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Research Article

ISOLATION AND IDENTIFICATION OF ANTI-ESBL (EXTENDED SPECTRUM β-LACTAMASE) COMPOUND FROM MARINE *STREPTOMYCES* SP. VITSJK8

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ABSTRACT

Multidrug resistant extended spectrum β-lactamse producing organisms are more frequently reported from urinary tract infected UTI patients. Recent reports have shown that ESBL's efficiently hydrolyze third generation cephalosporins and monobactams, thereby limiting the therapeutic options. The aim of the present study was to isolate and to identify anti-ESBL compound from marine *Streptomyces* sp.VITSJK8. It was isolated from marine sediment sample collected at Cheyyur beach, Bay of Bengal, Tamil Nadu, India. The ethyl acetate extract from the culture supernatant of *Streptomyces* sp.VITSJK8 was prepared and purified by silica gel column chromatography and HPLC. The purified compound was identified by using ^{1}H and ^{13}C NMR spectral data and based on the similarity index with reference compounds available in the mass spectra library of National Institute for Standards and Technology as 1, 2-benzene dicarboxylic acid, mono (2-ethylhexyl) ester. The anti-ESBL activity of the purified compound was tested against ESBL isolates according to the National Committee for Clinical Laboratory Standards guidelines and it was revealed that its MIC_{50} value ranged from 0.13 to 2.00 µg/mL. The results of the study suggest that *Streptomyces* sp.VITSJK8 is the potential source for anti-ESBL compounds.

Keywords: Extended spectrum β-lactamse, Multidrug resistance, *Streptomyces* sp. VITSJK8, 1, 2-benzene dicarboxylic acid, mono (2-ethylhexyl) ester.

1. INTRODUCTION

Extended spectrum β- lactamases (ESBLs) producing microbes are currently considered as emerging threat for humans worldwide. These organisms are multidrug resistant in nature and the mechanism of drug resistance is due to production of enzyme called extended spectrum β-lactamases (ESBLs). The β-lactamase enzymes produced by these organisms break down the structural beta-lactam ring of β-lactam antibiotics. Many genera of gram negative bacteria possess a naturally occurring, chromosomally mediated β-lactamase and also some are plasmid mediated β- lactamases [1]. Beta lactam family consisting of four major groups of antibiotics: penicillins, cephalosporins, monobactams and carbapenems. Extended-spectrum β lactamases (ESBLs), have the ability to hydrolyze and cause resistance to various types of the newer β-lactam antibiotics, including the extended-spectrum (or third-generation) cephalosporins (cefotaxime, ceftriaxone and ceftazidime) and monobactams (aztreonam), but not the cephamycins (cefoxitin and cefotetan) and carbapenems (imipenem, meropenem and ertapenem) [2]. The enzyme contains serine at the active site which reacts mainly with the carbonyl carbon of the β-lactam ring leads to opening of the ring (inactive β lactam) and regenerating the β lactamase [3]. ESBL enzymes are commonly found in *Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Escherichia coli Proteus mirabilis*, *Salmonella* species and other members of Enterobacteriaceae [4]. Occurrences of ESBL producing Enterobacteriaceae have also reported in South India and Central India [5]. Apart from urban cities, ESBL pathogens spread to the rural villages also. In the past two decades, gram negative bacilli producing ESBLs causes major problem in hospital setup. Currently 70% of the infections caused by the bacteria in hospitals that are resistant to at least one of the drugs commonly used for routine treatment purposes [6]. Microorganisms that produce ESBL's remain an important reason for therapy failure with cephalosporins and have serious consequences for infection control [7]. More than 95% of urinary tract infections are caused by *Escherichia coli*. The ESBL resistance is increasing among UTI pathogens due to usage of conventional drugs worldwide [8]. High levels of antimicrobial resistance in uropathogens were already reported in UTI patients [9]. Routine monitoring of antibiotic resistance provides data for antibiotic therapy and resistance control. Surveillance of antimicrobial resistance in frequently isolated human pathogens can help to develop rational therapies [10].

To overcome the potential emergence of drug resistance by ESBLs, a replacement of existing antibiotics is very much required. Marine actinomycetes are Gram positive bacteria having high G+C (> 55%) content in their DNA. About 80%

of antibiotics available in the market are from *Streptomyces* [11]. They produce numerous bio-active compounds that yielded valuable therapeutics such as enzymes, antibiotics etc.

2. MATERIALS AND METHODS

2.1. Isolation of ESBL Isolates

ESBLs were isolated from the urine sample of urinary tract infected (UTI) patients and confirmed as per CLSI-2010 guidelines. Positive ESBL was recorded when an enhanced zone of inhibition of β-lactamase inhibitor containing discs (Hi-media, Mumbai), cefotaxime/clavulanic acid (30µg/10µg) was ≥ 5mm than the cefotaxime (30µg) alone. ESBL production was also further confirmed by using E-Test strips and the strip was calibrated with MIC reading scales in µg/ml [12].

2.2. Isolation and Characterization of Actinomycetes Isolate

The potential actinomycetes isolate was isolated from marine sediment sample collected at Cheyyur beach (latitude-12 ° 66′, longitude-79 ° 54′), Bay of Bengal, Kanchipuram, Tamil Nadu, India. The culture of the isolate was maintaned on ISP1 (trptone yeast extract) media containing (g/L): Casein enzymic hydrolysate- 0.5; Yeast extract -0.3; and agar- 2.0 (pH - 7.0 ± 0.2).

2.3. Extraction and Purification of the Anti-ESBL compound

Matured colonies of actinomycetes isolate were gram stained to check for purity and then inoculated in to 500 ml ISP-1 broth prepared with 50% distilled water, 50% sea water at pH 8.0 in 1000 ml Erlenmeyer flask and fermented in an orbital shaker for 7 days at 28°C [13]. After fermentation the broth was centrifuged at 10000 x *g* for 15 minutes. Equal volume of ethyl acetate (1:1 v/v) solvent was added to cell free supernatant and kept in the shaker overnight for thorough mixing. All procedures were done under strict aseptic conditions. Aqueous phase was collected through the separating funnel. The ethyl acetate (EA) extract was then concentrated in rotary vacuum and lyophilized in a freeze dryer (Thermo, USA). The ethyl acetate (EA) extract (2.0 g) was dissolved in 3.0 mL methanol and loaded on the silica gel column (60-120 mesh, SRL, India) after filtration. Chloroform/methanol mixture (98:2) v/v) was used as solvents and 10 mL fractions were collected at a flow rate of 1 mL/min. All the fractions were concentrated using a rotary evaporator and screened for anti-ESBL activity. Those fractions that showed anti-ESBL activity were pooled, concentrated and lyophilized using a freeze dryer, redissolved in methanol, filtered through a 0.45 µm filter (Ranbaxy, India) and subjected to preparative HPLC purification.

Purification of the active fraction was carried out using preparative HPLC system (Schimadzu, Japan) with Zorbax Eclipse plus C_{18} column (4.6 x 250 mm, 5 µm) connected to a diode array detector and fraction collector. Ionised water and methanol were used as mobile phase. The sample (100 µL) was injected to the column and the flow rate was maintained at rate 1 min/mL with run time of 36 min [14]. The fractions obtained were continuously monitored at the wavelength 210-290 nm. All fractions were screened for anti-ESBL activity and the active fraction was subjected to spectrascopic studies.

2.4. Structure elucidation

Fourier transform infra-red spectroscopy. The KBr pellet (13 mm) prepared using the pure compound was used to obtain the 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}. The FTIR spectra were analyzed for the presence of various functional groups.

2.5. GC-MS analysis

The purified compound was analyzed by using GC Clarus 500 (Perkin Elmer, Singapore) equipped with an Elite-5MS column (30 x 0.25 mm x 0.25 µm) composed of 5% diphenyl and 95% dimethyl-polysiloxane). An electron ionization system with ionizing energy of 70 eV was used. For GC-MS the GC oven was held for 2 min at 110°C and then ramped from 110°C to 280°C at 10°C/min. Total run time was 36 min, InjA auto was 250°C at 2 µL volume and split 10:1. Helium was used as the carrier gas with a flow of 30 mL/min and the mass conditions included solvent delay 2 min, transfer and source temperature 150°C and scan from 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 of NIST (National Institute for Standards and Technology).

2.6. Nuclear magnetic resonance spectroscopy

The ^{1}H and ^{13}C NMR spectra were recorded for the isolated purified compound (Bruker, Germany, 400 MHz) using $CDCl_3$ as the solvent and tetramethylsilane as the internal reference. The structure of the anti-ESBL compound extracted from *Streptomyces* sp. VITSJK8 was established with the help of spectral data obtained from spectroscopic analysis. The 2D structure of the compound was obtained using Chem3D Draw Ultra software (Version 10) [15].

2.7. MIC_{50} value

MIC_{50} value of the bioactive compound was determined by broth dilution method recommended by National Committee of Clinical Laboratory Standards (NCCLS) guidelines. Stock solution of the standard antibiotic (Ertapenem) was prepared and taken as positive control. The compound was prepared by dissolving in sterile water to make a concentration of range 0.125-8.0 µg/mL [16]. ESBL clinical isolates *Klebsiella pneumoniae* (ATCC 700603), *E.coli* (VITEB1), *Klebsiella pneumoniae* (VITEB2), *Klebsiella pneumoniae* (VITEB5), *Klebsiella pneumoniae* (VITEB6) and *Klebsiella pneumoniae* (VITEB8) were used for anti-ESBL assay.

3. RESULTS AND DISCUSSION

3.1. Isolation, identification and characterization of VITSJK8

The screening of marine sediment sample collected from the Cheyyur beach, Bay of Bengal, Kanchipuram, Tamil Nadu, India for anti ESBL activity yielded a potential isolate named VITSJK8. The isolate was cultured on ISP1 culture medium and culturing conditions are given in Table 1. The growth of the isolate and surface morphology of the isolate under electron microscopy are given in Fig. 1. The isolate was identified according to Shirling and Gottlieb method [17] and designated as *Streptomyces* sp.VITSJK8 (KF289838). Blast search of the 16S rDNA sequence showed 99% similarity with *Streptomyces fradiae* (NR_043485).

Table 1. Cultural characteristics of Streptomyces sp.VITSJK8.

Morphological		Biochemical	
Gram staining	+	Citrate	+
Motility	-	Starch	+
Spore mass pink	+	Urease	+
Diffusible pigment	-	Oxidase	-
Spore surface	Smooth	H_2S production	-
Physiological		Casein	+
Growth at 28°C	+	Gelatin liquefaction	-
Growth at NaCl 1%(w/v)	+	Soyabean meal 1%(w/v)	+
Growth at pH 7	+	Ammonium chloride 1%(w/v)	+
		Mannitol 1%(w/v)	+
		Indole	-
		MR &VP	-
		Catalase	-
		Hemolytic activity	-
		Chitinase activity	-

+ positive: -negative

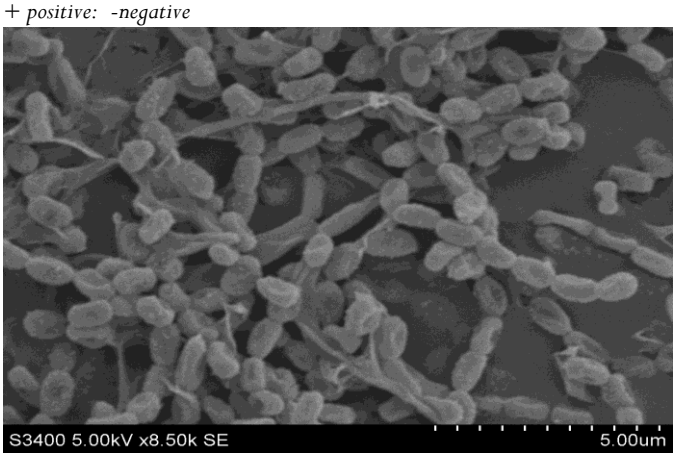

Fig. 1. Growth of Streptomyces sp. VITSJK8 on ISP medium. SEM image showing the arrangement of its spores

3.2. Purification and structure elucidation

The isolate was cultured in shaker flasks using ISP1medium and incubated at 28° C, for fourteen days under constant shaking (150 rpm). The EA extract (2g) was injected to column chromatography and eluted stepwise with chloroform-methanol as the eluent. Thirty three fractions were collected and screened for anti-ESBL activity. The active fraction was then injected in preparative HPLC for further purification. All fractions were collected and active fraction showed anti-ESBL activity against all ESBL clinical isolates, *Klebsiella pneumoniae* (ATCC 700603), *E.coli* (VITEB1), *Klebsiella pneumoniae* (VITEB2), *Klebsiella pneumoniae* (VITEB5), *Klebsiella pneumoniae* (VITEB6) and *Klebsiella pneumoniae* (VITEB8) was detected. One major fraction (I) with the retention time of 5.548 min found to have anti-ESBL activity. Two other minor fractions (II and III), with retention times of 14.203 and 17.186, respectively, were also identified but they did not show any anti-ESBL activity against tested ESBL clinical isolates. The chromatogram pattern of preparative HPLC is given in Fig. 2. The active fraction having anti-ESBL activity showed absorption maxima at 222 nm. Active fractions were collected, concentrated and lyophilized for further characterization. The structure of the compound was established with the help of spectral data obtained using FTIR, GCMS, ^{1}H and ^{13}C NMR.

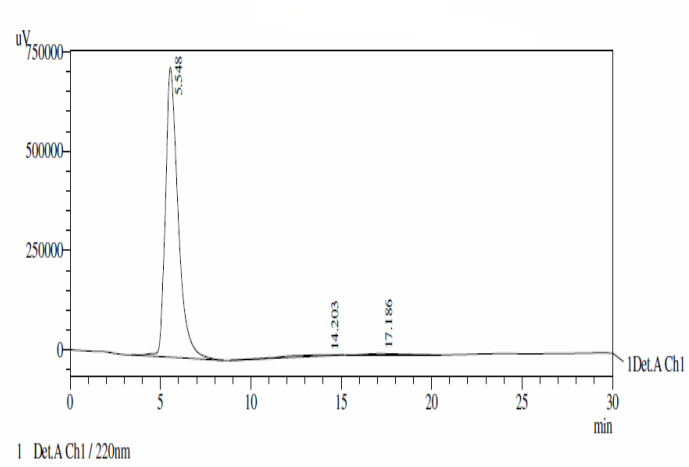

Fig. 2. Preparative HPLC chromatogram of the purified fraction of Streptomyces sp.VITSJK8.

3.3. Fourier transform infrared spectroscopy

FTIR spectrum of the purified compound showed peaks corresponding to standard library spectra (Fig. 3). The peak observed at 3130 cm^{-1} corresponds to alkenes C-C stretching. A peak at 2875.86 cm-1 represents aldehyde C–H stretching. A peak at 1404 cm^{-1} indicates the presence of aromatic C-C stretching. Peaks observed from 1298 cm^{-1} to 1124 cm^{-1} represents aliphatic C-N stretching. A single peak at 948 cm^{-1} indicates O-H carboxylic acids and other peaks from 858 cm^{-1} to 704 cm^{-1} corresponds to C-H stretching.

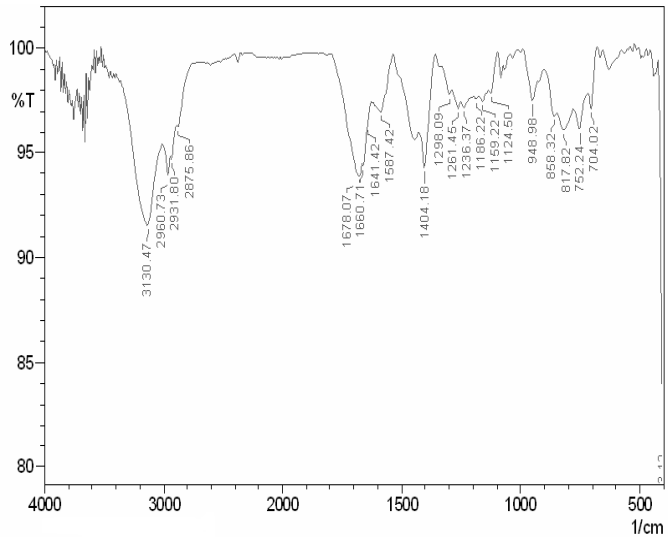

Fig. 3. FTIR spectrum of the purified compound from Streptomyces sp. VITSJK8.

3.4. Gas chromatography-mass spectrometry

The GC-MS spectra obtained for the extracted compound (Fig. 4) was matching with 1,2-benzene dicarboxylic acid, mono (2-ethylhexyl) ester (DMEHE) with a molecular weight of 279.24 using the NIST library.

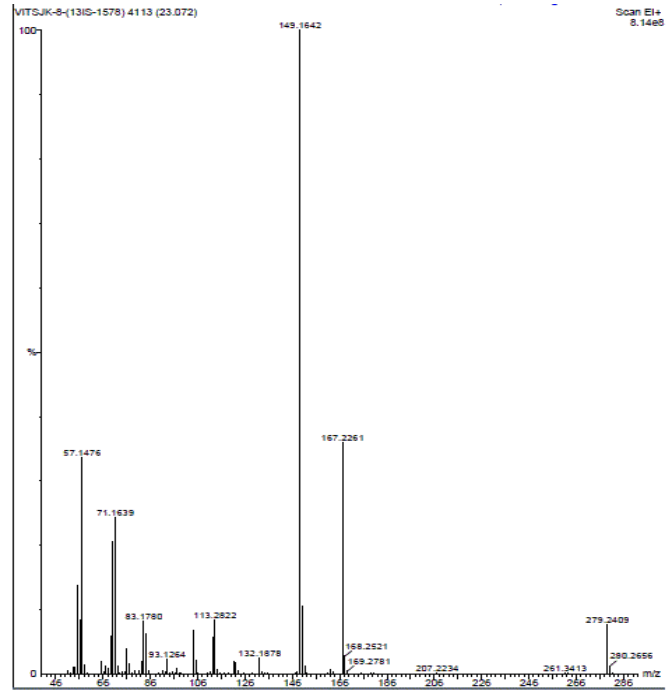

Fig. 4. GC-MS spectrum of the purified compound from Streptomyces sp. VITSJK8.

3.5. Nuclear magnetic resonance spectroscopy

The ^{1}H-NMR spectrum ($CDCl_3$; 400 MHz) with 0.8 -0.85 (m, 6H, CH3), 1.2 -1.4 (m, 9H, CH2, CH), 3.8 (d, 2H, CH2) and 7.56 -7.74 (m, Ar- 4H) is demonstrated in Fig. 5. The ^{13}C-NMR spectrum ($CDCl_3$; 400 MHz) with 170.1(COOH), 168.6 (C=O), 133.8 (Ar-c), 132.9 (Ar-c), 132.8 (Ar-c), 132.5 (Ar-c), 128.4 (Ar-c), 128.0 (Ar-c), 66.9 (CH2), 38.9 (CH), 31.9 (CH2), 29.3 (CH2), 23.7 (CH2), 23.0 (CH2), 14.1(CH3) and 11.6 (CH3) is given in Fig. 6. Based on the spectral data, the compound was identified as 1,2-benzene dicarboxylic acid, mono (2-ethylhexyl) ester with the molecular formula of $C_{16}H_{22}O_4$. The 2D structure of the purified compound (Fig. 7) was modeled using Chem3D Ultra software (version 10).

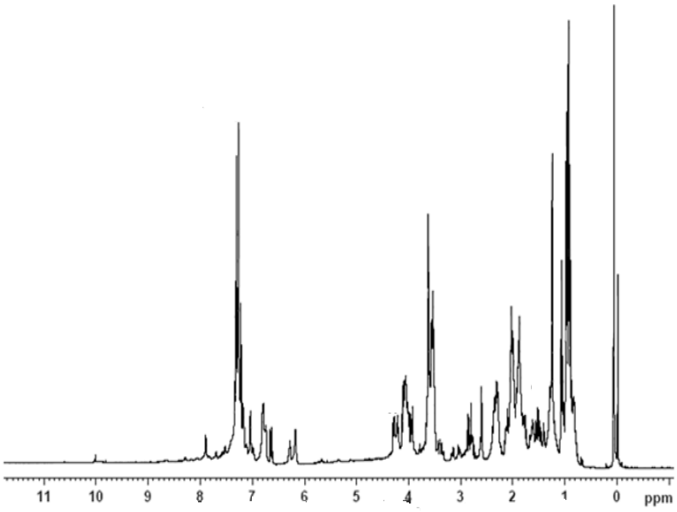

Fig. 5. ^{1}H NMR spectrum for the purified compound from Streptomyces sp. VITSJK8. Confirmation

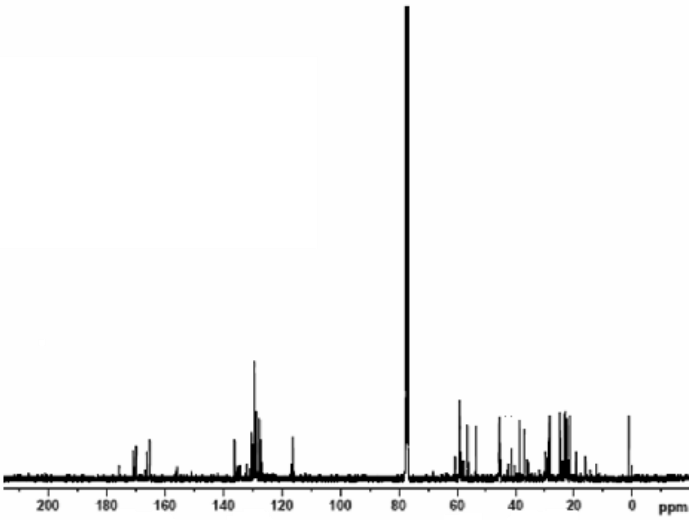

Fig. 6. ^{13}C NMR spectrum of the purified compound from Streptomyces sp. VITSJK8

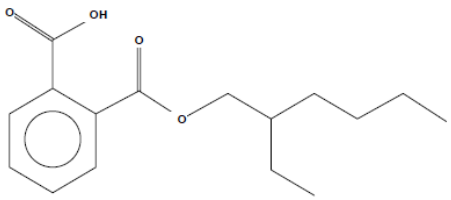

Fig. 7. Structure of the purified compound 1,2-benzene dicarboxylic acid, mono (2-ethylhexyl) ester from Streptomyces sp. VITSJK8 predicted based on the NMR spectrum (Chem3D Draw Ultra software-Version 10).

3.6. MIC_{50} value of the lead compound

The MIC_{50} values of anti-ESBL activity of the purified compound are given in Table 2. DMEHE showed the MIC value of 2.0 μg/mL against *Klebsiella pneumoniae* (ATCC 700603), 1.0 μg/mL against *E.coli* (VITEB1), 1.0 μg/mL against *Klebsiella pneumoniae* (VITEB2), 0.5 μg/mL against *Klebsiella pneumoniae* (VITEB5), 0.25 μg/mL against *Klebsiella pneumoniae* (VITEB6) 2.0 μg/mL against *Klebsiella pneumoniae* (VITEB7) and 0.13 μg/mL against *Klebsiella pneumoniae* (VITEB8). The ESBL isolate *Klebsiella pneumoniae* (VITEB8) was found to be highly susceptible to 1,2-benzene dicarboxylic acid, mono (2-ethylhexyl) ester (DMEHE). Ertapenem was used as the positive control. DMEHE showed better inhibition over (0.125 to 2.0 μg/mL) ESBL clinical isolates when compared to cefotaxime/clavulanic acid (30μg/10μg).

Table 2. MIC_{50} values of 1,2-benzene dicarboxylic acid, mono (2-ethylhexyl) ester isolated from Streptomyces sp.VITSJK8

ESBL isolates*	Eratapenum (μg/mL)	DMEHE (μg/mL)
Klebsiella pneumoniae (ATCC 700603 **)**	2	2.0
E. coli (VITEB 1)	2	1.0
Klebsiella pneumoniae (VITEB2)	0.5	1.0
Klebsiella pneumoniae (VITEB5)	2	0.5
Klebsiella pneumoniae (VITEB6)	1	0.25
Klebsiella pneumoniae (VITEB7)	4	2.0
Klebsiella pneumoniae (VITEB8)	2	0.125

**0.5 Mc Farlands/ 0.5 mL turbidity in 0.85% saline.*

Actinomycetes have produced several biomolecules as secondary metabolites and have been used as reservoir for bioactive secondary metabolites [18]. A β-lactamase inhibitory protein (BLIP-I) from *Streptomyces exfoliatus* SMF19 was reported to possess anti-ESBL activity against TEM-1 target protein of ESBL pathogens [19]. A sugar molecule produced by *Steptomyces hygroscopicus* subsp. ossamyceticus (strain D10) isolated from the soil sample collected at Thar desert, Rajasthan, India have been reported to possess anti-ESBL activity (zone of inhibition) against *Escherichia coli* (20 mm) and *Klebsiella* sp. (15 mm) [20]. The widespread clinical use of clavulanic acid as beta-lactamase inhibitor was proficient against resistant pathogens in the past. Unfortunately, the emergence of clavulanic acid-resistant variants of beta-lactamase producing organisms resulting in inactivation of clavulanic acid and compromises the efficacy of this combination. High degree of resistance to amoxicillin-clavulanic acid (93.4%) by ESBL isolates was already reported [21] A single amino acid change at Ambler position Ser130 (Ser-->Gly) is responsible for its failure in therapy [22]. Multi drug resistance exhibited by pathogens has a major contribution in complicating treatment options. Drugs from marine actinomycete have been advancing through clinical trials successfully and can be potent alternative with new targets to combat antimicrobial resistance patterns. Hence, we attempted to investigate the efficacy of *Streptomyces* sp. VITSJK8 derived compound against ESBL clinical idolates. The results of this study suggest that the compound 1,2-benzene dicarboxylic acid, mono (2-ethylhexyl) ester (DMEHE) is responsible for anti-ESBL activity against tested pathogens. To the best of our knowledge this is the first report on extraction, purification and identification of anti-ESBL compound 1,2-benzene dicarboxylic acid, mono (2-ethylhexyl) ester from *Streptomyces* sp. VITSJK8, isolated from marine sediment sample.

4. ACKNOWLEDGEMENTS

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