

Antimicrobial, Antioxidant and Cytotoxic Activity of Marine *Streptomyces parvulus* VITJS11 Crude Extract

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ABSTRACT

The main aim of the study was to evaluate the bioactive properties of ethyl acetate crude extract of *Streptomyces parvulus* VITJS11 with a view to assess their therapeutic potential. The biological activity of ethyl acetate extract was tested against fungal and bacterial pathogens. The free radical scavenging potential of the crude extract was determined by DPPH assay. The chemo preventive properties of *S. parvulus* VITJS11 ethyl acetate extract was examined by MTT assay on HepG2 cells. The morphological, physiological and the biochemical properties of the strain *S. parvulus* VITJS11 was confirmed by conventional methods. Genotypic characterization was done using 16S r-DNA partial gene amplification and sequencing. The authenticity of the crude chemical constituents were determined by the GC-MS. The ethyl acetate extract of VITJS11 showed maximum antifungal activity against three *Aspergillus* species and prominent antibacterial activity against two Gram positive and Gram negative bacteria at 20 mg/mL. The antioxidant potential of the crude extract exhibited strong reducing power activity at 5mg/ mL with 85% inhibition and the cytotoxic effect was found with IC₅₀ of 500µg/ mL on HepG2 cell lines. The GC-MS analysis and the chromatogram patterns revealed 16 peaks, indicating the presence of bioactive constituents, which included several important organic compounds, namely 9-(2',2'-dimethylpropanoilhydrazono)-3,6-dichloro-2,7-Bis-[2-(diethylamino)-ethoxy]fluorine (23.1) Dotriacontylpentafluoropropionate,(25.0) Octadecanoic acid, (20.0); Trans-2-methyl-4-n-butylthiane, S, S-dioxide.(19.0). The results showed the benefit of ethyl acetate extract from *S. parvulus* VITJS11 in treating microbial infections and indicated their broad spectrum of activity with beneficial virtues for therapeutic use.

Key words: Marine actinomycetes, *Streptomyces parvulus* VITJS11, antimicrobial activity, antioxidants, cytotoxicity, bioactive compounds

INTRODUCTION

The development of drug resistance by the pathogens is due to the concurrent usage of existing antibiotics. The search for new, safer, broad-spectrum antibiotic with greater potency has been progressing slowly (Gupte et al. 2002). Hence, to combat these drug resistant pathogens, new antibiotics are in need and should be developed. There is no satisfactory drug as of now to control the occurrence of cancer and it is known to be the leading cause of death worldwide

(Taylor et al. 2007). The limited availability to control cancer drug resistance along with the emergence of new pathogens has led the expansion of the search for novel drug candidates. In fact, the list of novel microorganisms, especially actinobacteria and their products found in microbiologically unexplored ecosystems. In the course of screening for new antibiotics, several studies are oriented towards the isolation of new bacterial species from different soils and ecosystems (Mellouli et al. 2003). Actinomycetes are ubiquitous in the marine environment and in,

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actinomycetes, *Streptomyces* species are one of the largest sources of bioactive natural products and they are taxonomically diverse and biologically active. The potential contribution of marine *Streptomyces* in the discovery of new bioactive molecules is increasingly challenging and the study of marine *Streptomyces* and their potential role in the production of metabolites is becoming an interesting new topic for research. Several investigations have shown an increasing number of biologically active and structurally unique compounds from it (Hardt et al. 2000; Marsgy et al. 2001). Indian coastal region is an ideal location for harvesting many valuable species producing bioactive compounds with numerous complex and novel chemical entities. Recently, much attention has been directed toward biologically active compounds from natural products as they exhibit fewer side effects in comparison to orthodox medical drugs. However, the potential beneficial effects of these natural compounds need to be confirmed in large, rigorous trials. These structures can be chemically modified and improved through knowledge of the structure-activity relationship, mechanism of action, drug metabolism, molecular modelling and combinatorial chemistry studies. The discovery of new active metabolites must be followed by adequate biological testing (Jemimah et al. 2014). Hence, the aim of this study was to investigate the bioactive potential of *S. parvulus* VITJS11 crude extracts from marine environment of Bay of Bengal, South coast of India for its antifungal, antibacterial, antioxidant and cytotoxic potential.

MATERIALS AND METHODS

Sample collection and isolation

Marine soil samples were collected from the south East coast of India, Ramanathapuram- Sethu Karai (Lat.9°50"N and 78°10'E) at the depth of 10-100 cm at littoral zone and the collected samples were stored at 4°C. The isolation was performed on selective media such as actinomycetes isolation agar (AIA), Kuster's agars, Bennett agar, Starch casein agar along with 25% marine water and 25% marine soil extract for effective isolation (Balagurunathan et al. 2001). All the plates were incubated at 30°C for 1 - 2 weeks. Emerging isolates were sub cultured on ISP2 agar and stored under refrigeration. The morphological and cultural characteristics were determined on

various ISP medium (Newman et al. 2003; Ravikumar et al. 2008).

Cross streak method

The *S. parvulus* VITJS11 was cross streaked on the modified nutrient glucose agar plates near the periphery against wide range of Gram-positive and Gram-negative bacteria at right angle and incubated for three days (Alexander et al. 1977; Brock et al. 1994). The zone of inhibition was measured after two days of incubation.

Scanning electron microscopy

Streptomyces parvulus VITJS11 was grown on starch casein agar at 27°C for seven days. The spore chain morphology and spore surface ornamentation was evaluated by scanning electron microscopy analysis.

Fermentation and extraction of antibacterial compound

The inoculum of *S. parvulus* VITJS11 was prepared on starch casein broth at a seed concentration of 100 mL in a 250 mL Erlenmeyer flask (pH 7.2) incubating at room temperature for seven days. For the isolation of bioactive compounds, extraction process was carried out successively with using ethyl acetate solvent with increasing polarity. The crude extract powder was weighed and studied for its bioactivity (Remya et al. 2007).

Test organisms

The pathogens, including *Aspergillus niger* MTCC No:872; *A. fumigatus* MTCC No:8877; *A. flavus* MTCC No:8790; *Pseudomonas aeruginosa* (MTCC No: 4676), *Staphylococcus aureus* (MTCC No: 7405), *Salmonella typhi* (MTCC No: 1167), *Escherichia coli* (MTCC No: 1588), were obtained from the Microbial culture collection, IMTECH, Chandigarh, INDIA.

Determination of antifungal activity

The antifungal activity was assessed by agar-well diffusion assay. The culture medium of sabouraud dextrose broth was inoculated with the fungal strains and was adjusted to 1×10^8 CFU/mL. Then 100 μ L of the inoculum was placed on the sabouraud dextrose agar and the wells were filled with 100 μ L of 20 mg/mL *S. parvulus* VITJS11 crude extract. The plates were incubated at 27°C for 72 h. The antifungal activity was evaluated by measuring the zone of inhibition of fungal growth.

Agar-well diffusion assay

The *in vitro* antibacterial activity of all the solvent extracts of *S. parvulus* VITJS11 was determined by agar-well diffusion method (Pandey et al. 2004). Log phase bacterial cultures of 10^8 CFU/mL were used. One hundred microlitre of various concentrations ranging 1.0 – 20 mg/mL of crude extract of *S. parvulus* VITJS11 were tested. Diameter of the zone was measured and expressed in mm.

Free radical Scavenging Activity

The antioxidant activity was determined by DPPH scavenging assay (Khalaf et al. 2008). Various concentration (0.1, 0.5, 1.0, 3.0 and 5.0 mg/mL) of *S. parvulus* VITJS11 crude extract was taken in separate tubes. Ascorbic acid was used as reference compound (0.1, 0.5, 1.0, 3.0 and 5.0 mg/mL). A freshly prepared solution of 0.002 % DPPH (2, 2, Diphenyl-2-Picryl hydrazyl) in methanol was added to each tube containing different concentrations of extracts (2.0 mL). The samples were incubated in dark at 37 °C for 20 min and read at 515 nm. The data were expressed as the percent decrease in the absorbance compared to the control. The percentage inhibition of radical scavenging activity was calculated.

Maintenance of cell cultures

The HepG2 cells were obtained from NCSS, Pune and cultured in RPMI-1640 medium on 10 cm tissue culture dishes (Greiner Bio-one™, Germany) supplemented with 10% heat inactivated fetal bovine serum. Cells were incubated in humidified incubator with 5% CO₂ at 37°C, and sub cultured when confluence was reached up to 80% (Freshney et al. 1982).

Cell viability assay

The 3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide) MTT assay, was used to assess the viability of the cells (Mosmann et al. 1983). The HepG2 cells (5×10^3) were seeded per well into 96-well plates containing 100 µL DMEM medium with 10% FBS incubated at 37°C for 24 h. Then, the cells were treated with 100 µL of the *S. parvulus* VITJS11 crude extract in triplicates with different concentrations ranging from 50 to 1000 µg/mL. Doxorubicin (5µg / mL) was used as internal positive control and 100 µL of DMEM was used as negative control; wells without any cells were considered as blank. After incubating in humidified incubator with 5% CO₂

at 37°C for 48 h, 20 µL of 5 mg/mL MTT diluted in PBS were added to each well and incubated for 4 h. One hundred microlitre of 10% SDS in, 0.01M HCl solution was added to each well to dissolve the formazan crystals formed. The plates were covered with aluminium foil and kept in an incubator for 12 h for dissolution of the formed formazan crystals. Amount of formazan was determined measuring the absorbance at 560 nm using a micro plate reader.

Molecular Characterization

Total genomic DNA was isolated using the phenol chloroform method (Nathan et al. 2004). PCR amplification of 16S r-DNA was carried out using the primers FC27 (5' to 3' AGAGTTTGATCCTGGCTCAG) and RC1492 (5' to 3' TACGGCTACCTT GTTACGACTT) (Rainey et al. 1996). The PCR product was detected by agarose gel electrophoresis. Sequencing was performed using big dye terminator cycle sequencing kit (Applied Bio Systems, USA). The sequence was subjected to homology search using BLAST programme of the National Centre for Biotechnology Information (NCBI) and the sequence data was submitted to the GenBank database under the accession number (KC961640).

Phylogenetic analysis

The acquired sequences was used for a gene homology search, with the 16S r-DNA sequences available in the public databases from BLAST and identified to the generic level (Altschul et al. 1997; Benson et al. 1999). Using the CLUSTAL-X Multiple Sequence Alignment Program (Strasbourg, France), the 16S r-DNA sequences of the strains were aligned with sequences of related organisms obtained from GenBank and a phylogenetic tree was constructed via the neighbor-joining method using the EvolView program (Huangkai et al. 2012). To validate the reproducibility of the branching pattern, a bootstrap analysis was performed.

GC-MS analysis

Streptomyces parvulus VITJS11 crude extract was subjected to GC-MS. The analysis was performed using GC SHIMADZU QP2010 system. NIST08 and WILEY8 database were used for the identification of the separated peaks and the relative percentage of each component was

calculated by comparing its average peak area to the total areas. (Jemimah et al. 2013)

RESULTS AND DISCUSSION

Recently, numerous pathogens have developed resistance due to the indiscriminate use of commercial antimicrobial drugs. Hence, there is an urge to search the novel drugs against such

pathogens. It has been envisaged that marine environment are extreme source to provide exciting new bioactive compounds. In the present study, an attempt was made to identify the bioactive potential of *S. parvulus* VITJS11. The morphology, biochemical and taxonomical characterization of *S. parvulus* VITJS11 represented the features of *Streptomycetaceae* family belonging to genera *Streptomyces* (Table 1).

Table 1 - Morphological properties, biochemical, cultural and process parameters of *Streptomyces parvulus* VITJS11.

Morphological properties								
Spore morphology	Spore surface		Colour of aerial mycelium	Colour of substrate mycelium		Gram staining	Motility	
Spiral	Smooth		Grey	Yellow		+	-	
Effect of Temperature			Effect of pH			Effect of NaCl Tolerance		
15 °C	-		5	-		0.5	++	
28 °C	++		6	-		1 %	+	
37 °C	+		7	+		2 %	-	
45 °C	-		7	++		3 %	-	
50 °C	-		8	+		4 %	-	
60 °C	-		8	-		5 %	-	
Carbon sources				Nitrogen Sources				
D-glucose			++	Cysteine		-		
Sucrose			+	Arginine		-		
D-galactose			+	Threonine		-		
Mannose			+	Alanine		-		
Maltose			+	Aspartic acid		+		
Lactose			+	Glycine		-		
Mannitol			+	Histidine		-		
L-Rhamnose			+	Lysine		-		
Arabinose			+	Phenyl alanine		++		
Biochemical Properties								
Melanin	Starch	Gelatin	Hemolysis	Tween	Casein	Esculin		
-	+	-	+	+	+	+		
Growth on ISP Media								
ISP 1	ISP 2	ISP 3	ISP 5	ISP 6	ISP 7	ISP 8	ISP9	
Good	Very good	Good	Good	Good	Very good	Good	Good	

The strain was studied on the basis of its morphology and recognized by their presence of aerial hyphae with grey coloration on starch casein medium. The frequent production of spreading substrate mycelia was observed and the colonies were found darkened during sporulation with spirals. The colonies with powdery appearance had a characteristic feature with smooth surface. The ornamentation of spore surface of *S. parvulus*

VITJS11 was observed under light microscopy. Scanning electron microscopy (magnification, 6,000) showed aerial mycelium bearing spores with 0.7 µm (Fig. 1).

The physiological characteristics of the *S. parvulus* VITJS11 was compared with *Streptomyces* species given in the key of Nonomura. The strain VITJS11 showed more similarity to the reference strain, *S. parvulus* (Table 2).

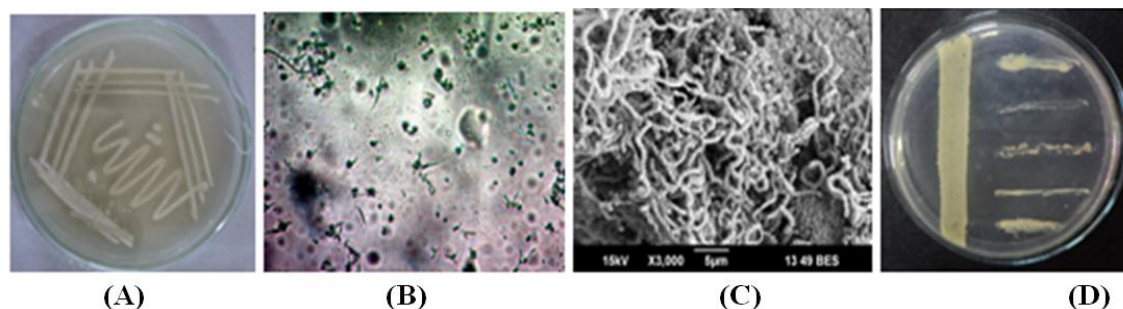


Figure 1 - *Streptomyces parvulus* VITJS11- (A) Pure culture plate of on starch casein agar; (B) Light microscopy at 40X; (C) Scanning electron microscopy; (D) Cross streak assay of the strain against the bacterial pathogens.

Table 2 - Comparison of morphological and biochemical characteristics of *Streptomyces parvulus* VITJS11 with reference strain.

	<i>S. parvulus</i> VITJS11	<i>S. parvulus</i>
Colour of aerial mycelium	Grey	Grey
Melanoid pigment	-	-
Reverse side pigment	-	-
Soluble pigment	-	-
Spore chain morphology	Spiral	Spiral
Utilization of carbon sources		
Arabinose	+	+
Xylose	+	+
Inositol	+	+
Mannitol	+	+
Fructose	+	+
Rhamnose	+	+
Sucrose	+	+
Raffinose	+	+

The antifungal activity of *S. parvulus* VITJS11 was examined against three pathogenic species of *Aspergillus*. The strongest inhibition of fungal growth was noticed against *A.niger* of 30 mm at 20 mg/mL concentration and *A.fumigatus* (25 mm), *A.flavus* (21 mm). The results could be useful for the better understanding of the biocontrol mechanisms of the fungal strains, indicating that the strain VITJS11 probably producing a bioactive potential with antifungal component (Fig. 2). The strain *S. parvulus* VITJS11 subjected to primary screening for its activity against bacterial pathogens was confirmed by the cross streak method. The crude extract of the isolate further subjected to agar well diffusion method comparatively exhibited potent activity for all the clinical pathogens tested. The most active antibacterial crude fraction was obtained by the solvent mixture of ethyl acetate. The antibacterial

effects of crude extract showed activity against the bacterial pathogens.

Antifungal activity of *Streptomyces parvulus* sp. VITJS11 crude extract

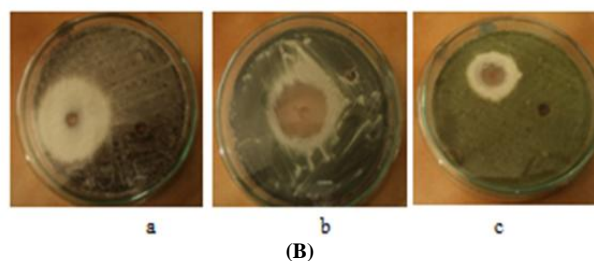
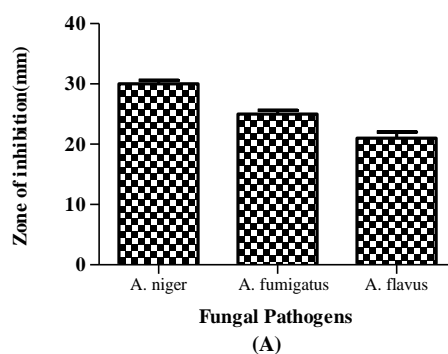


Figure 2 - (A) Graphical representation of antifungal activity of *Streptomyces parvulus* VITJS11 crude extract ; (B) Pictorial view showing the inhibition of fungal pathogens(a) *Aspergillus niger* [30 mm] (b) *Aspergillus fumigatus* [25 mm] (c) *Aspergillus flavus* [21 mm].

The maximum activity was found at 26 mm, which was observed against *S. typhi* and *E. coli*, followed by *S. aureus* 24 mm and *P. aeruginosa* with 20 mm diameter of inhibition at the maximum concentration of 20 mg/mL. The intensity of the inhibitory effects was comparable with the positive control (Fig. 3). The current results were

also supported by the findings of similar study on *Streptomyces* strains, which were showing better antifungal activity observed against *Aspergillus niger* and strong antibacterial activity against Gram positive and Gram negative bacteria (Laidi et al. 2007; Oskay et al.2009). The studies on marine *Streptomyces* sp exhibited good antimicrobial activity against clinical pathogens (Ponmurugan et al. 2008). The concurrent studies of *Streptomyces* sp PM-32 isolated from off-shore

sediments collected at the Bay of Bengal coast was reported to have antimicrobial activity against a group of bacterial and fungal pathogens (Manivasagan et al. 2009). Studies on antibacterial activity from *Streptomyces* sp SLO-105 was found against Gram-positive bacteria (*S. aureus*, methicillin-resistant *S. aureus* (MRSA), *Micrococcus luteus*) and antifungal activity against *Aspergillus niger* (Morakchi et al. 2009; Ko et al. 2010).

Antibacterial activity of *Streptomyces parvulus* sp. VITSJ11 crude extract

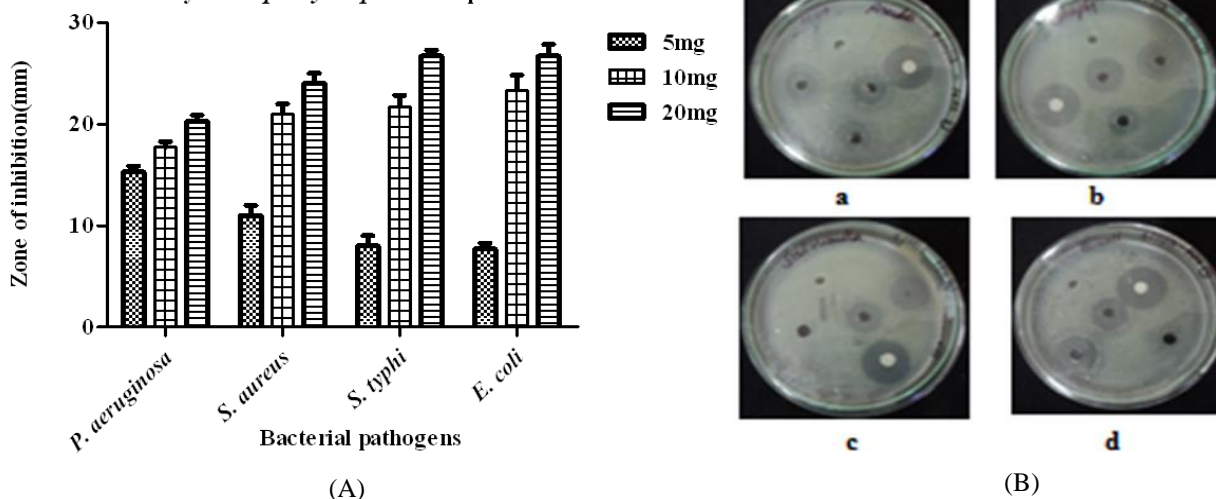


Figure 3 - (A) Graphical representation of antibacterial activity of *Streptomyces parvulus* VITJS11 crude extract; (B) Pictorial view showing the inhibition of bacterial pathogens at the maximum concentration 20 mg/mL (a) *Pseudomonas aeruginosa* [20 mm] (b) *Staphylococcus aureus* [24 mm] (c) *Salmonella typhi* [26 mm] (d) *Escherichia coli* [26 mm].

The present study correlated with the existing report where the strain SRB25 was more similar to *Streptomyces* with antimicrobial property against multidrug resistance *S. aureus* (Sathish et al. 2012). The active compound of *Streptomyces* KUAP106 was effective against Gram-positive, Gram-negative and unicellular filamentous fungi (Usha et al. 2010). The *Streptomyces* sp. isolated from saline farm-lands showed both antibacterial and antifungal activity (Sajid et al. 2009). The antioxidant activity of the VITJS11 crude extract showed maximum activity with 85% inhibition at 5.0 mg/mL concentration and its activity remained highly significant with the reference used (Fig.4).

Antioxidant potential of *Streptomyces parvulus* sp VITSJ11 crude extract

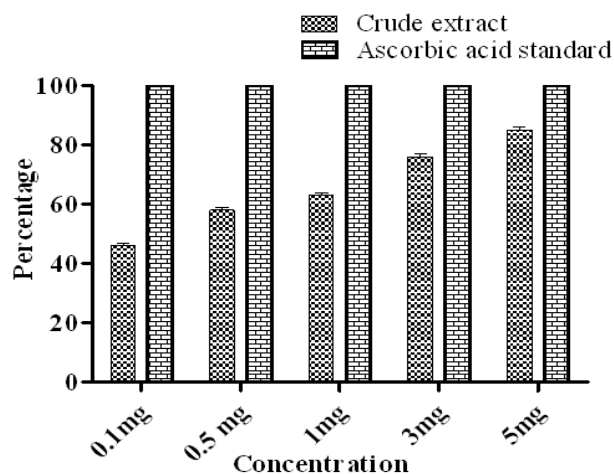


Figure 4 - Graphical representation of antioxidant property of *Streptomyces parvulus* VITJS11 crude extract.

Human hepatocellular liver carcinoma cell lines (HepG2) was used as a model system to examine chemo-preventive effect of *S. parvulus* VITJS11. The concentration of the extract varied from 50 to 1000 µg/mL. The IC₅₀ value was 500 µg/mL on HepG2 cells lines and exhibited substantial growth inhibition with 21% viability (Fig. 5). The HepG2 cell death resembled apoptosis, which was special evident with most notorious effect for strain VITJS11 crude extract. It was interesting to note that the strain VITJS11 crude extract resulted higher percentage of apoptotic cells. One possibility of the apoptotic phenomenon could be that the compounds in the extract interfering with

mitosis would have lead to apoptosis within short period of time. Actinomycin D is the most significant member of actinomycins, which are a class of polypeptide antibiotics isolated from *Streptomyces* genus (Hollstein et al. 1974). Actinomycin D decreases Mcl-1 expression and acts synergistically with ABT-737 against small cell lung cancer cell lines (Aishan et al. 2010). Among the members of actinomycetes genus, *Streptomyces* sp is a dynamic producer of functional, bio-effective metabolites with broad pharmaceutical range having antimicrobial, antihelminthic, antitumor and antiviral agents (Ravikumar et al. 2008).

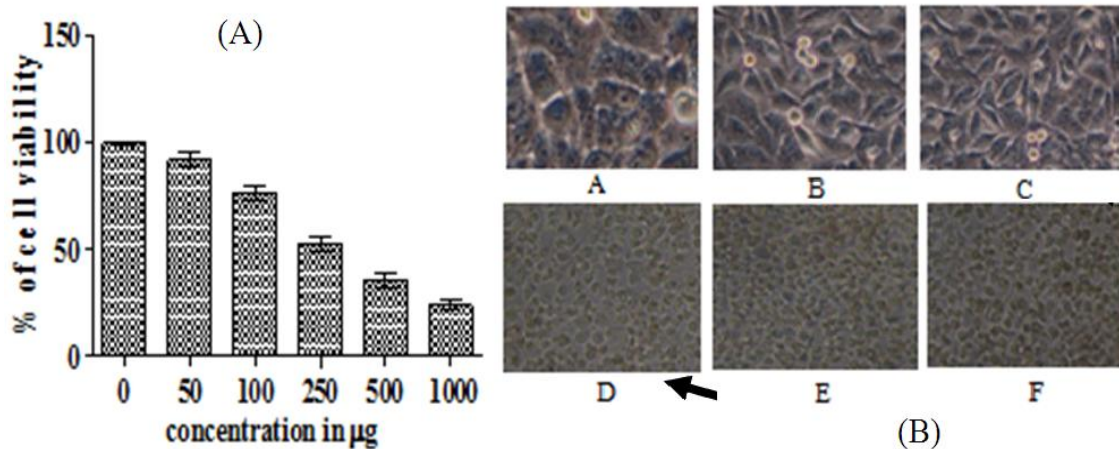


Figure 5 - (A) Percentage of cell viability shows the effective drug concentration toxic to HepG2 cell lines. (B) Morphological changes of HepG2 cell lines treated with crude extract of *Streptomyces parvulus* VITJS11. Cells were visualized under inverted light microscope. Detachment of cells from substratum; cell shrinkage, nuclear condensation and fragmentation were evident in cells treated.

The phylogenetic analysis of *S. parvulus* VITJS11 was identified based on the nucleotide sequences of their 16S r-DNA genes, which showed similarity values of 98% to 16S r-DNA genes of firmicutes in the NCBI database and the isolate revealed species level similarity to *S. parvulus* S10. Phylogenetic tree construction was conducted using neighbor joining algorithm with bootstrap values based on 1000 replications and the annotation, tree visualizing was performed using EVOLVIEW (a web application tool). The generated tree revealed that the isolate shared close affinity with *Streptomyces* species (Fig. 6).

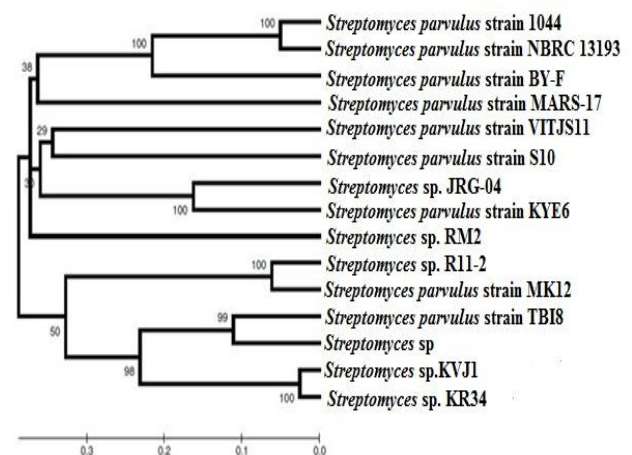


Figure 6 - Phylogenetic tree based on partial 16S r-DNA sequences, showing the relationship with other species belonging to the genus *Streptomyces*.

The presence of volatile chemical constituents of ethyl acetate crude extract were analyzed by the GC-MS analysis and the chromatogram patterns revealed 16 peaks, indicating the presence of bioactive constituents, which included several important organic compounds namely 9-(2',2'-dimethylpropanoilhydrazono)-3,6-dichloro-2,7-BIS-[2-(diethylamino)-ethoxy]fluorene(23.1) Dotriacontyl pentafluoropropionate (25.0) Octadecanoic acid (20.0); Trans-2-methyl-4-n-butylthiane, S, S-dioxide (19.0). Interpretation on mass spectrum of GC-MS was done using the database NIST08, WILEY8 (Fig. 7). In the present study, the partial characteristics of the active molecules indicated that bioactivity was likely to be due to the production of potential organic

substances. The chemical fingerprints of the crude extract was identified and further manifested in differentiation from the related chemical profiles. Hence, the poor availability of drugs and the increasing number of treatment failures have motivated current searches for therapeutic alternatives which can work effectively as potential antimicrobial agents (Pina-Vaz et al. 2004). Thus, the new antimicrobial marine *Streptomyces* derivatives could be useful alternatives for the treatment of fungal and bacterial infections. The advantages of using these bioresources and their and its natural compounds may reduce the risk of side effects and lower the cost.

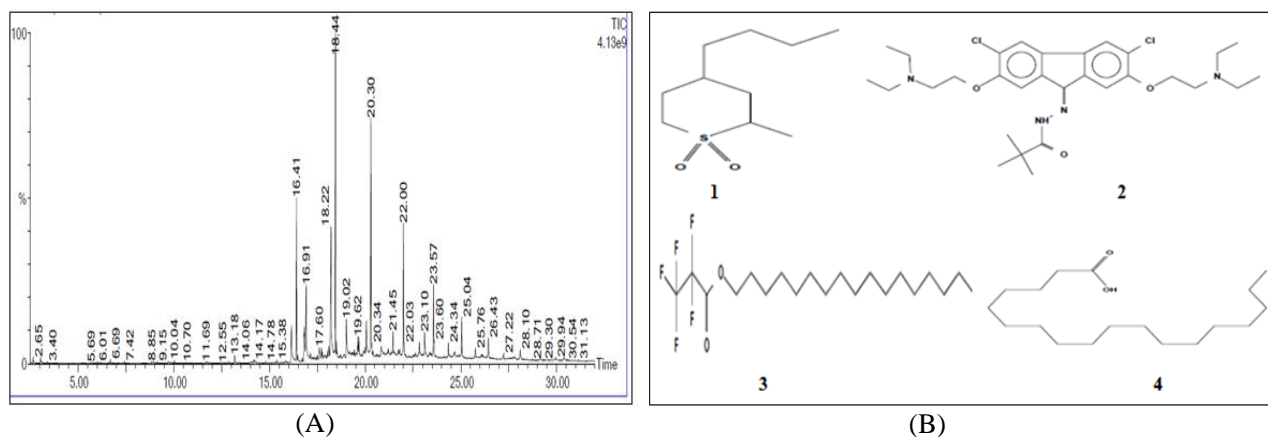


Figure 7 - *Streptomyces parvulus* VITJS11 crude extract (A) Chromatogram(B) GC-MS peaks of major volatile bioactive metabolites with molecular structure (1) 9-(2',2'-Dimethylpropanoilhydrazono)-3,6-Dichloro-2,7-Bis-[2 Diethylamino) Ethoxy]Fluorene; (2)Dotriacontylpentafluoropropionate; (3) Octadecanoic acid; (4) Trans-2-Methyl-4-N Butyl Thiane, S, S-Dioxide.

CONCLUSION

Streptomyces parvulus VITJS11 showed various bioactive properties, which highlighted its importance as potential pharmacological agents. Hence, there could be probability of new bioactive compound in the crude extract, which might provide a basis for further development of novel compound from *S. parvulus* VITJS11. This also provided a new insight towards the development of good candidates for pharmaceutical and bioactive natural products.

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