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RESEARCH ARTICLE

Isolation and Characterization of Marine Actinomycetes from West Coast of India for Its Antioxidant Activity and Cytotoxicity

P. Chidambara Rajan*[1], A. Mahalakshmi Priya[2], D. Jayapradha[1], S. Saranya Devi[3]

[1]*Dept. of Biotechnology, Dr. N.G.P. Arts and Science College, Kalapatti Road, Coimbatore 641 048, Tamilnadu*
[2]*Head and Assistant Professor, Department of Botany, Government College for women's, Salem, Tamilnadu*
[3]*Dept. of Biotechnology, Sengunthar Arts and Science College, Tiruchencode, Tamilnadu*

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ABSTRACT

The actinomycete isolates were isolated form marine environment and the secondary metabolites from this isolates were tested for cytotoxicity and antioxidant properties. A combination of physiological parameters, chemotaxonomic characteristics, distinguished 16S rRNA gene sequences and phylogenetic analysis based on 16s rRNA gene provided strong evidence for the actinomycetes and their novel isolates form marine environment. Three isolates were selected to determine antioxidant and cytotoxicity. Antioxidant activity for different concentrations of crude extract was tested by 1, 1-Diphenyl-pircrylhydrazyl (DPPH) free radical scavenging assay. Isolate 2 exhibited the scavenging activity at Ic_{50} 21.50µg/ml. To determine *in vitro* cytotoxicity, various concentrations of extract was tested on HeLa cancer cell line by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Secondary metabolites showed a significant and proliferative activity and a dose dependent effect observed. The *in vitro* cytotoxicity assay on human cervical cancer cell line (HeLa) revealed that the secondary metabolite had the strongest cytotoxicity with Ic_{50} 21.50µg/ml.

Keywords: DPPH, MTT, Streptomycetes, Antioxidant, 16S rRNA, Cytotoxicity, Actinomycetes.

INTRODUCTION

Cancer is a heterogeneous disease and can be characterized as the growth of a malignant cell population that eventually leads to the interference of normal physiological functions. Anticancer drug research focuses on inhibition of tumor cell growth and induction of apoptosis in the malignant cell population. Apoptosis or programmed cellular death [13], is characterized by the ability of a cell to undergo a step-by-step self suicide program without affecting neighbouring or adjoining cells. Actinomycetes, characterized by a complex life cycle, are filamentous Gram-positive bacteria belonging to the phylum *Actinobateria* that represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain bacteria [24]. Around 23,000 bioactive secondary metabolites produced by microorganisms have been reported and over 10,000 of these compounds are produced by Actinomycetes, representing 45% of all bioactive microbial metabolites discovered. Among Actinomycetes, around 7,600 compounds are produced by *Streptomyces* species. Many of these secondary metabolites are potent antibiotics, which has made streptomycetes the primary antibiotic producing organism exploited by the pharmaceutical industry. Members of this group are producers in addition, clinically useful antitumor drugs such as Anthracyclines (Aclarubicin, Daunomycin and Doxorubicin), Peptides (Bleomycin and Actinomycin D), Aurelic acids (Mithramycin), Enediynes (Neocarzinstatin), Antimetabolites (Pentostatin), Carzinophilin, Mitomycins and others. The searches for novel drugs are still a priority goal for cancer therapy, due to the rapid development of resistance to multiple chemotherapeutic drugs. In addition, the high toxicity usually associated with cancer chemotherapy drugs and their undesirable side effects increase the demand for novel antitumor drugs active against untreatable tumours, with fewer side effects and with greater therapeutic efficiency. The exploitations of marine actinomycetes as a source for novel secondary metabolites production is in infancy. Recent investigation using enrichment techniques, new

***Corresponding Author:** P. Chidambara Rajan**, Email:** prchida@gmail.com, **Phone No:** +91- 97892 90383

selected methods and media have led to the isolation of novel actinomycetes from sediment samples. Marine actinomycetes represent an under exploited sources for the discovery of novel secondary metabolites and their potential should not be overlooked [15]. There are very few reports on antimicrobial activity from marine actinomycetes from west coast of India [4, 16]. Some novel active compounds from marine actinomycetes are reported at a high frequency [17, 12] and some of them show strong antitumor activity, which suggests marine actinomycetes are promising source of antitumor drugs [9]. Some antitumor drugs from marine actinomycetes, such as thiocoraline [3], Salinosporamide A [6, 21] and ECO-4601 [5] are under clinical trials. Therefore, as a new source of anti tumor drug candidates, marine actinomycetes have attracted serious attention in the last decade [11, 8].

MATERIALS AND METHODS

Source of Actinomycetes

The actinomycetes strain was isolated from the marine soils from Kerala, India. Starch casein agar was used for the isolation of actinomycetes.

Identification of Actinobacteria

The colony morphology of actinomycete was noted with respect to colour, mycelium and pigmentation. Spore chain morphology was identified by cover slip culture technique. Actinomycete isolates were biochemically characterized. 16S rRNA sequencing was done for phenotypic identification.

Production of Secondary metabolites

Isolates were inoculated in 250ml Erlenmeyer flask containing 100ml of ISP 1 broth (Tryptone Yeast Extract broth)containing (g/L) Tryptone 5, Yeast Extract 3, pH 7.0 to 7.2 before autoclave, incubate at 28°C in shaker (200-250rpm) for 5 days. These inoculums were transferred to 500ml medium and incubated for 10-12days at 28˚C in shaker (200-250rpm). 13th day the broth was centrifuged at 6000rpm for 15 minutes and cell free supernatant was separated.

Extraction of metabolites

The supernatant was extracted with ethyl acetate. Ethyl acetate was added to filtrate at 1:1 ratio (v/v) and shaken vigorously for an hour to complete extraction. The ethyl acetate phase that contain antibiotic was separated from aqueous phase, it was then evaporated to dryness in water bath at 80°C -90˚C and the residue obtained was weighed.

Antioxidant activity by DPPH (1, 1-diphenyl-picrylhydrazyl) radical scavenging assay

The extracts were dissolved with ethyl acetate to prepare various sample solutions at 200,100 and 50µl. Each extract solution (2ml) was mixed with 1ml of ethyl acetate containing DPPH, with final concentration of 0.2mM DPPH. Mixed well and incubate in dark at 37°C for 30 minutes. The absorbance was measured at 517nm. The absorbance of the control was obtained by replacing the crude sample with ethyl acetate. Quercetin was used as standard reference. Incubation of free radical by DPPH in percentage (I %) was calculated using the following equation [14, 20].

$$I\%\ \textit{Cell Inhibition} = \frac{\text{A Blank} - \text{A Sample}}{\text{A Blank}} \text{ X 100}$$

Where A_{Blank} was the absorbance of control reaction and A_{Sample} is the absorbance of the test compound.

Cytotoxic activity MTT (3(4, 5-dimethylthazol-2-yl)-2-5-diphenyl tetrazolium-bromide)

The human cervical cancer cell line (HeLa) was obtained from National centre for cell science (NCCS) pune, India. Cells were grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS) and NIH 3T3 Fibroblasts were grown in Dulbecco's Modified Eagles Medium (DMEM) containing 10% FBS. For screening experiment, the cells were seeded into 96 well plates in 100µl of respective medium containing 10% FBS at plating density of 10,000 cells/well and incubated at 37°C, 5% CO_2, 95% air and 100% relative humidity for 24 hours prior to addition of extract. The extract was solubilised in Dimethyl Sulfoxide and diluted in respective serum free medium. After 24 hours, 100ml of the medium containing the extract at various concentration (6, 25, 12.5, 25, 50,100,200,400&800µg/ml) was added and incubated at 37°C, 5% CO_2,95% Air and 100% relative humidity for 48 hours. Triplicate was maintained and medium containing without extract were served as control. After 48 hours, 15µl of MTT (5mg/ml) in PBS was added to each well and incubated at 37°C for 4 hours. The medium with MTT was flicked off and the formed formazan crystals were solubilised in 100µl of DMSO and then measured the absorbance at 570nm using micro plate reader. The % cell inhibition was determined using following formula [18, 2, 10].

$$\%\ \textit{Cell Inhibition} = \frac{\text{Mean Absorbance of Sample}}{\text{Mean Absorbance of Control}} \text{ X 100}$$

RESULTS AND DISCUSSION

In this course of study 30 isolates were isolated from marine soils. The culture characteristics (Pigment production), morphological characteristics and biochemical characterization of isolates were present in (**Table 1).** Phenotypic and biochemical characteristics were presented in (**Table 2)**, for 3 isolates. Out 30, 10 isolates were identified as *Streptomyces* genus and *Streptomycetaceae* family. In this 10 isolates 3 isolates were selected for the Antioxidant and cytotoxicity activity. All these isolates were incubated in ISP 1 broth for the production of secondary metabolites (fermentation broth). The weight of crude extract after fermentation was 97mg, 89mg and 101mg for isolates 1, 2 & 3 respectively.

Free radicals are chemical species containing one or more unpaired electrons that make them highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability [1]. In recent years much attention has been devoted to natural antioxidant and their association with health benefits [1]. Antioxidant activity of isolates were observed and noted as isolate 2 exhibit potent scavenging activity (Ic_{50} 21.50µg/ml) compare to other two isolates (1 and 3) (**Fig 1**). Actinomycetes are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites, notably antibiotics, antitumor agents, immunosuppressive agents and enzymes [15].The cytotoxic activity (MTT Assay) was performed. The extract of isolate 2 shows about 68% of inhibition and 61% for isolate 1, 59% for isolate 3 in human cervical cancer cell line (HeLa) (**Fig 2**). Suthindhiran and Kannabiran (2009) have reported that Streptomyces train with moderate haemolytic and antimicrobial activity isolated from Marakkanam coast. Cytotoxic activity of Isolate 2 on HeLa cells suggest that the strain could be clinically important and need to be investigated further for the anticancer properties.

In this context the present study highlighted the antioxidant and cytotoxic activity of crude extract of marine actinomycetes. Further studies are needed to carry out the isolation of the active principle present in the crude extract and to characterize the biological activity.

Table 1: Cultural Characteristics of isolates

Isolates	Medium	Growth	Aerial Mass Color	Reverse color
Isolates 1	Starch Casein	Good	Dull White	Ivory
	ISP 1	Aundant	Dark gray	Ivory
	ISP 2	Good	Light gray	Khaki
	ISP 4	Good	White	Pale yellow
	ISP 5	Good	Light gray	Pale yellow
	ISP 7	Good	Gray	Ivory
	Nutrient	Moderate	White	Light Yellow
Isolates 2	Starch Casein	Moderate	White	Pale yellow
	ISP 1	Moderate	White	Ivory
	ISP 2	Good	Light gray	Yellow
	ISP 4	Good	White	Ivory
	ISP 5	Good	Light gray	Ivory
	ISP 7	Good	Gray	Ivory
	Nutrient	Good	White	Pale Yellow
Isolates 3	Starch Casein	Good	White	Ivory
	ISP 1	Good	Gray	Pale yellow
	ISP 2	Good	Gray / Cement	Light brown
	ISP 4	Good	Gray	Pale yellow
	ISP 5	Good	Gray	Ivory
	ISP 7	Good	Gray	Ivory
	Nutrient	Moderate	Dull White	Ivory

Table 2: Physiological and Biochemical characteristics of the isolates

Test	Isolates 1	Isolates 2	Isolates 3
Gram Stain	+	+	+
Aerial Mass	White to Gray	White	Gray
Colony color	Gray	White	Gray
Soluble color	-	-	-
Melanin	-	-	-
Starch hydrolysis	+	+	+
Gelatin hydrolysis	+	+	+
Urease	-	+	+
Citrate utilization	+	-	+
H_2S production	-	-	-
Effect of pH			
5	-	-	-
6	+	+	+
7	++	++	++
8	++	++	++
9	-	-	-
Effect of Temperature			
15˚C	-	-	-
28˚C	++	++	++
37˚C	+	+	+
45˚C	-	-	-
Effect of NaCl Concentration			
1	+	+	+
2	++	++	++
3	+	+	+
4	+	+	+

+ = Poor, ++ = Good, - = Nil

16s rRNA sequence

The nearly complete 16s rRNA sequence of strain *Streptomyces ngp1* was generated (Genebank accession No. JN675471).Phylogentic analysis of 16s rRNA sequences of the strain and related taxas showed almost complete 16s rRNA sequence of the strain had 99% similarity to that of *Streptomyces sps.* As a Result, strain

Streptomyces ngp1 was identified as native organism and termed as *Streptomyces chida1*

Aligned Sequence Data:

>**PCR_NGP3**

GATGAGCCCGCGGCTATCCACCTATTTGGGGAGGTAACGGCTCACCAAGGCGACAACGGG
TAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGCCCAGACTCCTACGG
GAGGCAGCAGTGGGAAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGA
GGGATGACGGCCCTTCGGGTTGTAAACCTCTTTCAGCAAGGATGATGAAGCGAAACAACT
CGACCCC

>FP_ACT1-II sequence exported from oRGANISM3.ab1

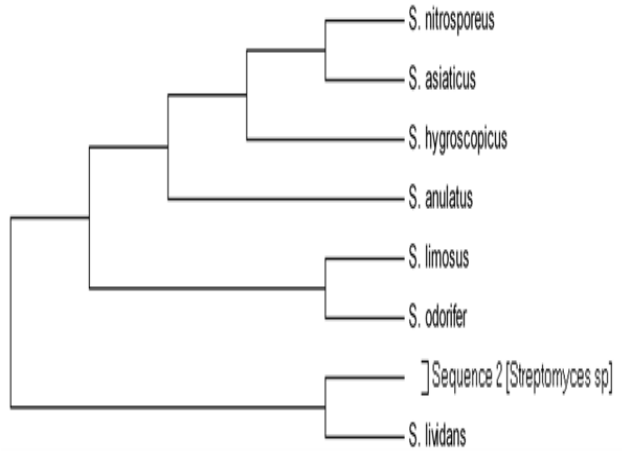


Fig 1: DPPH Assay of crude extract of isolates

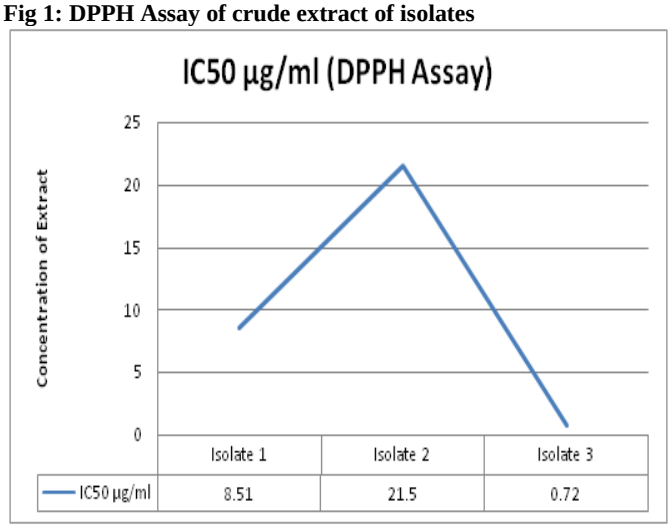
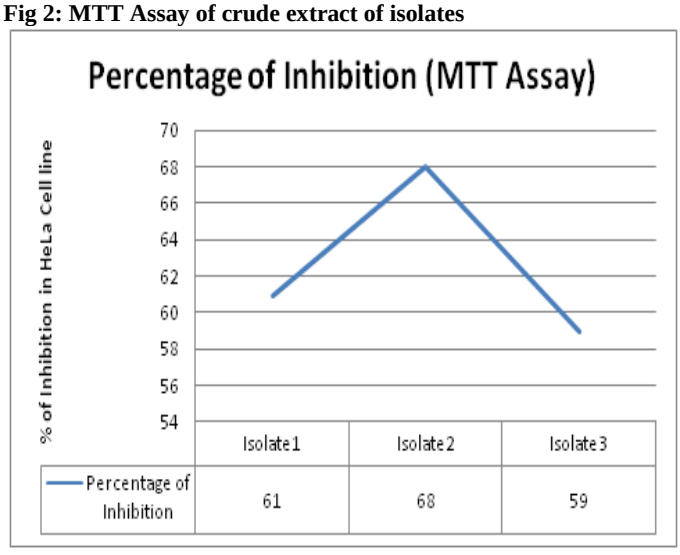


Fig 2: MTT Assay of crude extract of isolates



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