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ORIGINAL ARTICLE

Extraction of Quinone Derivative from *Streptomyces* sp. VITVSK1 Isolated from Cheyyur saltpan, Tamilnadu, India

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Abstract Extraction of pigments from natural sources is gaining momentum due to many biological applications. The aim of our study was to extract and to identify the pigment produced by *Streptomyces* species isolated from saltpan soil samples. The pigment-producing isolate was characterized by molecular taxonomy, identified as *Streptomyces* species, and designated as *Streptomyces* sp. VITVSK1. The isolate produced green color pigmentation upon solid substrate fermentation using parboiled rice as a media for 7 days at 37°C. The pigment derivative was extracted using methanol as solvent and purified by silica gel column chromatography and preparative thin layer chromatography using chloroform: methanol as solvent system. The purified compound was identified as 2,5-di-*tert*-butyl-1,4-benzoquinone (DTBBQ) based on similarity index with reference compounds available in the mass spectra library, NIST. Structure of the pure compound was also elucidated by ^{1}H and ^{13}C nuclear magnetic resonance spectra. The compound DTBBQ showed 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity with IC_{50} value of 0.6 μg/mL. DTBBQ also showed antimicrobial activity with a zone of inhibition of 21 mm against *Bacillus cereus.* The results of the present study showed that *Streptomyces* sp. VITVSK1 could be a promising source for the production of biologically active quinone-based pigments.

Keywords antimicrobial activity · 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity · quinone derivative · *Streptomyces* sp. VITVSK1 · 2, 5-di-*tert*-butyl-1, 4-benzoquinone

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Introduction

Actinobacteria are one of the most economically and biotechnologically useful microbes, and the secondary metabolites produced by these microbes possess a wide range of biological activities (Berdy, 2005). Quinones are secondary metabolites that are ubiquitous in nature found in bacteria, plants, as well as distributed in various other forms of life (Thomson, 1997). Naturally occurring quinones have captured human attention for thousands of years, for their bright colors with possible use as dyes and drugs (Bayen et al., 2007). There are many derivatives of quinones, which include 1,4-benzoquinone as a subunit that exhibit wide pharmaceutical applications such as antimicrobial (Koyama, 2006), antitumor (Gupta, 1994), and antimalarial (Silva et al., 2009). Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) was reported to possess biological effects that include antitumor and antioxidant properties (McErlean and Moody, 2007). Two free radical scavenging quinones, betulinans A and B were reported from the methanolic extract of *Lenzites betulina* (Lee et al., 1996). These free radical scavengers have the ability to act as protective agents against various diseases such as atherosclerosis, diabetes, rheumatoid arthritis, and cancer-initiation (Hammond et al., 1985). Himalomycins A and B are two quinone antibiotics produced from a *Streptomyces* isolate, B6921. They possess strong antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* (Fotso et al., 2003).

In the present study, we report the extraction and identification of the pigment produced by *Streptomyces* sp. VITVSK1 via UV-visible spectroscopy, fourier transform infrared spectroscopy (FTIR), gas chromatography and mass spectroscopy (GCMS), ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The antioxidant and antibacterial activities of the purified compound were also reported.

Materials and Methods

Isolation and characterization of the isolate. The saltpan soil samples were collected from the Cheyyur region, Kanchipuram district, Tamil Nadu, India. The collected samples were transported to laboratory and stored in refrigerator at 4°C. *Streptomyces* isolate producing pigment was selectively isolated by pour plate technique on ISP no. 4 media (g/L) (soluble starch, 10.0, K_2HPO_4, 1.0, $MgSO_4 \cdot 7H_2O$, 10.0, NaCl, 1.0, $(NH_4)_2SO_4$, 2.0, $CaCO_3$, 2.0, $FeSO_4 \cdot 7H_2O$, 0.1, $MnCl_2 \cdot 4H_2O$, 0.1, $ZnSO_4 \cdot 7H_2O$, 0.1, and distilled water) (Kharel et al., 2010). The isolate was subjected to morphological, chemotaxonomic, and physiological characterization (Shirling and Gottlieb, 1966). The spore chain morphology of the isolate was determined by scanning electron microscopic analysis (Hitachi, Japan; 3 KV, 10 μm). The genomic DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Germany) and subjected to polymerase chain reaction (PCR) amplification using a set of primers Forward (5'-CGCGGCCTATCAGCTTGTTG-3') and Reverse (5'-CCGTACTCCCCAGGCGGGG-3') (Stach et al., 2003). The amplified DNA was purified using NucleoSpin® PCR clean-up Gel extraction kit (MACHEREY-NAGEL GmbH & Co. KG, Germany). The 16S rDNA gene sequencing was carried out for the purified DNA, and the obtained 16S rDNA nucleotide sequence was sequenced using ABI3730xl sequencer (Applied Biosystems, USA) and subjected to BLAST search. Sequence similarity was estimated using ClustalW software (Saitou and Nei, 1987). The phylogenetic tree was constructed by neighbor-joining method using Tree view version software (Page, 1996).

Fermentation and extraction of pigment. Pure culture of the VITVSK1 isolate was used for preparation of seed culture. The seed culture was inoculated in 100 mL of ISP no. 4 media prepared with distilled water, with neutral pH and was incubated for 5 days in rotary shaker (200 rpm) at 37°C. Subsequently, 1% of the inoculum was inoculated on 25 g of parboiled rice and incubated for 7 days at 37°C. Fermentation was carried out for 1 week and extracted with methanol as solvent (SRL Chemicals, India). The solvent layer was collected and filtered using Whatmann paper No 3. The pigment-based compound present in the solvent phase (methanol) was concentrated to dryness using a rotary evaporator (Augustine et al., 2005). The extract was dried and stored at −20°C.

Chromatographic analysis. The methanol layer was concentrated, and the residual dry weight of extract (1.5 g) was chromatographed over a silica gel (60–120 mm mesh size) column and eluted with chloroform:methanol (10:0, 9.5:0.5, 9:1, 8.5:1.5, 8:2, 7.5:2.5, and 7:3).

UV-visible spectroscopy. The active fractions from the silica gel column chromatography were dried and the absorption spectra of the isolated pure compound dissolved in methanol were recorded in the wave length region of 200–700 nm using UV-visible spectrophotometer (ELICO double beam SL 210, India) with a 3-cm quartz cell path length.

Fourier transform infrared spectroscopy. The KBr pellet (13 mm) prepared using the pure compound was used to obtain the Fourier transform infrared spectroscopy (FTIR) spectra (Shimadzu IR affinity-1 FTIR spectrometer, Japan) in the scanning range of 400–4000 cm^{-1} at a resolution of 4 cm^{-1}.

Gas chromatography and Mass spectroscopy analysis. The pure compound separated was analysed by using GC-MATE-II gas chromatography-mass spectrometer (Agilent JEOL, USA). The gas chromatographic oven was held at 80°C (Initial temp) for 1 min, ramp 10°C/min to 280°C, hold 2 min, total run time: 24.00 min InjA auto = 250°C, Volume = 1 μL, Split = 10:1. Helium was used as the carrier gas at 30 mL/min, and the mass conditions include solvent delay 2.00 min, transfer temp = 150°C, source temp 150°C, scan: 50 to 600 Da. The identification of the compound was based on 90% similarity between the MS spectra of the unknown compound and reference compounds available in the MS spectra library, NIST (National Institute for Standards and Technology).

Nuclear magnetic resonance spectroscopy. The ^1H and ^{13}C NMR spectra were recorded for the isolated compound (UNITY-500, Switzerland, 500 MHz) using $CHCl_3$ as the solvent and tetra methyl silane as the internal reference. The structure of the compound extracted from the isolate VITVSK1 was established with the help of spectral data obtained from spectroscopic methods employed. The 2D structure of the compound was obtained using Chem3D Draw Ultra software (Version 10).

2,2-Diphenyl-1-picrylhydrazyl assay. The antioxidant activities of the extract and pure compound were ascertained by 2,2-diphenyl-1-picrylhydrazyl assay. The stock concentration of the sample was 1 mg/mL and working concentrations ranging from 10, 20, 30, 40, and 50 μg were taken, and volume was made up to 1 mL with distilled water. To this 3 mL of 0.16 mM DPPH in methanol was added. The contents were incubated at room temperature, for 30 min in dark, and absorbance was measured at 517 nm (Duan et al., 2006). The absorbance of the DPPH control (containing no sample) was also noted. The percentage inhibition is calculated with blank using the formula given below (Yan and Chen, 1995).

$$\% \text{ inhibition} = ((\text{Blank OD} - \text{Sample OD})/\text{Blank OD}) * 100$$

Agar diffusion assay. The antimicrobial activity of the pure compound was evaluated against *Bacillus cereus* (MTCC-1168). A fresh culture (0.2 mL) of each organism was inoculated into 5 mL of sterile nutrient broth (Hi Media) and incubated for 3–5 h to standardize the culture to McFarland standards (10^6 CFC/mL). Three replicates of respective microorganism were prepared by spreading 100 μL of revived culture on Mueller Hinton Agar (Hi Media) with the help of spreader. One hundred micrograms of pure compound extracted from the isolate VITVSK1 was added to a well (7 mm), and streptomycin disk (10 μg) was used as a positive control. The Petri plates were incubated at 37°C for 24 h. The zone of inhibition (mm) surrounding the well was measured (Holder and Boyce, 1994).

Table 1 Culturing conditions and chemical characteristics of *Streptomyces* sp. VITVSK1.

Tests	Result
Grams stain	+
Aerial mycelium	Green
Motility	-
Spore chain	Smooth
Methyl red test	+
Voges proskauer test	+
Triple sugar iron test	-
Citrate utilization test	+
Carbon source (1% w/v)*	
D-glucose	+
D-maltose	+
D-galactose	+
D-mannose	+
D-sucrose	+
Nitrogen source (1% w/v)*	
Peptone	+
Yeast extract	+
Ammonium sulphate	+
Potassium nitrate	+
Casein	-
Aminoacid sources (1% w/v)*	
Histidine	+
Glycine	+
Tryptophan	+
Tyrosine	-
NaCl concentration (w/v)*	
3%	+
5%	+
7%	+
9%	-
Temperature for growth (C)*	
Range	20-45
pH for growth*	
Range	6.0-7.5
Chemical characteristics	
Cell wall aminoacids	Meso-DAP
Phospholipids	Type III
G+C content (mol %)	65

*Growth of the isolate was measured as dry weight of the mycelium

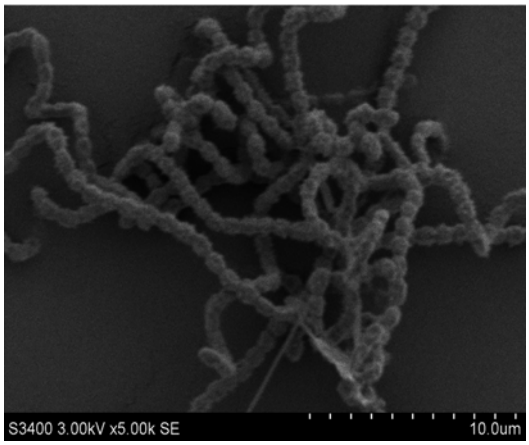

Fig. 1 Scanning electron microscopic image of *Streptomyces* sp. VITVSK1 strain. Spore chain morphology at 10-μm size.

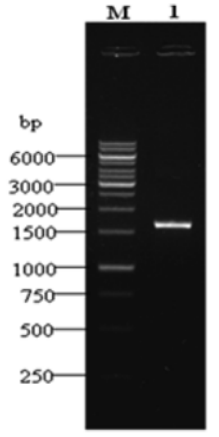

Fig. 2 PCR gel picture showing amplification of 16S rDNA of the *Streptomyces* sp. VITVSK1.

Results

Isolation and identification of actinomycetes. The isolated strain is Gram-positive and aerobic bacteria. The cultural, biochemical, and chemotaxonomic characterization studies of the isolate is provided in Table 1. The spore chain morphology of the pigment-producing isolate was determined by SEM analysis. The aerial mycelium of the isolate with branched hyphae, bearing non-motile spores with smooth surface was observed under electron microscope (Fig. 1). The isolate was identified by molecular taxonomic approach. The 16S rDNA of the isolate was evaluated by PCR amplification (Fig. 2) and subsequent construction of phylogenetic tree (Fig. 3). Using the BLAST search tool in the NCBI data bank, sequences homologous to our isolate were collected and aligned using ClustalW (DDBJ). Based on the molecular taxonomy and phylogeny, the isolate was identified to belong to the genus *Streptomyces* and designated as *Streptomyces* sp. VITVSK1. The 16S rDNA nucleotide sequence was submitted to GenBank, NCBI with the accession ID JQ429445.

Production medium. Solid substrate fermentation was carried for the growth of the VITVSK1 isolate, and parboiled rice was used as the production medium. The methanolic extract gave higher DPPH scavenging activity.

Identification of the compound. The fractions obtained from the column chromatography were screened for DPPH scavenging activity, and the active fractions were collected, concentrated, and

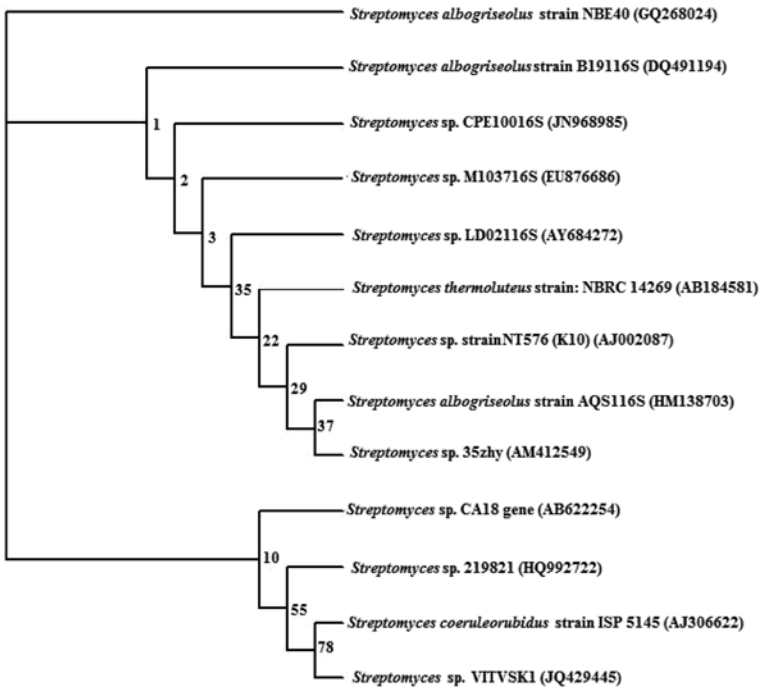

Fig. 3 The taxonomic position of the *Streptomyces* sp. VITVSK1 shown using neighbour-joining method based on Tree view version software. Bootstrap values are presented at the nodes of the tree.

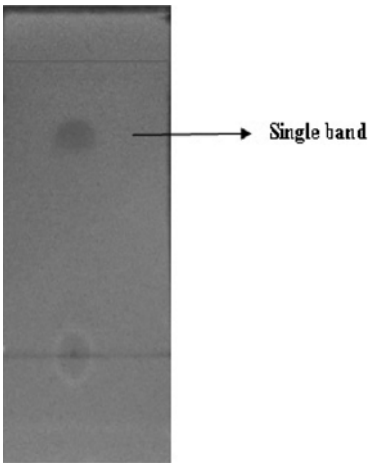

Fig. 4 TLC separation of the pure compound showing a single spot in chloroform/methanol (9:1) as mobile phase.

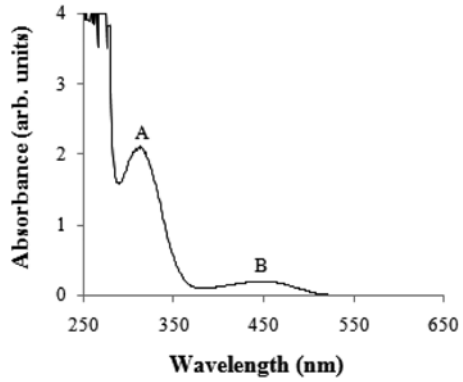

Fig. 5 UV spectrum of the pure compound extracted from *Streptomyces* sp. VITVSK1. (A) Benzene peak, (B) Quinone group peak

further separated by thin layer chromatography (TLC). Solvent system (chloroform: methanol in 9:1 ratio) had been used to elute the pure compound. TLC separation showed one distinct spot corresponding to single purified compound with an Rf value of 0.7 cm (Fig. 4).

Ultraviolet absorption spectroscopy. The λ_{max} of the separated compound determined using UV-visible spectrophotometer was found to be 290 and 450 nm. The UV spectrum showed two peaks representing a peak for benzene group (290 nm) and quinonoid groups (400–500 nm) (Fig. 5).

Fourier transform infrared spectroscopy. FTIR spectra of the pure compound showed peaks corresponding to standard library spectra (Fig. 6). A broad peak observed at 1616 cm^{-1} was due to the presence of aromatic C=C stretching. A peak observed at 2972 cm^{1} can be assigned to aliphatic C–H stretching frequency. A peak observed at 3064 cm^{-1} indicated the presence of aromatic C-H stretching frequency. A peak observed at 842 cm^{-1} indicated the presence of para di-substituted compound.

Gas chromatography-Mass spectrometry. The GC-MS spectra obtained for the extracted compound (Fig. 7) was matching with quinone derivative 2,5-di-*tert*-butylcyclohexa-2,5-diene-1,4-dione also commonly known as 2,5-di-*tert*-butyl-1,4-benzoquinone with

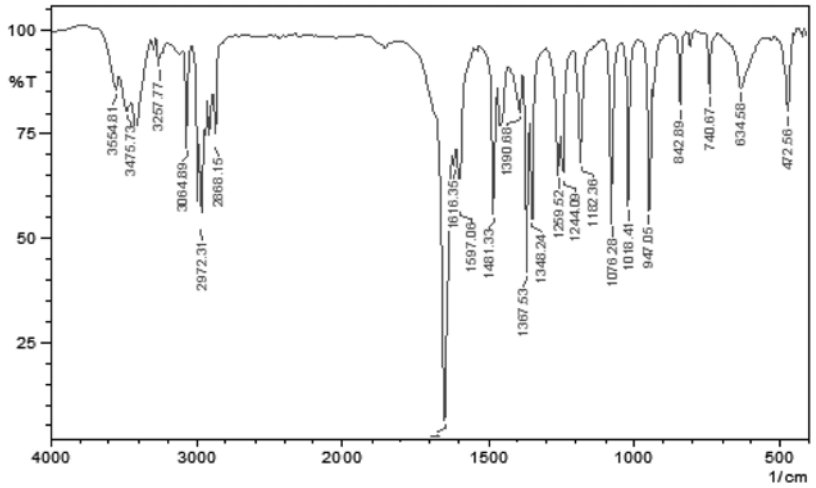

Fig. 6 FT-IR spectra of the pure compound extracted from *Streptomyces* sp. VITVSK1.

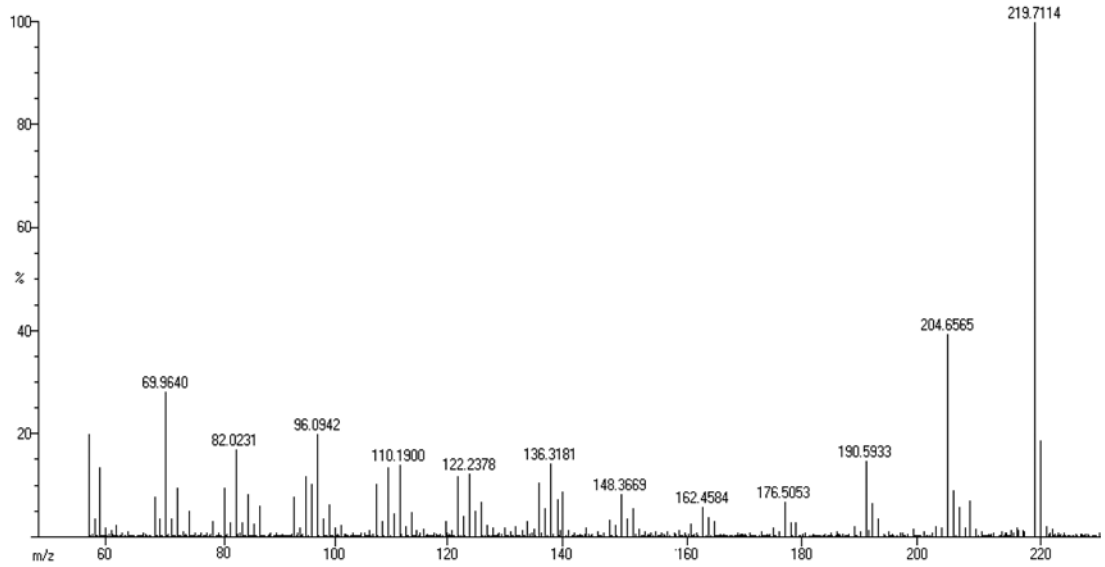

Fig. 7 GC-MS spectra of the pure compound extracted from *Streptomyces* sp.VITVSK1

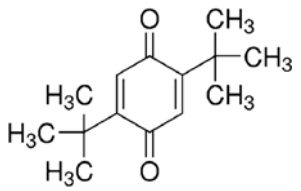

Fig. 8 Structure of the pure compound predicted based on the NMR spectral data.

a molecular weight of 220.3 using the NIST library.

Nuclear magnetic resonance spectroscopy. The pure compound was pale yellowish in colour. The ^{1}H-NMR spectra ($CHCl_3$, Me4Si, 500 MHz) 1.3 (s, 18H, CH_3) 6.5 (s, 2H, CH): ^{13}C-NMR: (500 MHz, $CHCl_3$) 29.3, 34, 133, 154 and 187 ppm. Based on the spectral data, the molecular formula was established as $C_{14}H_{20}O_2$. The 2D structure of the pure compound was modelled using Chem3D Ultra software (version 10) (Fig. 8).

DPPH assay. The DPPH scavenging activity (IC_{50} value) of the methanol extract was found to be 27.19 μg/mL, and the pure compound 2, 5-di-*tert*-butyl-1, 4-benzoquinone (DTBBQ) showed IC_{50} value of 0.6 μg/mL compared with the standard ascorbic acid which showed IC_{50} value 2.65 μg/mL (Fig. 9).

Agar diffusion. The pure compound DTBBQ extracted from *Streptomyces* sp. VITVSK1 showed antibacterial activity against *B. Cereus* (21 mm) (Fig. 10B). The standard antibiotic streptomycin showed a zone of inhibition of 18 mm (Fig. 10A). The solvent dimethyl sulphoxide (DMSO) did not showed any zone of inhibition (Fig. 10C).

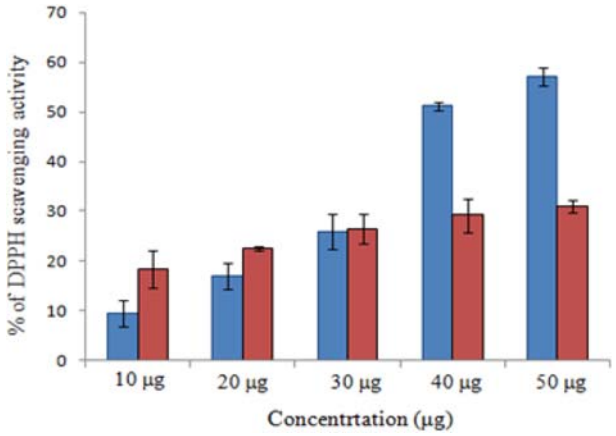

Fig. 9 DPPH radical scavenging activity of the methanolic extract and the pure compound.

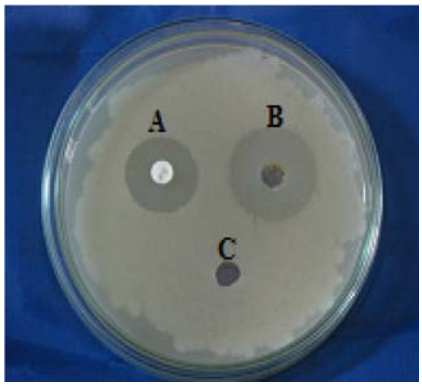

Fig. 10 Zone of inhibition of the pure compound extracted from *Streptomyces* sp. VITVSK1 strain against *Bacillus cereus*. (A) Streptomycin, (B) Pure compound, and (C) Control- DMSO solvent.

Discussion

Microbial metabolites are of tremendous potential due to their wide range of pharmacological activities (Soliev et al., 2011). Several quinone-based pigment compounds that had been reported include an ultramarine blue pigment actinorhodin produced by *Streptomyces coelicolor* (Zhang et al., 2006). Katorazone is a new yellow pigment produced by *Streptomyces* sp. IFM 11299 (Abdelfattah et al., 2012). Benzoquinone ansamycin antibiotics contain quinone group, which include geldanamycin that had been reported from recombinant *Streptomyces autolyticus* JX-47 (Dai et al., 2011). Benzoquinone derivatives, 8-demethylgeldanamycin and 4,5-epoxy-8-demethylgeldanamycin, were found to posses various pharmacological properties such as antibacterial, antitumor, and cytotoxic activities (Buchanan et al., 2005). The antioxidant property of 1,4-benzoquinones has been reported (Lisa et al., 1972). Green aminobenzoquinone derivative called blennione was isolated from toad stool (mushroom) *Lactarius blennius* (Spiteller and Steglich, 2002). In the present study we report the extraction and identification of pigmenting compound 2,5-di-*tert*-butyl-1,4-benzoquinone from *Streptomyces* sp. VITVSK1. The pigment nature of the compound may be due to the presence of carbonyl carbon and quinoid group in DTBBQ as well as the influence of culture conditions. The conjugated π electrons have the tendency to delocalize, creates energy difference between highest occupied molecular orbital and lowest unoccupied molecular orbital. The energy difference will create a particular wavelength of light to be absorbed. The remaining light is reflected that gives rise color pigment (Charkoudian et al., 2010).

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