 16 µg/ml.

Conclusion: This study clearly proves that the marine sediment derived actinomycetes with bioactive metabolites can be expected to provide high quality biological material for high throughout biochemical and anticancer therapy programs.

Keywords: *Streptomyces avidinii* strain SU4, HPLC, Cytotoxic activity.

INTRODUCTION

Marine microorganisms continue to provide pharmacologically important secondary metabolites which are unique and novel chemical compounds. Marine microbes are continuously explored for drug discovery. Apart from microbes all other marine sources have also provided valuable chemical diversity [1]. Marine actinomycetes have been traditionally a rich source for biologically active metabolites. Although heavily studied over the past three decades, actinomycetes continue to prove themselves as reliable sources of novel bioactive compounds. Among the well-characterized pharmaceutically relevant microorganisms, actinomycetes remain major sources of novel, therapeutically relevant natural products [2]. The majority of these compounds demonstrate one or more bioactivities many of them developed into drugs for treatment of wide range of diseases in human, veterinary and agriculture sectors [3]. Searching for novel actinomycetes constitutes an essential component in natural product-based drug discovery. Analytical methods continue to improve to allow the rapid elucidation of structures and make natural products valuable components of modern drug discovery.

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

As part of our ongoing research of microbial metabolite, we isolated a new strain of *Streptomyces* from marine sediment. 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 this new species.

MATERIALS AND METHODS

Sample collection and isolation of actinomycetes:

The soil samples collected from from Pulicat, Ennore, Muttukadu, and Veerampattinam were carefully transferred to the lab using sterile polythene bags. Actinomycetes were isolated using Starch

Casein Agar medium by following the method of [7]. 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.

Extraction of cell free crude extracts

A loopful of isolate was inoculated into 50ml of SS medium in 250ml of Erlenmeyer flasks containing the sea water 50 %, distilled water 50%, pH 7.5 and incubated for 2 days in rotary shaker incubator (200 rpm) at 28°C. These seed inoculums (10%) were transferred into 200 ml production medium in 1 L Erlenmeyer flask. The inoculated cultures in the production medium were incubated for 72 h on a rotary shaker (2000 rpm) at 28 C. after fermentation the broth was centrifuged at 4000 rpm for 10 min and the filtrate was separated and stored at -20°C for further use [8].

Extract preparation

The stock solution was performed by dissolving 20 mg of extracts in 1 ml of Dimethyl sulphoxide (DMSO) to get the concentration of 20mg/ml [9].

Screening of active strain:

Brine shrimp lethality assay

Dried cysts of *Artemia salina* were incubated in natural seawater (1g.1-1) at 28-30°C under constant aeration for 48hrs. After hatching, active naupli free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Different concentrations of stock solutions were prepared by dilution with dimethyl sulphoxide (DMSO) so as to obtain 31.25, 62.5, 125, 250, 500 and 1000 µg.ml⁻¹ of ten isolated actinomycete cell free extracts. Ten artemia naupli were added into each concentration of extract in 96 microtitration well plate. Control was maintained with 0.2% of DMSO [10] instead of extract. After 24 hrs, dead shrimp was counted using microscope. Larvae did not exhibit any internal or external movement during several seconds of observation was calculated as dead and the percentage of mortality was calculated and the data were transformed to the probit analysis for the determination of IC₅₀ of the crude extract.

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 VERO cell lines 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 VERO 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 5X10³ 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 (LC₅₀) were determined by calculating the % of viability:

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

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

$$R_f = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent}}$$

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) [10]. The morphological characters of the isolate PCL-1 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 of [11].

Molecular Sequencing

Genomic DNA was isolated from cells as described by [12]. The 16S rRNA gene of strain PCL-1 was amplified by polymerase chain reaction, using two universal bacterial primers, 1492R (5'-GGTTACCTTGTTAC GACTT-3') and Eubac27F (5'-AGAGTTTGATCTGGCTC AG-3') [13]. 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.

RESULTS AND DISCUSSION

Isolation of actinomycetes

By selective isolation procedure 52 isolates were selected by distinct colony morphology, and 10 random isolates were subjected to Brine shrimp lethality assay to screen the cytotoxic active isolate (**Fig:1**). Meyer [14] reported that, if the brine shrimp lethality assay displayed LC 50 < 1000 µg/ml of natural derived products was known to contain physiologically active principles. One of the promising isolate PCL-1 exhibited strong cytotoxic activity against brine shrimp was selected for further study.

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 PCL-1 exhibited LC50 in 64.5 concentration against Hep-2 tumor cell lines (**Fig 2**).

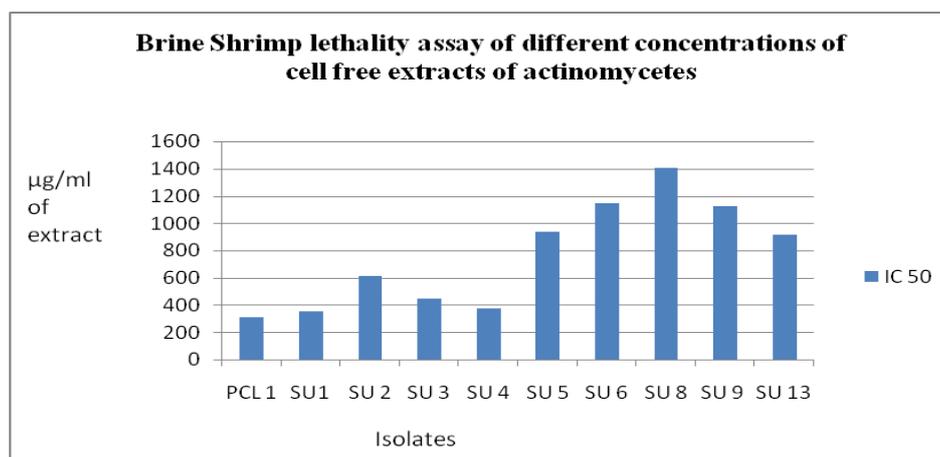


Fig. 1: Brine shrimp lethality assay for different concentrations of selected actinomycetes Methyl Thiazole Tetrazolium Cell Viability Test (MTT)

In order to evaluate the biocompatibility on normal cell lines (VERO), 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 PCL-1 exhibited IC₅₀ value against VERO cell lines

as 250µg/ml. As established by the American National Cancer Institute (NCI) is in IC₅₀ < 30 µg /ml, and for all standard antitumor agents the IC₅₀ value was less than 25 µg/ml Zheng [15].

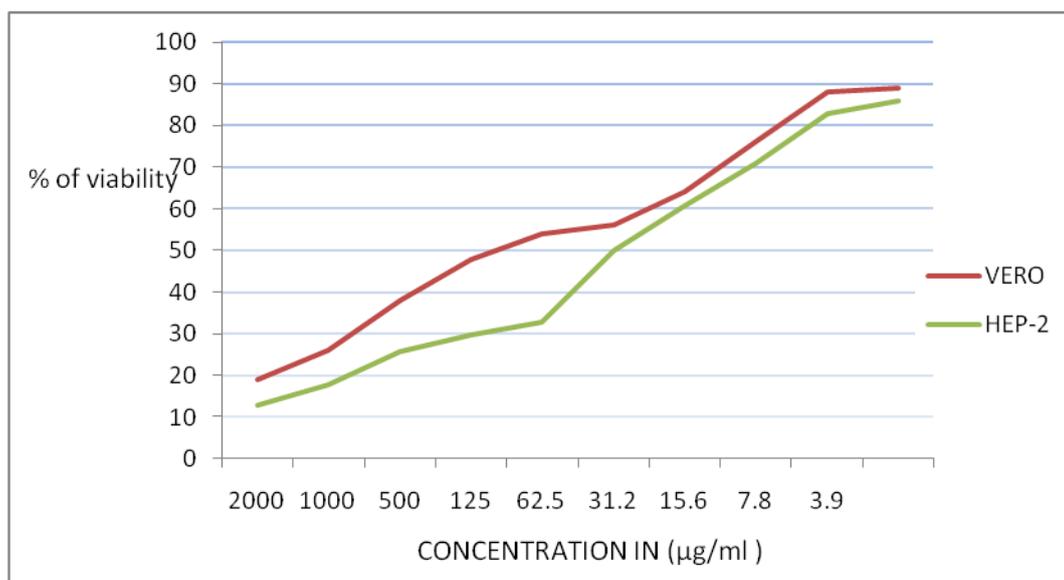


Fig. 2: MTT Cytotoxicity for the crude extract on Hep- 2 cell line and VERO cell lines

Purification of Crude Extract: Thin Layer Chromatography

The crude extract of the isolate PCL-1 was purified by the technique TLC. At first the compounds present in it were confirmed by performing precoated TLC and the Fig. 3 shows the presence of compounds through many fractions and its R_f values were calculated for different visualization- iodine, short UV and long UV as 0.108, 0.162, 0.297, 0.621, 0.729, 0.810, 0.947 and 0.135, 0.189, 0.270, 0.594, 0.75mean6, 0.864, 1.0 and 0.297, 0.513, 0.648, 0.891

respectively. By performing Diphenyl picryl hydrazyl (DPPH) method the mean R_f value was calculated as 0.605 and manual TLC was carried out to get the purified compound (Fig.4) and it was confirmed by Precoated TLC technique. The band was eluted with ethyl acetate and the presence of active compound was detected by using UV spectrophotometer at 200-800 nm (Fig. 5). The active compound have UV visible maximum absorption at 270-280 nm in methanol. It is reported that the most of peptide antibiotic exhibit maximum absorbance at 210-230 nm and 270-280 nm [16-18].

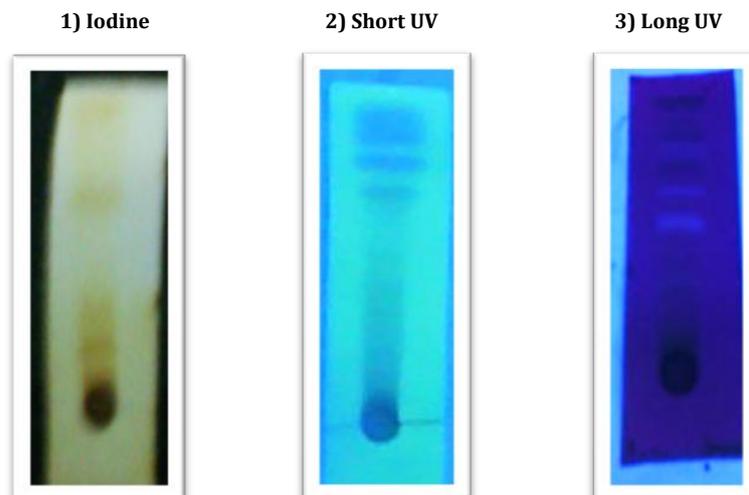


Fig. 3: Precoated TLC was viewed under short and long UV and it showing the bands of the crude extract.



Fig. 4: Manual TLC was viewed under long UV and it showing the band of the crude extract

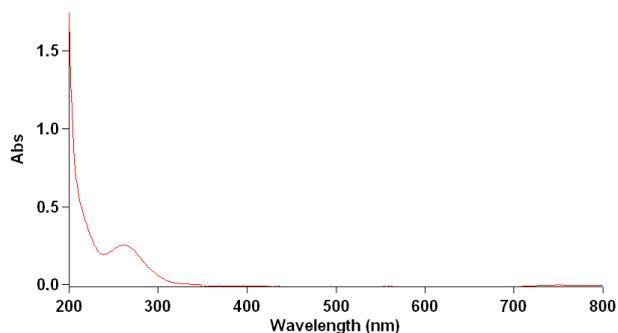


Fig. 5: UV visible spectrum of active compound of TLC fraction

Purification of the Compounds by High Performance Liquid Chromatography (HPLC)

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. 6). The active compound was obtained in sufficient quantity for spectral analysis and biological testing. The active compound was eluted at time 4.292 min with 100 % purity.

The purified HPLC fraction was tested against both human laryngeal carcinoma cell line and VERO cell line. The compound 1 was found to be cytotoxic toward Hep-2 with IC_{50} of 3.84 μ g/ml and showed moderate in vitro cytotoxicity against VERO with IC_{50} of 12 μ g/ml.

The potent antibiotic producing strain PCL-1 was characterized following the procedures recommended by International *Streptomyces* Project (ISP) (Table: 1). The strain grew well on all the media and produced grey aerial mycelium and light yellow substrate mycelium in all the medium used. 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:7). The isolate PCL-1 grow well at 30-40°C, pH 7-11 and shows a wide range of carbon compound utilization. The *Streptomyces* are already reported for its sorbital and sucrose utilization [19].

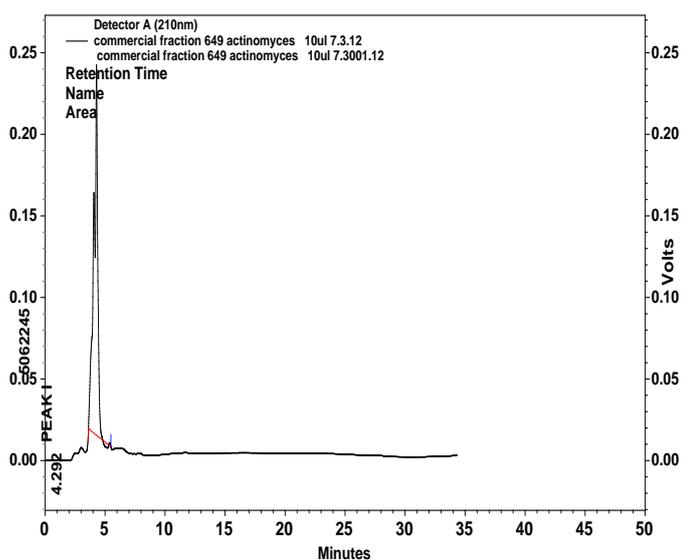


Fig. 6: HPLC Chromatogram of Fraction collected peaks PCL-1 sample

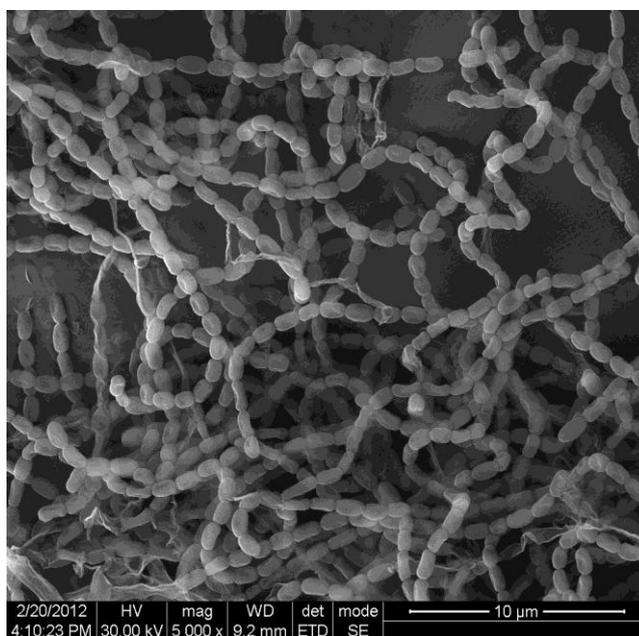


Fig. 7: Scanning electron micrograph of *Streptomyces avidinii* strain SU 4

Table 1: The cultural, biochemical and physiological characters of isolate PCL-1

Tests	Results
Grams stain	+
Diffusible pigmentation	Nil
Melanin production	Nil
Aerial mycelium	Grey
Substrate Mycelium	Light yellow
Motility	Nil
Colony color	Grey
Spores	Smooth, retinaculum apertum (RA)
Spore arrangement	Terminal
Carbon source (1% w/v) utilization	
Arabinose	+
Mannitol	+
Inositol	+
Fructose	+
Temperature for growth (o C)	
Range	10-50
Optimum	30-40
pH for growth	
Range	5-11
Optimum	7-11

A BLAST search of the 1450 bp 16S-rRNA gene sequence of the isolate showed 98% homology to *Streptomyces avidinii*. The percentage of similarity between the actinomycetes and database suggests it as novel strain. Thus, the novel strain was named as *Streptomyces avidinii* strain SU4 and made publically available in GenBank with an assigned accession number JF730120.

CONCLUSION

In our search for cytotoxic compound producer from marine soil, an Actinomycete PCL-1 was isolated, which was capable of producing cytotoxic compound. The strain was identified as *Streptomyces avidinii* strain SU4, 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 VERO 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 270-280 nm. Spectral studies are needed to identify the compound.

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REFERENCE

- Kelecom A Secondary metabolites from marine microorganisms. AnAcad. Bras. Cienc 2002; 74: 151-170. DOI: 10.1590/S0001-37652002000100012.
- Jensen PR, Fenical W: Marine Microorganisms and Drug Discovery: Current Status and Future Potential. In: Drugs from the Sea. N. Fusetani (Ed.): Karger, Basel, pp: 6-29, 2000.
- Bernan VS, Greenstein M, Maise WM Marine microorganisms as a source of new natural products. Adv. Applied Microbiol 1997; 43: 57-90. DOI: 10.1016/S0065-2164(08)70223-5.
- Newman DJ, Cragg GM SnaderKM Natural products as sources of new drugs over the period 1981-2002. J Nat Prod. 2003; 66: 1022-1037.
- Me'ndez C, Salas JA Altering the glycosylation pattern of bioactive compounds. Trends Biotechnol 2001; 19: 449-456.
- Argoudelis A, Baczyneky DL, Duo MT Laborde, A. L., Sebek, O.K., Truesdel, S.K. J. Antibio 1987; 40: 750-760.
- Ellaiah P, Kalyan D, Rao VS, Rao BV Isolation and characterization of bioactive actinomycetes from marine sediments. Hindustan Antibiotics Bulletin 1996; 38: 48-52.
- Karthick L, Gaurav Kumar KV, Bhaskara rao Diversity of marine actinomycetes from nicobar marine sediments and its antifungal activity. Int J Pharm Pharm sci. 2010;2 (1): pp 100-201.
- Solis PNC, Wright W, Anderson MM, Gupta MP, Phillison JD A microwell cytotoxicity assay using artemia salina. Planta Medica 1993; 59:50-252.
- Shirling EB, Gottlieb D Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol 1966; 16: 313-340.
- Vengadesh prabhu K, Sundaramoorthi C, Gupta saurabh, Karthick K, Tamilselvi N. International Research journal of Pharmacy 2011; 2 (4): 114-118.
- Hopwood DA, Bibb MJ, Chater KF, Kieser T, Bruton CJ, Kieser HM: Genetic manipulation of Streptomyces: a laboratory manual. The John Innes Foundation: Norwich (UK), 1985.
- Jiang HL, Tay JH, Tay STL Bacterial diversity and function of aerobic granules as a microbial response to high phenol loading. Appl Environ Microbiol 2004; 70: 6767-6775.
- Meyer B N, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols, McLaughlin JL Brine shrimp: A convenient general bioassay for active plant constituents. J Med Plant Res 1982; 45:31-34.
- Zheng Z, Huang W, Yang Y, Li Z, Cai J, Su H Detection of antitumor and antimicrobial activities in marine organism associated actinomycetes isolated from the Taiwan Strait, China. Microbiol Lett 2000; 188:87-91
- Motta A, Brandelli D Characterization of an antimicrobial peptide produced by *Brevibacterium linens*. J Applied Microbiol 2006;52(6):357-63.
- Besson F, Peypoux F, Michel G, Delcambe L Identification of antibiotics of Iturin group in various strains of Bacillus subtilis. J Antibiot 1978;31: 284-288.
- Kurusu K, Ohba K New peptide antibiotics LI-F03 and characterization. J Antibiot 1987;40 (11): 1506-1514.
- Swapnil S Desai, Atul A Phatak Study of growth pattern of Actinomycetes species in various nutrient medium. Int J Pharm Pharm sci 2010; 2 (4): pp 43-45.