ANTIPROLIFERATIVE ACTIVITY OF *RHODOCOCCUS* VITSNK6 ISOLATED FROM MARINE SEDIMENT SAMPLES COLLECTED AT THE PUDUCHERRY COAST, BAY OF BENGAL, INDIA

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Summary

Actinomycete strains isolated from marine sediment samples collected at the Bay of Bengal coast of Puddcherry, India was screened for antiproliferative activity on three cancer cell lines by trypan blue staining and MTT assay. The potential isolate having antiproliferative activity was cultured on ISP –Veg media supplemented with sea water, pH 7.2, and the inoculated broth was maintained at 28° C for 7 days. The media and cultural conditions for maximal growth have been optimized under shake- flask conditions by measuring the dry weight of the mycelium. The maximal growth was attained with the use of optimized production medium, pH of 7.2 and incubation temperature of 27°C. Based on molecular taxonomic and phylogenetic characterization, the strain was identified as *Rhodococcus* and designated as *Rhodococcus* sp. VITSNK6. The 16 S rDNA nucleotide sequence from the 16 S rRNA partial gene sequence of the isolate was deposited in the GenBank under the sequence of the accession number FJ 973467. The ethyl acetate extract prepared from the supernatant of the culture broth was used for testing the antiprolifertive activity against HeLa (human cervical carcinoma), MDA-MB 231 (human breast adenocarcinoma), HepG2 (hepatocellular carcinoma) cells, HEK (Human embryonic kidney) and Vero (green monkey kidney) cell lines. The susceptibility of cancer cell lines to the extract was in the order of Hep G2> MDA-MB 231>HeLa with the IC_{50} values 22 µg/ml, 35 µg/ml and 45 µg/ml respectively. The extract treated cancer cells showed apoptosis mediated cell death and the viability of cancer cells was inversely proportional to the concentration of the extract used. The normal cell lines were less susceptible to inhibition than cancer cells. The results of this study showed that the secondary metabolite produced by the isolate has marked antiproliferative activity against cancer cells.

Keywords*:* Actinomycetes, *Rhodococcus* sp. VITSNK6, cancer cells, antiproliferative activity.

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Introduction

Marine microorganisms are emerged as a promising new source for natural products discovery with potent biological activities (1). Most actinobacteria of medical or economic significance are in the subclass Actinobacteridae, belongs to the order Actinomycetales, and has been traditionally a focus for industrial and academic groups for more than 60 years. The secondary metabolites produced by these organisms are long and its impact is widespread across many therapeutic areas, from infectious diseases to oncology, immunosuppression, atherosclerosis and others (2).The search for more effective anti-cancer agents is on warfoot from various natural sources, which includes marine microorganisms and marine invertebrates. Actinomycetales, characteristic of its high G+C DNA content plays an important role among the bacterial communities of marine origin because of its diversity and ability to produce novel chemical compounds of high commercial value. Several cytotoxic compounds have been reported from marine actinobacteria (3) Maskey et al, 2003; (4) Feling et al, 2003; (5) Hayakawa et al, 2006).

Rhodococcus, an actinomycete capable of producing several secondary metabolites extensively reported in literature for cleansing of oil spills. It is a natural reservoir of new biosurfactants and the strain *Rhodococcus erythropolis* (3C-9 strain) has been reported to be the producer of biosurfactants, free fatty acids and glycolipids (6). An oil-degrading biosurfactant-producing bacterium TW53 was reported to be isolated from deep-sea sediment, and was identified through 16S rDNA analysis as belonging to the genus *Rhodococcus* (7). *Rhodococcus erythropolis* PR4 produces a mucoidan, an acidic extracellular polysaccharide (EPS) capable of degrading alkanes including pristanes has been reported (8). Several reports are available on the oil degrading activity of *Rhodococcus* sp. (6, 9, 10). Isolation of nitrile-hydrolysing *Rhodococci* was also reported from sediments of North West Pacefic Ocean (11).

In the present investigation an actinomycete strain having antiproliferative activity on cancer cell lines under *in vitro* conditions was reported.

Materials and methods

Sample collection and processing

Marine sediment samples (8 Nos.) were collected from the near salt pans of Marakkanam (Latitude (N) 12°20′; Longitude (E) 79°95′) and Puducherry (Latitude (N) 11°56’; Longitude (E) 79°53) coast, southern India at the depth of 50-300 cm. The collected sediments were maintained at ambient temperature with seawater and transported to laboratory. The sediment samples were dried in laminar air flow for 8-12 hrs and then kept at 42°C for 10-30 days in a sterile Petri dish and these preheated samples were used for the isolation of actinomycetes.

Isolation of actinomycetes

A diluted sample was pour plated on Starch Casein Agar (SCA) media and incubated for one week at room temperature. After incubation, actinomycetes isolates were distinguished from other microbial colonies by characteristic white powdery colonies (12). Based on the colony morphology, eight strains of actinomycetes were isolated. The isolates were then purified by streaking on ISP 1 agar plate (Tryptone yeast extract agar).

Well grown slant cultures of the isolates inoculated into 50 ml medium in 250 ml Erlenmeyer flasks containing the production medium and incubated for 2 days in rotary shaker (200 rpm) at 28^{o}C. After incubation the secondary metabolites were extracted with ethyl acetate and cytotoxicity was determined by MTT assay on HeLa, MDA-MB 231 and Hep G2 cells. The potential isolate having antiproliferative activity was cultured on ISP – Veg media supplemented with sea water, pH 7.2, and the inoculated broth was maintained at 28° C for 7 days.

Molecular taxonomic characterization of the potential isolate

The morphological, cultural, physiological and biochemical characterization of the potential isolates were carried out as described in International *Streptomyces* Project (ISP) (13. The molecular taxonomy and phylogenetic analysis were performed as described earlier (14. The sequencing was carried out in both the sense and antisense directions. The similarity and homology of the16S rDNA sequence was analyzed with the similar existing sequences available in the data bank of National Center for Biotechnology Information (NCBI) using BLAST search. The DNA sequences were aligned and phylogenetic tree was constructed by neighbor joining method using ClustalW software (15). The secondary structure and the restriction sites in the 16s rDNA sequence of the isolate were predicted using the bioinformatics tools Genebee and NEBCutter (version 2.0) and bioinformatics tool available online www.genebee.msu.su/services/rna2_reduced.html

Cell lines

HeLa (Human cervical carcinoma), HEK (Human embryonic kidney) Vero (green monkey kidney), cell lines obtained from ATCC and MDA-MB 231(Human breast adenocarcinoma), HepG2 (hepatocellular carcinoma) obtained from NCCS, Pune, India were maintained in RPMI 1640/DMEM/L-15 (Himedia/Gibco, Mumbai, India) medium supplemented with 10% FBS (v/v) and 100 mg/l streptomycin and 100 IU/ml penicillin (Himedia, India), at 37°C in 5% carbon dioxide.

Morphological and MTT cell proliferation assay

The cells incubated with the extract (100 µg/ml) for 24 hrs, stained with trypan blue and observed morphological changes and viability. The antiproliferative activity of the extract (5 to 100 µg/ml) on various cell lines (1X10^{5} cells/well) was determined as per user manual (CellQuanti-MTT cell viability assay kit, Bioassay Systems). The IC_{50} values were calculated by non-linear regression analysis using the Prism software.

Detection of apoptosis in cancer cells

After 48 hrs of incubation of cells (5X10^{5} cells/ml) with the extract, DNA was isolated by the method of Allen *et al*. (16) and DNA fragmentation was analyzed by using agarose gel (1.5 %) electrophoresis in both control treated cells. After electrophoresis, the gels were stained with ethidium bromide and visualized under UV light. Alternatively the morphology and the DNA fragmentation was visualized under inverted fluorescent microscopy.

Results

The isolate showed excellent growth and abundant aerial mycelium formation at pH 7.2, temperature 28°C and less growth and aerial mycelium formation at lower, higher pH values and temperatures used on the ISP –Veg media supplemented with sea water for 7 days. However the yield of secondary metabolite was maximal with the use of production medium. The blast search of the 16S rDNA sequence (1293 base pairs) of the isolate showed maximum (96%) similarity with Uncultured *Rhodococcus* sp. clone CBFOS-04(EU371586.1) and 90 % with bromate-reducing bacterium B7 (AF442523.1). The phylogenetic tree was constructed with bootstrap values (Fig.1). Due to non availability of physiological, biochemical and cultural conditions of the closest phylogenetic neighbours, we are unable to compare the characteristic features of the isolate with others. Based on the molecular taxonomy and phylogeny the strain was identified as *Rhodococcus* sp. and designated as *Rhodococcus* sp.VITSVK6. The RNA secondary structure (Fig 2) of 16s rRNA gene of *Rhodococcus* sp.VITSVK6 showed the free energy of the predicted structure is-265.6 kkal/mol. The restriction analysis of the sequence is given in Fig, 3 The restriction sites present on the bacterial 16S rDNA showed 44 restriction sites and 54 % and 56 % GC and AC contents, respectively. The nucleotide sequence of 16s rDNA of 16 S rRNA gene partial sequence was deposited in the GenBank, National Centre for Biotechnological Information, USA under the accession number (FJ 973466).

The extract treated cells stained with trypan blue were observed under microscope for morphological changes and viability of cells (Fig 4). It was observed that the extract treated cells were detached from the surface of the culture flask and floated in the media. Moreover cells were in a distorted shape and in some cells, protrusions are noticed which seem to be emerging from the cytoplasm which indicates a broken cell membranes.

Table1. Effect of extract from *Rhodococcus* sp.VITSVK6 on cancer and normal cells viability

Extract (µg/ml)	% Viable cells					
	Tomoxifen	Vero	HeK	HeLa	MDA-MB 231	HepG2
5	77	94	97	98	91	65
15	65	92	91	91	88	63
25	52	91	88	75	62	54
50	40	84	81	55	45	34
100	34	83	76	45	37	28

Values are mean of three individual experiments

The effect of various concentrations (5 to 100µg/ml) of the ethyl acetate extract on the viability of different cell lines is given in Table 1. Ethyl acetate extract from *Rhodococcus* sp.VITSVK6 (100µg/ml) inhibited the viability cells maximally in a concentration dependent manner. The liver cancer cells were more susceptible (72%) to inhibition by the extract followed by breast cancer cells (63%) and He La cells (65%).

The extent of inhibition of the viability of normal (control) cells was minimal when compared to cancer cells. The Vero cells were less affected (17%) when compared to HeK cells (24%). Based on the proliferation inhibition of the cancer cells by the extract the IC_{50} values were calculated by MTT assay. The IC_{50} value of liver cancer cells was 22 µg/ml followed by breast cancer cells (35 µg/ml) and He La cells (45 µg/ml).

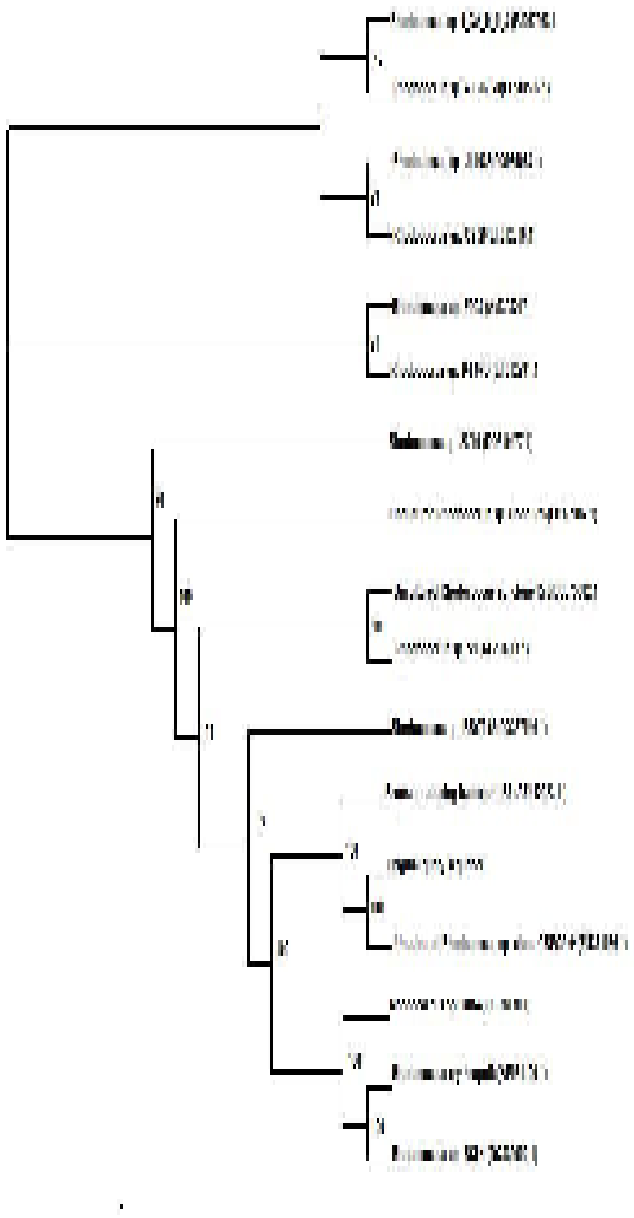

Figure 1. 16S rDNA-based phylogenetic tree of *Rhodococcus* sp.VITSVK6 with other *Rhodococcus* sp. sequence obtained from NCBI data base using Neighbor joining method

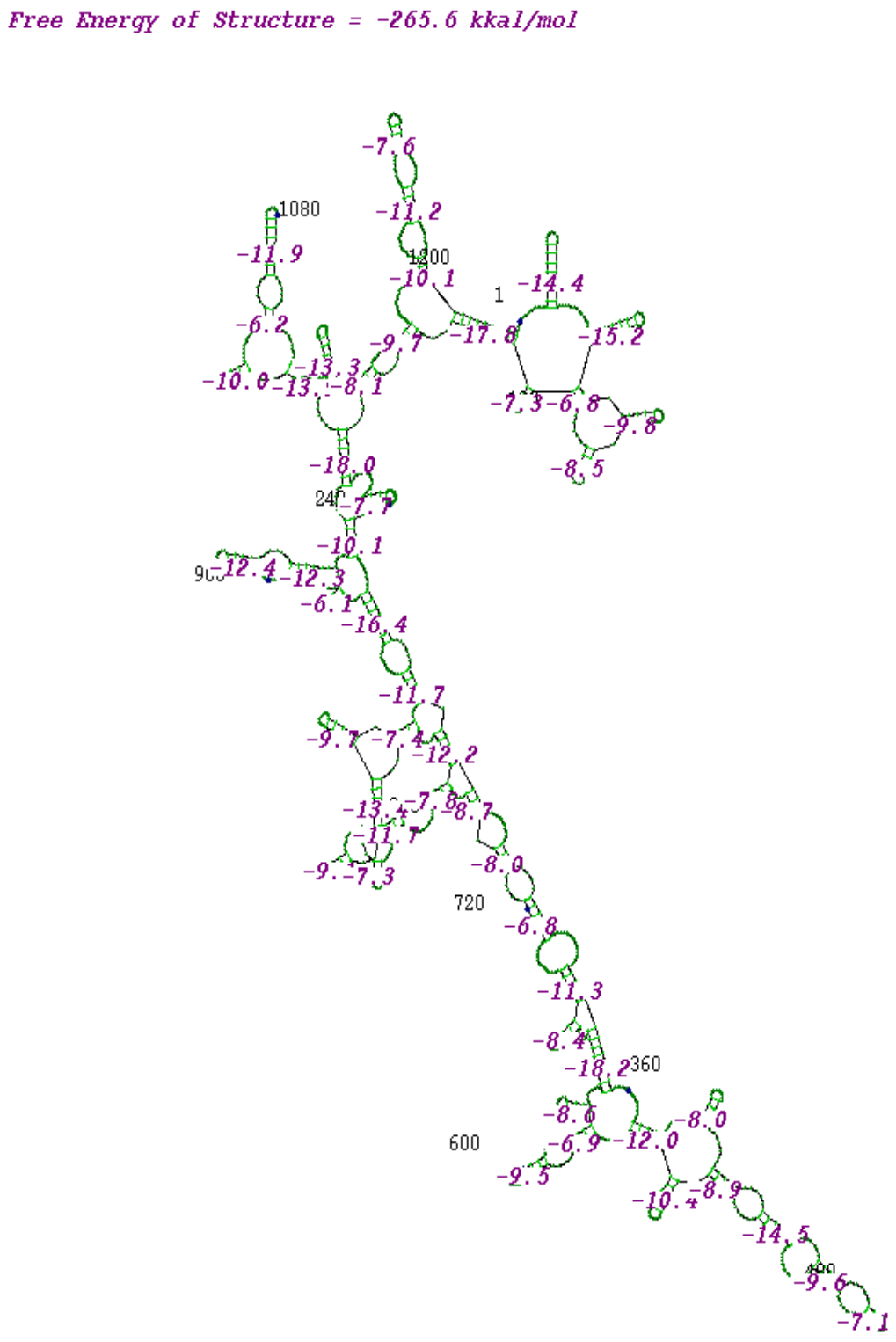

Figure 2. Secondary structure of 16S rDNA of isolate *Rhodococcus* sp.VITSVK6 using

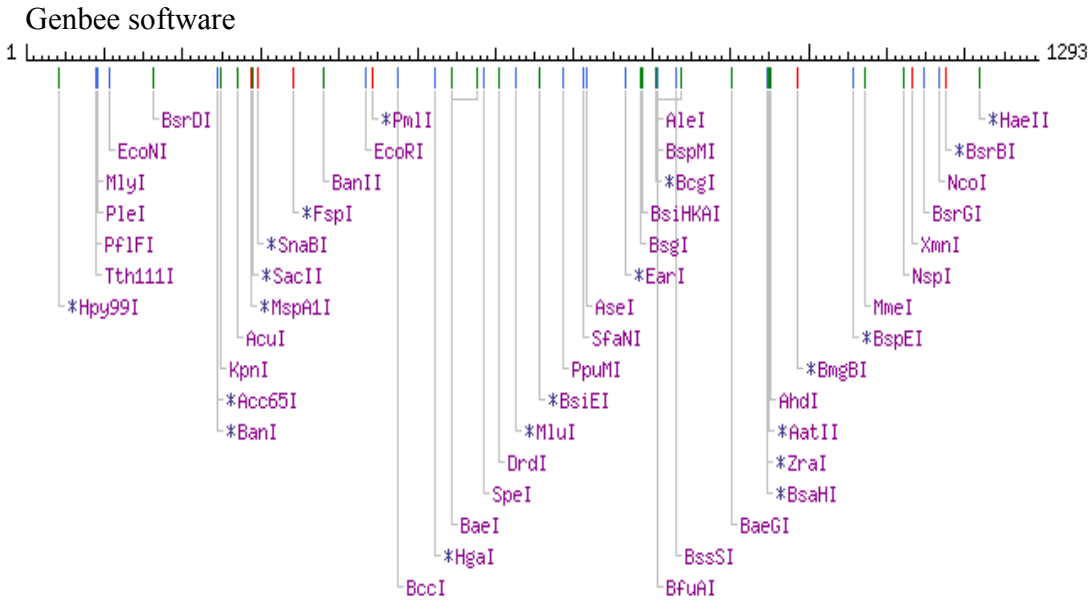

Figure 3. Restriction site analysis of 16S rDNA of isolate *Rhodococcus* sp.VITSVK6 using NEB Cutter program.

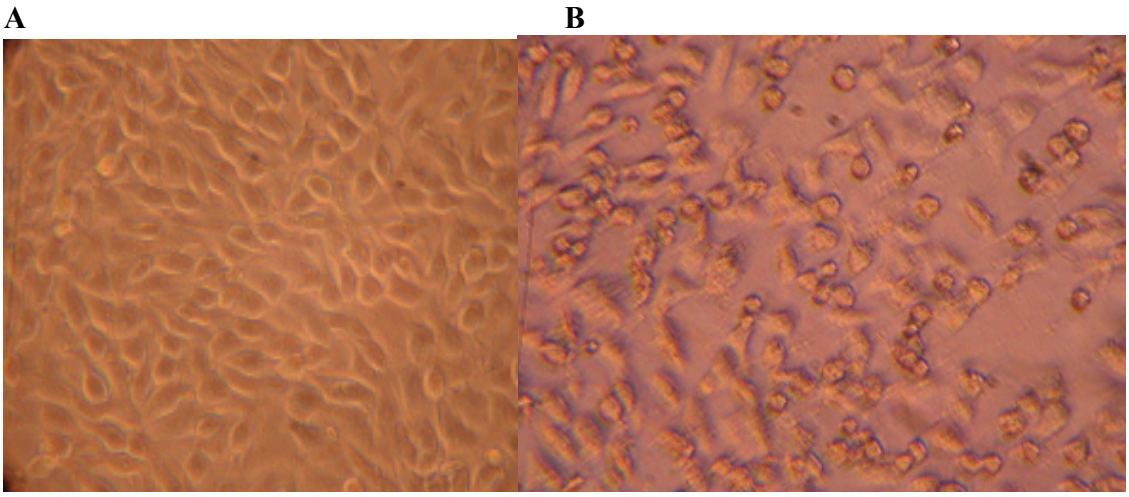

Figure 4. Effect of ethyl acetate extract of *Rhodococcus* sp.VITSVK6 on the viability He La cells stained with trypan blue. Photomicroscopic picture of HeLa cells A) control and B) treated with extract (100 µg/ml) after 24 hrs of incubation at 5% CO_2 in a CO_2 incubator stained with trypan blue and observed under Microscope (100 X magnification).

Discussion

In our systematic screening of marine actinomycetes from the sediment samples collected at the Marakkanam, the Puddcherry coast of Bay of Bengal, India resulted in isolation of an actinomycete having antiproliferative activity on three cancer cell lines under *in vitro* conditions. The strain was isolated characterized and identified as *Rhodococcus* sp. and designated as *Rhodococcus* sp.VITSVK6. Rhodococcus is a genus of aerobic, non-sporulating, non-motile gram positive bacteria closely related to mycobacteria and corynebacteria. It is available in broad range of environments; its genome contains 9.7 mega base pairs and rich in G+C content (67%). It is capable of producing bioactive steroids, acryl amide and acrylic acid and has been involved in fossil fuel biodesulfurization and can be easily distinguished from each other by robotyping (17).

The 16 S rDNA of isolated strain *Rhodococcus* sp.VITSVK6 is phylogenitically similar (96%) to *Uncultured Rhodococcus sp. clone CBFOS-04*(EU371586.1), 90% similar to *romate-reducing bacterium B7* (AF442523.1), 84% similar to *Rhodococcus sp. Dui-4* (EF028130.1) and 80% similar to *Rhodococcus sp. EP08* (AM398217.1). But the details of none of the strains are available in puplic data bases to compare with *Rhodococcus* sp.VITSVK6.

The secondary metabolite (100µg/ml) obtained from the ethyl acetate extract of the culture both showed significant antiproliferative activity against cancer cells in the order of HepG2> MDA-MB 231> HeLa cells. The inhibition of proliferation of cancer cells treated with extract was comparatively higher (2 fold) than normal cells. The normal cells are less susceptible to inhibition by the extract. Further extract treatment induces apoptosis in cancer cells that lead to DNA fragmentation and strand breaks. Apoptosis mediated DNA fragmentation has been reported to be major cause of cancer cell death. Significant cytototoxic activities of metacycloprodigiosin (1) and undecylprodigiosin (2) were reported against five different tumor cells (18). Proximicin A, B and C, novel aminofuran anticancer compounds from actinomycete *Verrucosispora* have been reported against adenocarcinoma, breast carcinoma and heptocellular carcinoma cells (19). Several strains of actinomycetes have already been reported to possess anticancer cytotoxic activity under *in vitro* conditions (18, 20).

From the results it can be concluded that *Rhodococcus* sp.VITSVK6 is a potential isolate capable of producing anticancer secondary metabolites. To the best our knowledge this is the first report that isolation of antitumor *Rhodococcus s*pecies from marine sample of Indian Peninsula. However, further studies are needed to isolate, to purify, to identify the active secondary metabolite and to study its antiproliferative and cytotoxic activity on other cancer cell lines and also *in vivo* animal model studies to establish its efficacy and anticancer potential.

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