Actinomycetes From Marine Sediment: Screening For Cytotoxicity, Identification and Analysis Of Bioactive Constituents By Gas Chromatography - Mass Spectrometry

S. SudhaSrikesavan¹, M. Masilamani Selvam²

Abstract— Recent research has increased our knowledge and understanding of the cytotoxic effect of marine derived Actinomycetes. In the present study, we had taken an initiative to isolate culturable halophilic actinomycetes and to screen the cytotoxic potential by brine shrimp lethality assay. The cytotoxicity was evaluated on Hep-2 and VERO cells by MTT assay. The isolates PCL-1, SU-2, SU-4, SU13 showed IC₅₀ value on Hep-2 cell line of 64.5, 32.1, 125, 16.5 μg mL⁻¹ and on VERO cell lines the IC₅₀ value of 94.6, 64.5, 250, 250 by MTT assay. The taxonomy of the active strains PCL-1, SU-2, SU-4, SU13 were evaluated by phenotypic and phylogenetic analysis. The 16S rRNA was sequenced and phylogenetic relationship with the closest related species were studied. Based on taxonomy the active isolates PCL-1, SU-2, SU-4, and SU13 were identified as Streptomyces avidinii, Streptomyces cacaoi, Streptomyces coelicolor, and Streptomyces cavoensis respectively. The 16S rRNA sequence of the isolate PCL-1, SU-2, SU-4, SU13 showed 98%,98%,98%,97% similarity with Streptomyces avidinii, Streptomyces cacaoi, Streptomyces coelicolor, Streptomyces cavoensis respectively. Based on the phylogenetic and phenotypic evaluation the isolate was designated as Streptomyces avidinii strain SU4, Streptomyces cacaoi strain SU2, Streptomyces coelicolor strain SU6, Streptomyces cavoensis strain SU3. These strains were made publically available through GENEBANK with the accession number JF730120, JF730119, JQ828940, JF730121 respectively. The ethyl acetate extract of the active strains were subjected to GC-MS analysis. The presence of some of the constituents in the actinomycetes extract provides the scientific evidences for eliminate tumor cells on bone marrow, purging agent in autologous bone marrow transplantation, cytotoxic activity, stimulates adipogenesis and glyceroneogenesis, affects the differentiation of Human Liposarcoma.

Keywords— Actinobacteria, Brine shrimp lethality, Cytotoxic activity GC-MS.

I. INTRODUCTION

ACTINOBACTERIA represents one of the largest taxonomic units among the 18 major lineages currently recognised within the domain Bacteria, including five subclasses and 14 suborders [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]. The isolated compounds from marine actinomycetes has a broad spectrum of biological activities such as antibiotic, antifungal, toxic, cytotoxic, neurotoxic, antimitic, antiviral and antineoplastic activities [4]. Recently, new targets have been added to the general screening like AIDS, immunosuppression, anti-inflammation, Alzheimer disease, ageing processes, some tropical diseases and resulted in discovery of several drugs [5].

Discovering novel anticancer agents both synthetically and naturally have become increasingly important as many cancers do not respond to current chemotherapy [6]. These techniques are individually useful in particular situations and when combined, they offer a more efficient treatment for tumors. Antitumor antibiotics produced by actinomycetes are among the most important cancer chemotherapeutic agents including members of the anthracycline, bleomycin, actinomycin, mitomycin and aureolic acid families. Several methods have been developed to identify Streptomyces species. 16S RNA sequence data have proven invaluable in streptomyces systematics, in which they have been used to identify several newly isolated streptomyces [7].

The aim of the study was to describe coastal inhabiting actinomycete exhibits cytotoxicity and identification of active strains by both traditional and advanced techniques. The active compounds responsible for cytotoxicity were analyzed by GC-MS analysis.

II. METHODOLOGY

A. Isolation of actinomycetes

Actinomycetes were isolated from marine soil samples collected from Pulicat, Ennore, Muttukadu, Veerampattinam...
and Parangipettai, Tamil Nadu, South India followed by the procedure described by [8] using starch casein agar medium with 50% seawater.

B. Extraction of cell free crude extracts

A loopful of ten actinomycetes strains was inoculated separately into 150ml of ISP2 medium (4.0g.1^{-1} of glucose, 10.0 g.1^{-1} of malt extract and 4.0 g.1^{-1} of yeast extract, pH 7.2±2°C made up with 50% sea water) for 10 days under continuous shaking (100 rpm). After that, cell free broth was adjusted to pH 5.0 with 1N 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.

C. 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 nauplii 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 nauplii were added into each concentration of extract in 96 microtitration well plate. Control was maintained with 0.2% of DMSO [9] 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 IC_{50} of the crude extract.

D. Cytotoxicity assay

The human laryngeal cancer cell line (Hep-2) and VERO cell line were obtained from Verinary College, 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 VERO 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 100μl of serum free medium containing various concentrations (3.87, 7.75, 15.5, 31.25, 62.5, 125, 250, 500, 1000 and 2000 μg/ml) of actinomycete extracts at 24 hrs and 48 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 microtiter 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. IC_{50} values were determined by calculating the % of viability:

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

E. Identification of Actinomycetes

The isolates were identified by 16s rDNA molecular sequencing.

F. Molecular sequencing

Genomic DNA was isolated from cells as described by [10]. The 16S rRNA gene of strain SU 5 was amplified by polymerase chain reaction, using two universal bacterial primers, 1492R (5'-GGTTACCTTGTAC GACTT-3') and Eubac27F (5'-AGAGTTTGATCCTGCGT CAG-3'); [11]. 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.

G. GC - MS analysis:

Preparation of extract: 2 μl of the ethyl acetate extract of selected isolate was employed for GC/MS analysis. The compound was characterized by using GC-MS [SHIMADZU QP2010] instrument at GC column oven Temperature 70°C, Injector Temperature 200°C at split mode ratio 40 with a flow rate of 1.51 ml/min. The MS with Ion source temp 200 °C, Interface temp: 240°C, Scan range: 40 – 1000 m/z, Event time: 0.5 sec, Solvent cut time: 5mins, MS start time : 5 (min), MS end time : 35 (min), Ionization : EI (-70ev).

Identification of components: Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

III. RESULTS AND DISCUSSION

In the course of screening cytotoxic actinomycetes, 52 isolates were obtained from the coastal soil samples of Pulicat, Ennore, Muttkadu, Veerampattinam and Parangipettai, Tamil Nadu, South India. In the present investigation, among the 52 isolates the 10 ten random isolates were used for Artemia lethality assay to screen for cytotoxic activity. Brine shrimp lethality test was conducted on each of the extracts at six different concentrations 31.25, 62.5, 125, 250, 500 and 1000 μg/ml. [12] 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.

In the present study among the ten isolates the four isolates PCL-1, SU1, SU4, and SU13 showed relatively high levels of toxicity with the IC 50 in < 500 μg/ml and the isolates SU2, SU3 and SU 5 showed IC 50 in > 500 μg/ml.

Sundaram et al., [13] reported that actinomycetes extracts exhibited the IC50 values in less than 1000 μg/ml. The extracts of the actinomycete isolates PCL-1, SU1, SU4, and SU13 shows IC 50 in < 500 μg/ml. The isolates SU2, SU3
and SU5 showed IC 50 in > 500 µg/ml and the isolates SU 6, SU 8, and SU 9 showed the IC 50 in > 1000 µg/ml (Fig: 1).

Among the seven biologically active isolates the DNA of only four isolates was isolated and the ITS region of 16s rRNA was amplified by polymerase chain reaction, using two universal bacterial primers, 1492R (5'-GGTTACCCTGTTAC GACTT-3') and Eubac27F (5'-AGAGTTTGATCCTGAGGCTC AG-3'). 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 nucleotide sequences retrieved from GenBank/EMBL/DDBJ database by using CLUSTAL W version 1.81 programs. Evolutionary tree was constructed using the neighbor-joining method [14] in the MEGA version 2.1 program [15]. The isolates PCL-1, SU-2, SU-4, SU-13 were found as the divergence of the species Streptomyces avidini, Streptomyces cacaoi strain SU2, Streptomyces coelicolor strain SU6, Streptomyces cacoensis strain SU3 and made publically available through GENEBANK with the accession number JF730120, JF730119, JQ828940, JF730121 respectively.

The MTT assay for the crude extracts of the isolates PCL-1, SU-2, SU-4, SU13 showed IC50 value on Hep-2 cell line of 64.5, 32.1, 125.6, 16.5 µg mL−1 and on VERO cell lines the IC50 value of 94.6, 64.5, 250.250. These values are very close to the criteria of cytotoxicity activity for the crude extracts, as established by the American National Cancer Institute (NCI) in IC50 < 30 µg /ml. (Fig: 2).

On GC-MS analysis of the crude extract the major constituent of isolate PCL-1 was found to be 1,2-Benzenedicarboxylic acid, bis(2-methyl propyl) esters (12.7%) and Isooctyl phthalate (15.29%) with the retention time 15.642, 21.606. The GC-MS spectrum of isolate SU 4 shows Diisobutyl phthalate (31.84%), Monoethylhexyl phthalate (20.33%) at 15.635, 21.606 retention time. The crude extract of isolate SU 13 showed Diisobutyl phthalate (13.26), 1,2-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester. (14.13%) at retention time 15.643, 21.614. (Table: I)

The major component in all the isolate was phthalate [16] reported Dibutyl phthalate, diisobutyl phthalate were active against partially four selected immortal cell lines. In a similar study the natural occurrence of 1, 2-Benzenedicarboxylic acid bis(2-ethylhexyl) phthalate has been isolated from a marine alga, Sargassum weightii, and apart from its plasticizing ability it was also found to have antibacterial effect on a

---

**TABLE I: ACTIVITY OF IDENTIFIED CHEMICAL CONSTITUENTS IN THE ETHYL ACETATE EXTRACT OF THE IDENTIFIED ACTINOMYCETES ISOLATES BY GC-MS ANALYSIS**

<table>
<thead>
<tr>
<th>ISOLATES</th>
<th>RETENTION TIME</th>
<th>NAME OF THE COMPOUND</th>
<th>ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL-1</td>
<td>15.642</td>
<td>1,2-Benzenedicarboxylic acid, bis(2-methyl propyl) esters (12.7%)</td>
<td>Antibacterial activity</td>
</tr>
<tr>
<td></td>
<td>21.612</td>
<td>Isooctyl phthalate (15.29%)</td>
<td>Inhibiting Melanogenesis</td>
</tr>
<tr>
<td>SU-2</td>
<td>15.635</td>
<td>Diisobutyl phthalate (31.84%)</td>
<td>Eliminate tumor cells on bone marrow, purging agent in autologous bone marrow transplantation, Cytotoxic activity.</td>
</tr>
<tr>
<td></td>
<td>21.606</td>
<td>Monoethylhexyl phthalate (20.33%)</td>
<td>Stimulates Adipogenesis and Affects the Differentiation of Human Liposarcoma</td>
</tr>
<tr>
<td>SU-4</td>
<td>15.635</td>
<td>Diisobutyl phthalate (31.84%)</td>
<td>Eliminate tumor cells on bone marrow, purging agent in autologous bone marrow transplantation, Cytotoxic activity.</td>
</tr>
<tr>
<td></td>
<td>21.606</td>
<td>Monoethylhexyl phthalate (20.33%)</td>
<td>Eliminate tumor cells on bone marrow, purging agent in autologous bone marrow transplantation, Cytotoxic activity.</td>
</tr>
<tr>
<td>SU-13</td>
<td>15.643</td>
<td>Diisobutyl phthalate (13.26)</td>
<td>Eliminate tumor cells on bone marrow,</td>
</tr>
<tr>
<td></td>
<td>21.614</td>
<td>1,2-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester. (14.13%)</td>
<td>Antibacterial activity</td>
</tr>
</tbody>
</table>

---

**Fig: 1. Brine Shrimp Lethality Assay of Different Concentrations of Cell Free Extracts of Costal Inhabitant Actinomycetes**

**Fig: 2. IC 50 For The Crude Extract On Hep-2 Cell Line And VERO Cell Lines**
number of bacteria [17]. Bis (ethyl hexyl) phthalate reported from Streptomyces bangladeshiensis show anti microbial activity against gram positive bacteria and some pathogenic fungi [18]. Adipogenesis and glycereoneogenesis activity of Monoethylhexyl Phthalate in human adipocytes was reported by [19]. [20] stated that mono-(2-ethylhexyl) phthalate affects the differentiation of human liposarcoma cells (sw 872). Diisooctyl phthalate isolated from Nigella glandulifera Freyn. was identified as inhibiting melanogenesis [21].

IV. CONCLUSION

Based on the results of our investigation, the isolated strains PCL-1, SU-2, SU4 and SU 13 were identified as Streptomyces avidinii strain SU4, Streptomyces cacaoi strain SU2, Streptomyces coelicolor strain SU6, Streptomyces cavernosus strain SU3, which were produces an extracellular bioactive metabolite, which inhibits the proliferation of Hep-2 cells. On GC-MS analysis, the presence of some of the constituents in the actinomycetes extract provides the scientific evidences for eliminate tumor cells on bone marrow, purging agent in autologous bone marrow transplantation, cytotoxic activity, stimulates adipogenesis and glycereoneogenesis, affects the differentiation of Human Liposarcoma. Further studies on purification and characterization of the pure compound from the strain were ongoing. The results of this study suggested that the marine actinomycetes from the unexplored Indian coast could provide lead compounds of therapeutic value.

ACKNOWLEDGMENT

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

REFERENCES