

Original Research Article

<http://dx.doi.org/10.20546/ijcmas.2016.507.049>

Purification of Actinomycin D from *Streptomyces parvulus* Isolated from Mangrove Ecosystem of Kerala, India

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ABSTRACT

Keywords

Streptomyces,
actinomycin D,
HPLC,
cytotoxicity,
mass spectrum.

Article Info

Accepted:
15 June 2016
Available Online:
10 July 2016

The present study was conducted to purify and characterize the active compound produced from *Streptomyces parvulus* for evaluating its cytotoxic potential against tumor cell line. The *Streptomyces parvulus* CBJ1, isolated from mangrove ecosystem of Poovar found to be an efficient producer of bioactive compound. The isolate produced yellow color in ISP2 production media during submerged fermentation. The orange color compound was obtained from mycelia extract using thin layer chromatography and purity was checked using reverse phase HPLC. The purified compound was identified as actinomycin D on the basis of ultraviolet-visible spectroscopy, mass spectroscopy and by comparison with published reference compounds. The antitumor activity of purified compound, actinomycin D analyzed on A549 cell line by MTT assay. The actinomycin D showed cytotoxicity on A549 cell line depending on the dose and incubation time. It showed 77% cytotoxicity at concentration of compound 1.25 µg/ml with IC₅₀ value of 0.52 µg/ml.

Introduction

Approximately half of the devastating effects in humankind are due to infectious disease (Walsh and Warren, 1974). Cancer still remains one of the most serious health problem in both developing and developed countries. Therapeutic methods for cancer treatment has enabled us to focus on novel bioactive secondary metabolites from natural source for effective drug development. Actinomycetes are prokaryote with the highest recorded chemical diversity produce many secondary metabolites of novel chemical structures and versatile bioactivities (Watve, 200; Aroonsri *et al.*, 2012). Among the actinomycetes genus

50% of the soil actinomycetes are constituted by *Streptomyces*, provide 70-80% of secondary metabolites. *Streptomyces* have been reported to produce several bioactive compounds that include antibiotics, antioxidants, immunosuppressive agents and cytotoxic compounds (Newman and Cragg, 2007).

Mangrove ecosystem is called home land of microb *et al.*, due to the presence of rich source of nutrients. This ecosystem situated between the terrestrial and marine environment which are highly productive ecosystem and abode to large unexplored

microbial diversity (Ghosh *et al.*, 2010). The actinomycetes diversity from mangrove ecosystems have been proven as excellent source for discovering secondary metabolites which have application medical field. The present study was designed to purify and characterize the compound produced from *Streptomyces parvulus* CBJ1 for investigating the cytotoxic potency against tumor cell line.

Materials and Methods

Isolation

During screening of bioactive actinomycetes, *Streptomyces parvulus* CBJ1 was isolated from the mangrove soil of Poovar (8.3177⁰ N, 77.0708⁰ E) located in Thiruvananthapuram district in Kerala, South India. The isolate was identified by morphological, phenotypic and phylogenetic data. The sequence is available in GenBank database with an accession number KT833783. The culture of isolate was grown and maintained on Yeast extract Malt extract agar (ISP2) plates (composition: D-glucose 4gm/L malt extract 10 gm/L, yeast extract 4gm/L and agar 20gm/L) and stored at 4⁰C for further use.

Fermentation and extraction of active compound

Spores of the strain CBJ1 was inoculated in 100 ml Erlenmeyer flask containing 25ml of ISP2 broth followed by incubation at 30⁰C on a rotary Shaker at 120rpm (New Brunswick) for 2 days and pH was adjusted to 7.5. Subsequently, 5% of inoculum was transferred in to an 100ml ISP2 production media in a 250ml Erlenmeyer flask growing under the same above conditions for 5 days. After the incubation period the fermented broth was centrifuged at 8000 rpm for 10 minute at 4⁰C. Finally mycelium was

extracted with methanol (HPLC grade, Merck) two times for 15 minutes for recovering the crude active metabolites in to methanol phase. The extracts were pooled and residual methanol was evaporated using rotary vacuum evaporator (Heidolph, Germany) at 50⁰C at 100rpm and vacuum 334mbar. The crude extract was weighed and stored at 4⁰C for further analysis.

Purification of active compound

The extract was spotted on silica gel thin layer chromatography (TLC) plates, eluted using hexane:ethylacetate (6:4v/v) as mobile phase and retention factor of active compound was measured. The purity of active compound was checked using reverse phase HPLC (Shimadzu, USA) equipped with C18 column with 55% acetonitrile in water as the eluting solvent both run isocratically at a flow rate of 1ml/min for 45minutes and retention time noted and compared with reference compound.

Characterization of purified compound

Characterization of pure active compound was carried out using different spectrometric analysis. The ultraviolet-visible spectroscopy of active compound was taken using spectrophotometer UV- 1700 (Shimadzu, Japan). The mass spectra was recorded using JEOL JMS 600 H mass spectrometer (JOEL, USA).

Cytotoxicity assay

The cytotoxic activity of pure compound against human tumor cell line A549(lung carcinoma) was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay (Wilson, 2000). Briefly 1×10⁴ cells per well were seeded at log phase in 96 well plates. After cultivation for 24hrs the media was replaced with fresh

media containing different concentration (0.01-1.25µg/ml) compounds dissolved in 0.1% DMSO and treated for 24, 48 and 72 hr. After incubation at 37°C in 5% CO₂, 100µl of medium containing 20µl of MTT solution (5mg/ml in PBS) was added to each well and cells were incubated for 4hrs at 37°C. At the end incubation, medium was removed and 100µl of DMSO was added to each well for formazan crystals to dissolve. The absorbance was measured at 570nm using Mullikan GO Micro plate Spectrophotometer (Thermoscientific). The growth inhibition was calculated from the formulae $(1-A/B) \times 100\%$ where A and B corresponds to the mean absorbance of treated and control wells. The concentration of the compound that gives 50% inhibition was expressed as the IC₅₀ (ug/ml).

Results and Discussion

Extraction and purification of compound

The submerged fermentation of *Streptomyces parvulus* CBJ1 was carried out under optimum condition of 30°C and 120rpm for 5 days in a production media. The culture both was harvested on fifth day for further extraction and purification. The mycelium was separated and extracted with methanol. Organic solvents always provide a higher efficiency in extracting bioactive compounds compared to water based compounds (Masuda *et al.*, 1997). Gyimesi *et al.*, (1970) and Kumar *et al.*, (2014) have reported that methanol was used for the extraction of antimicrobial metabolites from mycelia of *Streptomyces* sp.

The crude extract obtained was separated by TLC using solvent system

hexane:ethylceteate in the ratio 6:4 indicated the presence one distinct orange colored compound with an R_f value of 0.38cm (Fig.1). The separated compound had UV-visible spectra at 254nm and 442nm in methanol similar to that of actinomycin class of compounds. The purity of yellow compound was confirmed using reverse HPLC analysis, indicating that the separated compound showed a chromatogram at 441nm with a retention time of 37.656 minute similar to the pure actinomycin D purchased (Merck) (Fig.2). The results of UV-visible spectra and reverse HPLC analysis of purified compound indicates the fact that the compound have absorption maxima similar to amino phenoxazinone chromophore present in actinomycin family (Mauger and Stuart, 1990). There are examples of phenoxazinone metabolites like chandrananimycin D from *Streptomyces griseus* and venezueline C from *Streptomyces venezuelae* having anticancer activity (Gomes *et al.*, 2010; Ren *et al.*, 2013).

Mass spectrum of the pure compound showed the molecular ion peak at m/z of 1255 (M+H)⁺. The characterization data support the fact that the compound belongs to actinomycin D (Fig 3). Mass spectral data were compatible with actinomycin D, as recorded by Lackner *et al.*, (2000). Around 19 species of *Streptomyces* (Kurosawa *et al.*, 2006; Praveen, 2008), *Micromonospora* (Wagman, 1976) and *Nocardia* (El-Refai, 2011) reported are capable of producing various forms of actinomycins.

Fig 1. TLC Analysis of purified compound

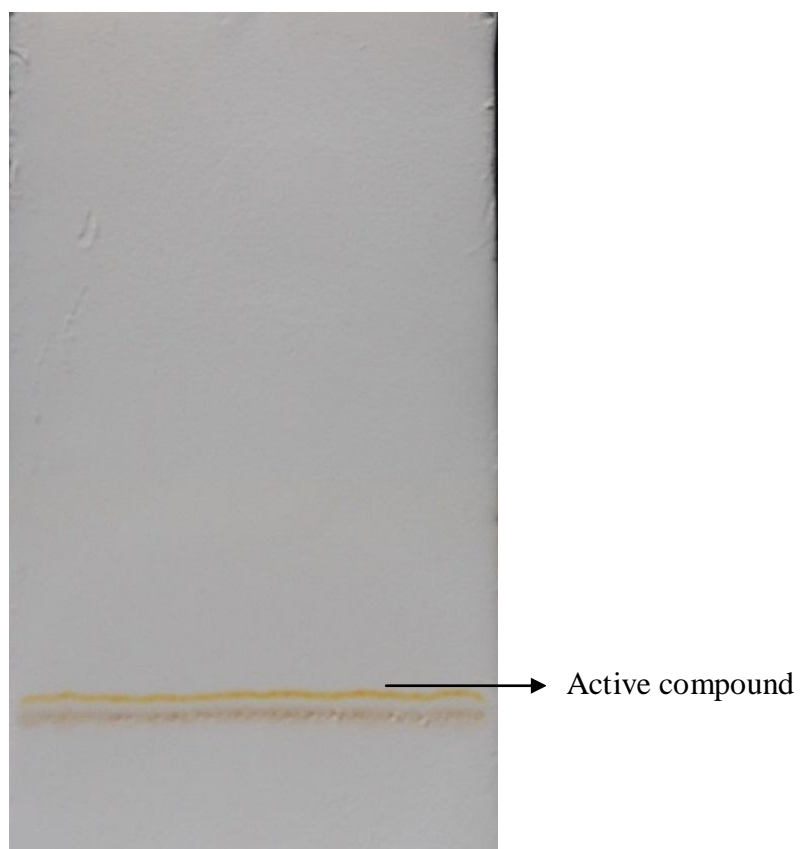


Fig. 2 HPLC analysis of purified compound

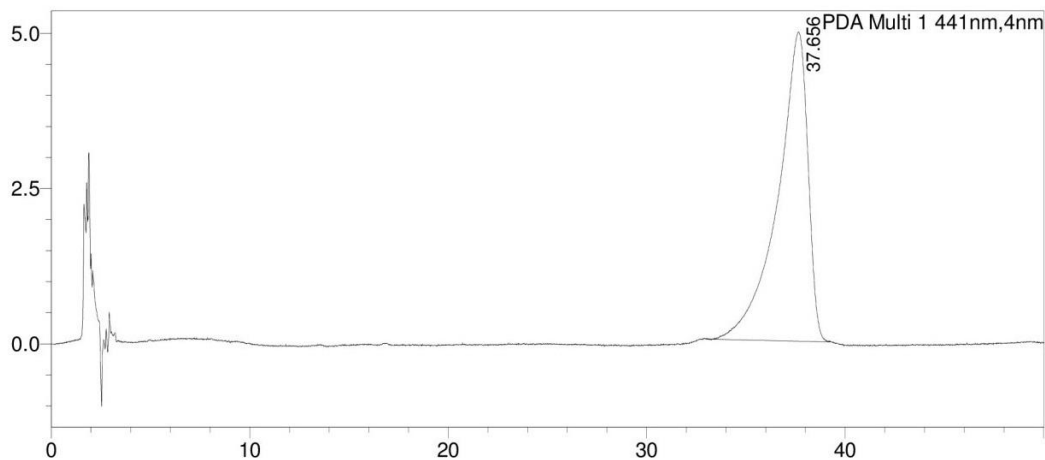


Fig.3 Mass spectrum of purified compound

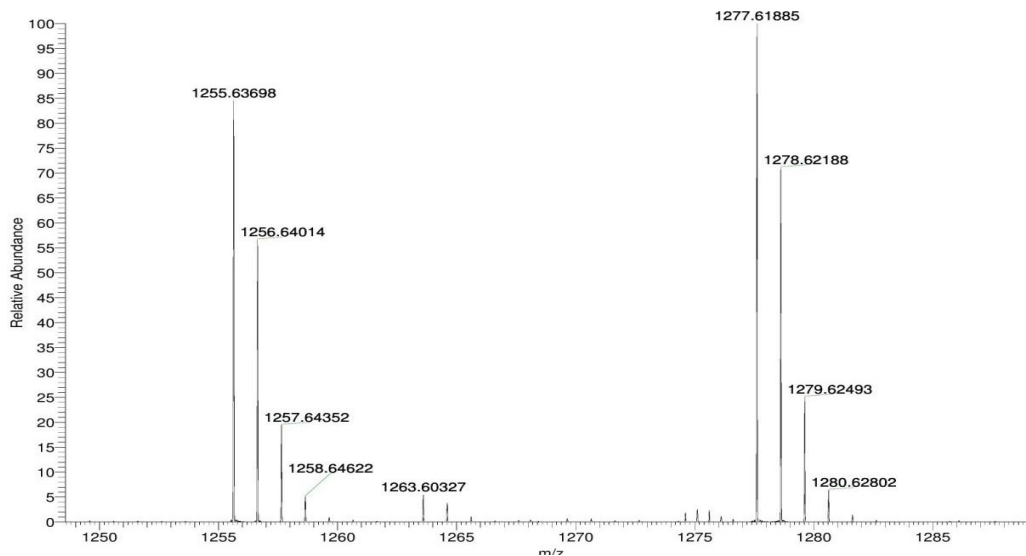
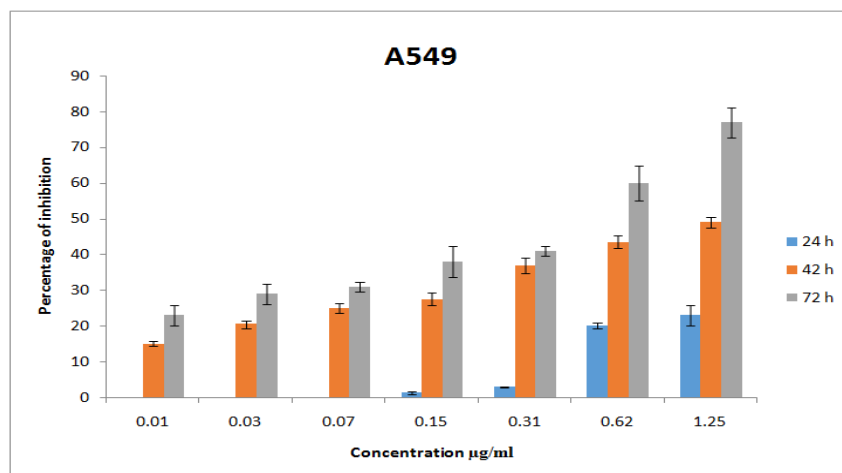


Fig.4 A549 cells treated with actinomycin D



Cytotoxicity assay

To determine the cytotoxic activity of purified actinomycin D to tumor cell line, the cells were treated with increasing concentration of actinomycin D. The MTT cytotoxicity assay revealed that compound decreased the cell viability significantly ($P < 0.05$) in a concentration dependent manner. As shown Fig.4 Actinomycin showed cytotoxic activity against A549 cells. It showed an IC₅₀ of 0.52 µg/ml after 72 hrs of incubation with a inhibition rate of 77% at 2.5 µg/ml. The results agree with the previous reports that the actinomycin D inhibited the proliferation of pancreatic cancer cells by inducing apoptosis (Kleeff *et al.*, 2000). Kim *et al.*, (2016) recently reported that manumycin A isolated from *Streptomyces parvulus* have chemotherapeutic effect on malignant pleural mesothelioma.

In conclusion, the compound isolated from *Streptomyces parvulus* identified as actinomycin D inhibited the proliferation of tumor cells. The application of actinomycin D in cancer treatment enhanced the requirement for the production of this compound in large scale in future for marketing in cost effective manner.

Acknowledgement

We are thankful to the University of Kerala for the research fellowship and infrastructure provided by Department of Biotechnology, University of Kerala is also acknowledged.

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How to cite this article:

Cibi, R., and Jayakumaran Nair, A. 2016. Purification of Actinomycin D from *Streptomyces parvulus* Isolated from Mangrove Ecosystem of Kerala, India. *Int.J.Curr.Microbiol.App.Sci*. 5(7): 461-467. doi: <http://dx.doi.org/10.20546/ijcmas.2016.507.049>