Oxid Antioxid Med Sci 2012; 1(1):51-57 *ISSN: 2146-8389*

GESDAV

Oxidants and Antioxidants in Medical Science

available at www.scopemed.org

Original Research

Antimicrobial and antioxidant properties of marine actinomycetes *Streptomyces* sp VITSTK7

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Received March 21, 2012
Accepted April 27, 2012

Published Online May 18, 2012

DOI 10.5455/oams.270412.or.005

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Key Words
Antimicrobial activity;
Antioxidant activity;
Aspergillus fumigatus;
Streptomyces sp VITSTK7

Abstract
Marine actinomycetes are potential provider of novel bioactive metabolites and currently emerged as an important source for natural products with unique chemical diversity. The aim of the present study was to evaluate antimicrobial and antioxidant potential of the actinomycetes isolate *Streptomyces* sp VITSTK7 isolated from marine sediments collected at the Bay of Bengal coast of Puducherry, India. The culturing conditions such as pH, temperature, NaCl, carbon source and nitrogen source were optimized for maximal growth by dry weight method. The ethyl acetate (EA) extract showed a significant antifungal activity against *Aspergillus fumigatus* with the MIC value of 0.25 mg/ml. However, EA extract showed moderate antibacterial activity against selected bacterial pathogens. The antioxidant potential of the extract was assessed by DPPH• (1,1-Diphenyl-2-picrylhydrazyl) scavenging activity, Fe^{+3} reducing assay, metal chelating assay and DNA inhibition protection assay. The DPPH• scavenging activity was 43.2% at 10 mg/ml of EA extract and compared with ascorbic acid standard. The Fe^{+3} reducing assay showed 0.37 absorbance at 700 nm at 10 mg/ml of EA extract. The EA extract at 10 mg/ml showed 51% metal chelation. The ability of EA extract to protect DNA (pBR322) against UV-induced photolysed H_2O_2-oxidative damage was also analyzed. The EA extract was able to protect the DNA significantly from oxidative damage. The results of this study indicate that the ethyl acetate extract of the isolate possess both free-radical scavenging and antimicrobial activity.

INTRODUCTION

The marine environment of Indian peninsula is rich in microbial diversity. However, the wealth of marine micro-flora has not been fully investigated till date [1]. Natural products remain to be the most propitious source of antibiotics [2]. There are approximately 32,500 natural products reported from microbial sources (AntiBase database) including about 1000 derived from marine microbes [3]. Natural compounds are the source of numerous therapeutic agents. The tremendous biochemical diversity of marine microorganisms and their biotechnological potential is becoming more and more recognized, not only by microbiologists but also by the pharmaceutical industry [4].

Several antibiotics were derived from marine actinomycetes [5] and at present, two thirds of antibiotics available are obtained from marine actinomycetes [6]. Screening of marine actinomycetes for novel antimicrobial secondary metabolites is gaining momentum in recent years. It is known that the antimicrobial activity of the metabolic products of aquatic bacterial strains is not weaker than the corresponding activity of soil strains [7]. Among the 140 described actinomycetes genera, only a few are responsible for the majority of over 20,000 microbial natural products identified so far [8]. In particular, the genus *Streptomyces* accounts for about 80% of the actinomycetes natural products reported to date [2]. Actinomycetes are best known for their ability to produce antibiotics and are Gram positive bacteria which comprise a group of branching unicellular microorganisms. They produce branching mycelium of two types, substrate mycelium and aerial mycelium. Members of the actinomycetes, which live in marine

environment, are poorly understood and only few reports are available pertaining to actinomycetes from mangroves [9-12].

Free radicals and oxidants play a dual role as both toxic and beneficial compounds, since they can be either harmful or helpful to the body. It has been implicated in the development of many human diseases. A few of them include arthritis, inflammatory diseases, kidney diseases, cataracts, inflammatory bowel disease, colitis, lung dysfunction; pancreatitis; drug reactions, skin lesions, and aging. Free radicals are also associated with liver damage due to alcohol consumption and the development of emphysema due to cigarette smoking [13]. They are produced either from normal cell metabolism *in situ* or from external sources (like pollution, cigarette smoke, radiation and medication). When an overload of free radicals cannot gradually be destroyed, their accumulation in the body generates a phenomenon called oxidative stress. This process plays a major part in the development of several chronic and degenerative illness [14].

Moreover, it has been shown that antioxidants and free radical scavengers are crucial in the prevention of pathologies, in which reactive oxygen species (ROS) or free radicals are implicated [11]. Synthetic antioxidants have been used in stabilization of foods [15]. But their use is being restricted nowadays because of their toxic and carcinogenic effects [16]. Thus, interest in finding natural antioxidants, without any undesirable effect, has increased greatly [17].

Oxidative stress is ultimately involved in endothelial dysfunction, a condition which is evident in adults suffering from various cardiovascular diseases including thalassemia [18-20]. Antioxidant and other supportive therapies protect red blood cells against oxidant damage [21, 22]. Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelating therapy reduces iron-related complications and thereby improves quality of life and overall survival [18, 19]. It is well known that the generation of free radicals happens because of microbial infection which leads to DNA damage [23].

Hence a study was planned to screen marine actinomycetes for antimicrobial and antioxidant secondary metabolites. The strain *Streptomyces* sp VITSTK7 (GQ499369) was isolated from marine soil sample of Bay of Bengal coast, Puducherry. Molecular characterization reveals that the strain *Streptomyces* sp VITSTK7 is a novel strain [8]. Our aim is to optimize the culture condition for better growth and to evaluate its antimicrobial and antioxidant activity.

MATERIALS AND METHODS

Isolation and preparation of crude extracts

The potential strain *Streptomyces* sp VITSTK7 was isolated from marine sediments collected at the Bay of Bengal coast of Puducherry, India. It was inoculated in Kuster's broth and incubated for 7 days at 25ºC. It was centrifuged for 15 minutes at 10,000 rpm and the supernatant collected was mixed with an equal volume of ethyl acetate and kept for overnight in rotary shaker (100 rpm). The crude extract was obtained by removing solvents using rotary evaporator, dissolved in water and lyophilized to a powder form [8]. The stock concentration was prepared as 100 mg/ml.

Physiological characterization

In order to optimize the incubation temperature *Streptomyces* sp VITSTK7 was inoculated in ISP-1 broth (Tryptone Yeast extract Agar medium) and maintained in different incubation temperatures (10, 20, 25, 30, 35, 40 and 45ºC). After incubation the tubes were centrifuged at 8000 rpm for 10 minutes. The pellet was measured for dry weight and tabulated. Then, *Streptomyces* sp VITSTK7 was inoculated again in ISP-1 broth maintained at different pH ranges (4, 5, 6, 7, 8, 9, 10, 11, 12 and 13). After incubation the culture was centrifuged and the dry weight was calculated. Different concentration of NaCl (5, 10, 15, 20 and 25%) were supplemented to ISP-1 broth and *Streptomyces* sp VITSTK7 was inoculated. After seven days of incubation the culture tubes were centrifuged and the dry weight was calculated.

Optimization of carbon source

The utilization of various carbon sources arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose by *Streptomyces* sp VITSTK7 was tested by adding 1% of carbon source separately to the tubes containing sterile ISP-1 broth. The strain *Streptomyces* sp VITSTK7 was inoculated into the broth supplemented with the respective sugars and the culture broth was kept in a shaking incubator for 7 days at 27ºC. Control was maintained without sugar supplement and *Streptomyces* sp VITSTK7 was inoculated. After incubation the tubes were centrifuged at 8,000 rpm for 10 minutes. Supernatant was discarded and the dry weight was calculated.

Optimization of nitrogen source

The utilization of various nitrogen sources like peptone, yeast extract, casein, ammonium sulphate, ammonium nitrate, ammonium citrate, sodium nitrate, cystein and histidine was tested by adding the nitrogen source (1%) to ISP-1 broth separately. The *Streptomyces* sp VITSTK7 was inoculated and incubated at shaking condition for 7 days at 27ºC. After incubation the tubes were centrifuged at 8000 rpm for 10 min. Supernatant was discarded and the dry weight was calculated.

 DOI 10.5455/oams.270412.or.005

Estimation of biomass

The biomass from the culture filtrate was separated by filtration using a pre-weighed Whatman filter paper and it was dried in an oven at 55ºC for overnight and the dry weight was measured. Growth in terms of biomass accumulation was expressed as mg/ml of the culture medium.

Minimal inhibitory concentration

Minimal inhibitory concentration was evaluated by micro broth dilution method using the standard protocol (CLSI M07-A7 for bacteria, CLSI M38-A for fungi) against five bacterial strains: *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853) *Bacillus cereus* (MTCC 430), *Klebsiella pneumoniae* (ATCC 10273), and three *Aspergillus* strains as *Aspergillus niger* (MTCC 281), *Aspergillus fumigatus* (MTCC 343), *Aspergillus flavus* (MTCC 277). DMSO (16 mg/ml) stock of the ethyl acetate (EA) extract was serially diluted with distilled water to get the varying concentration and 100 µl of each concentration was placed in the well. The drug-free positive control was also included for comparison. For each fungal isolates, 100 µl of Conidia culture was added into the respective wells. Microtitre plates were incubated for 48 h at 37ºC in a moist chamber and the MIC was calculated.

Antioxidant activity

DPPH radical scavenging assay

Different concentrations (0.1, 0.5, 1, 5, and 10 mg) of EA extract were dissolved in methanol and ascorbic acid (standard) was used as positive control. DPPH• (1,1-Diphenyl-2-picrylhydrazyl, 0.002%) in methanol was used as free radical. In a test tube, 2 ml of DPPH• solution was mixed with 2 ml of crude extract and standard separately. The tubes were incubated at room temperature in dark for 30 min and the optical density was measured at 517 nm. The absorbance of the control, DPPH• alone (containing no sample), was also noted [24, 25].

The scavenging reaction between DPPH• and an antioxidant (H-A) can be written as follows:

DPPH• + H-A → DPPH-H + A•
(Purple) (Yellow)

Antioxidants react with DPPH•, which is a stable free radical and is reduced to the DPPH-H and as consequence the absorbance's decreased from the DPPH• radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability [26].

The percentage of radical scavenging activity of the extract against the stable DPPH• was calculated using following equation (OD = optical density):

Radical Scavenging Activity (%)
= (Control OD – Sample OD) / Control OD x 100

Fe^{+3} reducing assay

Different concentrations (0.1, 0.5, 1, 5, and 10 mg) of EA extract and tannic acid in 1 ml of methanol were mixed in separate tubes with 2.5 ml of phosphate buffer (200 mM, pH 6.6), and 2.5 ml of 1% potassium ferricyanide and the mixture was placed in a water bath for 20 min at 50ºC, cooled rapidly and mixed with 2.5 ml of 10% trichloroacetic acid and 0.5 ml of 0.1% ferric chloride. The amount of iron(II)-ferricyanide complex formed was determined by measuring the formation of Perl's Prussian blue at 700 nm after 10 min. The increased absorbance of the reaction mixture at 700 nm indicates increased reducing power [27].

Substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm:

Potassium ferricyanide + ferric chloride
Antioxidant ↓
Potassium ferrocyanide + ferrous chloride

Metal chelating assay

The chelating effect on ferrous ions by EA extract was estimated as reported earlier [28]. Briefly, 200 µl of different concentrations of the extracts and 740 µl methanol were added into 20 µl of 2 mM $FeCl_2$. The reaction was initiated by the addition of 40 µl of 5 mM ferrozine into the mixture, which was then shaken vigorously and left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm [29]. The ratio of inhibition of ferrozine-Fe^{2+} complex formation was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{(Control absorbance – Sample absorbance)}}{\text{Control absorbance}} \times 100$$

DNA damage inhibition efficiency

Potential DNA damage inhibition by EA extract of VITSTK7 was tested by photolysing H_2O_2 by UV radiation in presence of pBR322 plasmid DNA and performing agarose gel electrophoresis with the irradiated DNA [30]. Aliquots (1 µl) of pBR322 (200 µg/ml) were taken in two polyethylene micro-centrifuge tubes. EA extract (50 µg) was added in one tube. Another tube was left untreated as the irradiated control. 3% H_2O_2 (4 µl) was added to two tubes which

were then placed directly on the surface of a UV transilluminator (300 nm). The samples were irradiated for 10 min at room temperature. After irradiation, 4 µl of tracking dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol) was added. The samples in all tubes were then analyzed by gel electrophoresis on a 1% agarose gel in TBE buffer (pH 8). Untreated non-irradiated control was made only with plasmid DNA, untreated irradiated control and treated irradiated test sample were loaded in the respective three wells of agarose gel. The bands were observed for protection of DNA by damaging [31].

Statistical analysis

Results are presented as mean ± SD of three independent tests. All tests were carried out in an identical condition.

RESULTS

The isolated actinomycetes strain *Streptomyces* sp VITSTK7 was optimized for culturing conditions such as temperature, pH, NaCl concentration, utilization of carbon sources and nitrogen sources. The optimized temperature was found to be 25ºC for maximal growth and the dry weight was calculated as 4.45 mg/ml (Fig.1). The optimized pH was found to be 7.0. The dry weight of the strain was calculated as 5 mg/ml (Fig.1). The optimized NaCl concentration was found to be 2% and the dry weight of that was calculated as 4.9 mg/ml (Fig.1). Arabinose and sucrose were the two carbon sources effectively utilized by the strain and the dry weight yields were calculated as 4.15 mg/ml and 4.1 mg/ml, respectively.

There was no growth when mannitol and rhamnose were supplemented in the media (Fig.2). Xylose, Inositol, fructose and raffinose were moderately utilized by the strain. Peptone and sodium nitrate were the two nitrogen sources utilized well by the strain and the dry weights were calculated as 4.8 mg/ml and 4.4 mg/ml, respectively (Fig.2).

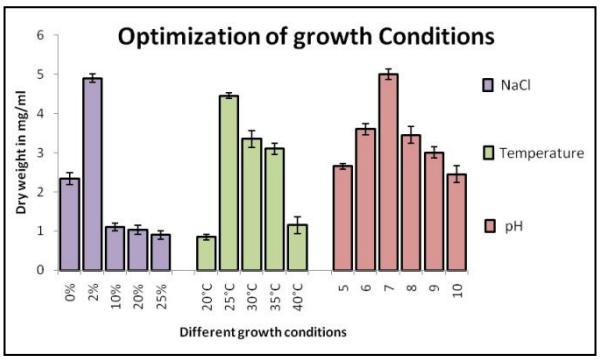

Figure 1. Optimization of growth conditions of *Streptomyces* sp VITSTK7 with different NaCl concentrations, different incubation temperatures and different pH conditions.

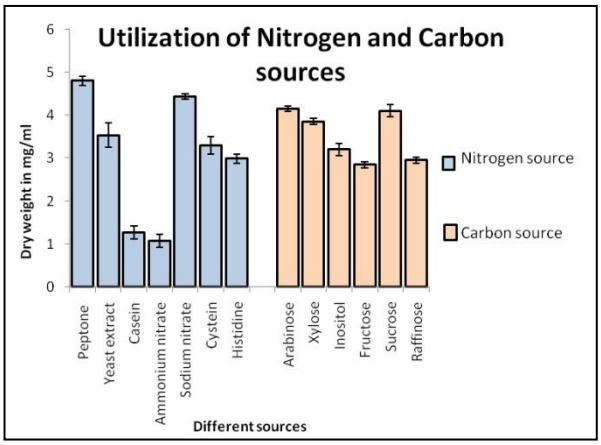

Figure 2. Influence of different carbon sources and nitrogen sources on the growth of *Streptomyces* sp VITSTK7. One percent of the respective sources are supplemented with the culture media.

Table 1. Effect of the ethyl acetate extract of *Streptomyces* sp VITSTK7 on bacterial and fungal pathogens

Strains	MIC value of EA extract	MIC value of antibiotics
Bacteria		(Chloramphenicol)
Escherichia coli	4	0.005
Staphylococcus aureus	4	0.015
Pseudomonas aeruginosa	2	0.015
Bacillus cereus	6	0.025
Klebsiella pneumoniae	6	0.03
Fungi		(Nystatin)
Aspergillus niger	1	0.004
Aspergillus fumigatus	0.25	0.008
Aspergillus flavus	0.8	0.016

The MIC values were given in mg/ml for both test and antibacterial/antifungal standards.

 DOI 10.5455/oams.270412.or.005

There was a moderate growth in the presence of yeast extract, casein, ammonium nitrate, cystein and histidine. However there was no growth in the presence of Ammonium sulphate and Ammonium citrate. The EA extract showed maximal activity against *P.aeruginosa* with the MIC value of 2 mg/ml when compared to chloramphenicol (0.015 mg/ml). It also showed activity against *E.coli* (MIC of 4 mg/ml), *S.aureus* (MIC of 4 mg/ml), *B.cereus* (MIC of 6 mg/ml) and *K.pneumonia* (MIC of 6 mg/ml). The EA extract was found to be very effective against opportunistic fungal pathogens. It showed the MIC value of 0.25 mg/ml against *A.fumigatus*, 0.8 mg/ml against *A.flavus* and 1 mg/ml against *A.niger*. However, the standard antibiotic nystatin showed the MIC value of 0.008 mg/ml, 0.016 mg/ml and 0.004 mg/ml against *A.fumigatus*, *A.flavus* and *A.niger*, respectively (Table 1).

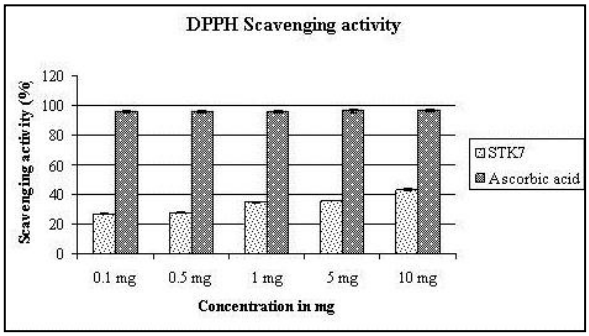

Figure 3. DPPH• scavenging activity of ethyl acetate extracts of *Streptomyces* sp. VITSTK7 and ascorbic acid.

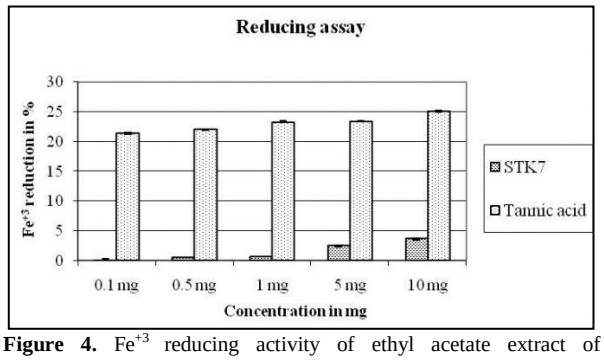

Figure 4. Fe^{+3} reducing activity of ethyl acetate extract of *Streptomyces* sp VITSTK7.

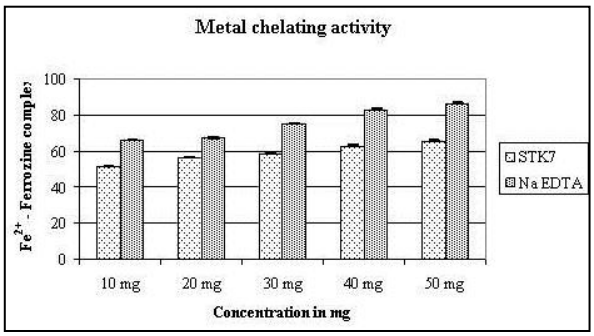

Figure 5. Metal chelating activity of ethyl acetate extract of *Streptomyces* sp VITSTK7

The EA extract of the isolate showed 43.2% DPPH• free radical scavenging activity and compared to standard ascorbic acid which showed 96.7% at 10 mg/ml concentration (Fig.3). The IC_{50} value was calculated as 0.6464 mg/ml using graph pad prism version 5.03. In Fe^{+3} reducing assay the EA extract showed 3.65% and compared to the standard tannic acid which showed 25.06% at 10 mg/ml (Fig.4). The EA extract (10 mg/ml) showed 51% metal chelation (inhibition of ferrozine-Fe^{2+} complex formation) and compared to the standard Na-EDTA which showed 66% (Fig.5). The IC_{50} value for this assay was found to be 43.57 mg/ml.

The DNA damage inhibition efficiency of EA extract is given in Fig.6. The electrophoretic pattern of pBR322 DNA was shown in lane 1. The UV-induced photolysis with H_2O_2 induced oxidative damage on pBR322 was shown in lane 3. The effect of EA extract on irradiated DNA was shown in lane 2. The untreated plasmid DNA pBR322 showed two bands on agarose gel electrophoresis. UV-photolysis of H_2O_2 in lane 3, damages the entire DNA as seen in Fig.6. However EA extract (50 µg) partially protected the DNA damage induced by UV-photolysis of H_2O_2. The faster moving band represented the native form of super coiled circular DNA and the slower moving band corresponded to the open circular form [30]. The UV-induced photolysis of H_2O_2 generates OH• radical and the radical formed causes DNA damage by binding with DNA strand. It was reported that the end products formed as result of ROS reaction attaches to the DNA and it releases mutagenic adducts [32].

Antioxidants are found to play important role in protecting DNA from various ROS mediated damages and may be useful in the treatment of human diseases where oxygen free-radical production is particularly implicated.

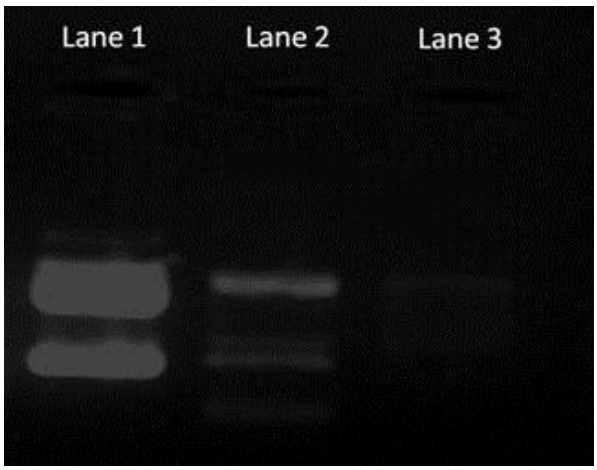

Figure 6: DNA damage inhibition efficiency of EA extract of *Streptomyces* sp VITSTK7. [Lane 1, untreated DNA (control); Lane 3, DNA treated with H_2O_2; Lane 2, DNA treated H_2O_2 with EA extract]

DISCUSSION

Out of 9 maritime states in Indian peninsula only few states were extensively studied for actinobacterial diversity and biological activity, but the vast diversity of actinobacteria in the Indian coast has yet to be fully studied for potential bioactive compounds. *Streptomyces* sp PM-32 isolated from offshore sediments collected at the Bay of Bengal coast has been shown to possess antimicrobial activity against a group of bacterial and fungal pathogens with moderate activity against *Aspergillus* species [33]. Antagonistic activity against different bacterial and fungal pathogens by *Streptomyces* sp isolated from soil samples has been reported [34]. Anti-*Aspergillus* (*A.niger*) activity of *Streptomyces* species isolated from coastal water of Dhanushkodi, Tamil Nadu, India has been reported earlier [35].

Totally 164 strains were isolated from 39 sediment samples collected from the Bay of Bengal coast of Puducherry and Marakkanam and the isolate VITSVK9 showed antibacterial activity against *Bacillus subtilis* (18 mm) and antifungal activity against *Aspergillus niger* (17 mm)[36]. We have reported the antifungal activity of the novel strain; similarly the isolate *Streptomyces s*pVITSTK7 from the Bay of Bengal coast of Puducherry showed anti-*Aspergillus* activity against *Aspergillus fumigatus* (37 mm)[37]. Zhong *et al* have recently reported that the EA extract (1 mg/ml) of mycelia of actinomycetes, *Streptomyces* strain Eri12 isolated from rhizosphere of Rhizoma curcumae Longae and collected from the Ya'an city of Sichuan province, Southwest of China, showed DPPH• radical scavenging activity (51.87%)[38]. DPPH• free radical scavenging activity of actinomycetes USF-TC31 isolated from marine algae was reported by Sugiyama and Hirota in 2009; all the six compounds extracted from the isolate showed good scavenging activity and the ED_{50} of those compounds was calculated as 1434.8, 10.3, 14.6, 1801.8, 14.4, and 13.0 μM, respectively [39]. The compound isolated from 5-(2,4-dimethylbenzyl)pyrrolidin-2-one (DMBPO) (10 μg/ml) from marine actinomycetes *Streptomyces* sp VITSVK5 has been shown to exhibit 59.32% scavenging activity against DPPH• of free radical at the concentration of 10 μg/ml [40].

The antioxidant activity (DPPH• scavenging) of butanol extract of the culture broth of *Streptomyces* sp No 1 and 2 isolated from soil sample collected from Western Ghats of Agumbe, Karnataka, was reported [16]. The isolates 1 and 2 (0.1 mg/ml) showed DPPH• scavenging activity of 51.63% and 53.36%, respectively. At the same concentration, these isolates were capable of reducing Fe^{+3} ions also. Among isolates, *Streptomyces* sp No 2 was found to possess marked antioxidant efficacy than *Streptomyces* sp No 1. The observed activities of the isolates might be due to active principles present in the solvent extracts [16].

From our study we conclude that *Streptomyces* sp VITSTK7 possess significant antimicrobial activity as well as DPPH• free radicals scavenging, Fe^{+3} reducing, metal chelation activity, and inhibition of DNA damage. However, further studies are needed to extract and to identify the active compounds present in the isolate.

ACKNOWLEDGEMENT

The authors thank the Management of VIT University, Vellore, India, for providing the necessary facilities to carry out this work.

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