Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Der Pharmacia Lettre, 2015, 7 (6):244-252
(http://scholarsresearchlibrary.com/archive.html)

Der Pharmacia Lettre
Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

Antibiotic potency of 2,4-dichloro-5-sulfamoyl benzoic acid extracted from marine bacterium *Streptomyces* Sp. VITBRK3 against methicillin resistant *Staphylococcus aureus*

Benita Mercy Rajan and Krishnan Kannabiran*

Division of Biomolecules and Genetics, School of Biosciences and Technology, VIT University, Vellore, India

---

ABSTRACT

Marine Streptomyces sp.VITBRK3 isolated from the sediment sample collectedat the Marakkanamcoast located in the Southeast coastal region of Bay of Bengal, India. The ethyl acetate extract was prepared from the culture supernatant of Streptomyces sp.VITBRK3.The anti-MRSA compound was purified by silica gel column chromatography and HPLC. The purified compound was identified by using ^{1}H and ^{13}C NMR spectral data. The anti-MRSA compound was compared with the reference compounds available in the mass spectra library of National Institute for Standards and Technology (NIST).The anti-MRSA activity was carried out according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines. Analysis of spectral data resulted in identification of anti-MRSA compound as 2,4-dichloro-5-sulfamoyl benzoic acid. The anti-MRSA activity of the purified compound showed the MIC_{50} value ranged from 0.8to 4.00 µg/mL against the Staphylococcal strains. The results of the study suggest that Streptomyces sp.VITBRK3 is the potential source for the anti-MRSA compound.

Keywords: *Streptomyces* sp.VITBRK3,anti-MRSA activity, 4-dichloro-5-sulfamoyl benzoic acid.

INTRODUCTION

Microbial pathogens exhibiting multi drug resistance is emerging as a serious problem in health care sector worldwide. The improper usage of antibiotics in humans is responsible for developing drug resistance by pathogenic microbes. Microorganisms acquire resistance towards common antibiotics by altering their metabolism and genetic structure[1,2]. There is an incessant need to find novel efficient drug molecules against multi drug resistant microbes. *Staphylococcus aureus* is a virulent pathogen that is responsible for a various infectious disease including sore throat, pneumonia, osteomyelitis, endocarditis, pimples and bacteremia. *S. aureus* has been reported to be resistant towards methicillin and vancomycin[3]. MRSA was first reported in Britain, 1961 and identified as one of the important organism causing nosocomial infection [4]. Due to the development of drug resistance in *S. aureus*, treatment of this bacterial infection has becomea serious problem[5]. Recently, new antimicrobial agents like daptomycin, linezolid and streptogramin combination(quinupristin/dalfopristin) are useful to treat MRSA[6,7].However, due to certain defects and development of drug resistance, emphasizes the need for more antimicrobial agents having potential activity against Gram positive bacteria[8].

Natural products are boundless source for important novel compounds having antagonistic activity against pathogenic organisms. Marine environment covers almost 70% of the earth surface[9]. Organisms present in these

environments are extremely rich source for bioactive compounds[10,11]. The ocean remains as an unexploited source for many drugs and pharmacologically active substances[12].

Actinomycetes are potential provider of bioactive compounds with wide range of biological activities. They are gram positive, filamentous bacteria known for their ability to produce numerous antibiotics till date [13]. Among them, *Streptomyces* are the richest source for bioactive compounds and nearly 80% of the known antibiotics are derived from *Streptomyces* species [14]. Therefore, it is important to find novel remedies from various niches which is less explored or unexplored.The aim of the study was to test the anti-MRSA activity of the compound extracted from *Streptomyces* sp.VITBRK3 isolated from marine sediment sample.

MATERIALS AND METHODS

Isolation and characterization of actinomycetes isolate

The isolate *Streptomyces* sp.VITBRK3 (GU223624) was isolated from marine sediment sample collectedat the Marakkanamcoast of Bay of Bengal, India. The culture of the isolate was maintaned on Actinomycete Isolation Agar medium contained (g/L): sodium caseinate – 2; L-Asparagine – 0.10; sodium propionate – 4; K_2PO_4 – 0.50; $MgSO_4.7H_2O$ – 0.10; $FeSO_4.7H_2O$ – 0.001; agar – 15; pH - 7.2. The spore chain morphology of the isolate was determined by scanning electron microscopic analysis (Hitachi, 3KV, 10 µm). The genomic DNA was isolated using QIAamp DNA Mini Kit (Qiagen) and subjected to PCR amplification using a set of primers (Forward (5'-CGCGGCCTATCAGCTTGTTG-3') and Reverse (5'-CCG TACTCCCCAGGCGGG G-3') [15]. The amplified DNA was purified using NucleoSpin® PCR clean-up Gel extraction kit (MACHEREY-NAGEL GmbH & Co. KG). 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), subjected to BLAST search. Sequence similarity was estimated using Clustal W software [16]. The phylogenetic tree was constructed by neighbour-joining method using Tree view version software [17].

Extraction and purification

The isolate *Streptomyces* sp. VITBRK3 was grown under submerged culture in 250 mL flasks containing 50 mL of the production medium containing (g/L): Casein enzymic hydrolysate - 5, Yeast extract - 3, pH 7.2. The culture was grown for 3 days at 30ºC in rotary shaker at 150 rpm. Then inoculum (2 mL) was transferred to production medium (1 L) and incubated for 5 days at 30ºC. The isolate was subjected to large scale fermentation with total volume being 20 L at 30ºC for 5 days in rotary shaker of 150 rpm. Then the culture broth was centrifuged at 10,000 rpm for 15mins and the supernatant was extracted using equal volume of ethyl acetate (1:1 v/v). It was concentrated using vacuum rotary evaporator and stored at 4ºC [12]. The ethyl acetate (EA) extract (1.2 g) was dissolved in 2.5 mL methanol and loaded on the silica gel column (60-120 mesh silica gel, SRL, India) after filtration. Chloroform/methanol mixture (10:0, 9.5:0.5, 9:1, 8.5:1.5, 8:2, 7.5:2.5 and 7:3 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-MRSA activity. Those fractions showed anti-MRSA activity were pooled, concentrated and lyophilized using a freeze dryer. It was 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 (HP 1090 Liquid Chromatograph) with Nucleosil-100 C-18 column (125 mm × 3.0 mm, 5 µm) connected to a diode array detector (DAD) and fraction collector. Phosphoric acid and acetonitrile were used as mobile phase. The sample (5 µL) was injected to the column and the flow rate was maintained at the rate of0.85 mL/min. The fractions obtained were continuously monitored at the wavelength 210, 230, 260, 280, 310, 360, 435, 500 nm. All fractions were screened for anti-MRSA activity and the active fraction was subjected to spectral studies.

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}.

GC-MS analysis

The purified compound was analyzed by using GC Clarus 500 Perkin Elmer (Singapore) equipped with an Elite-5MS fused silica capillary column (30 x 0.25 mm x 0.25 µm). An electron ionization system with ionizing energy of

70 eV was used. GC oven was held for 3 min at 105°C and then ramped from 108'C to 270°C at 10°C/min. Total run time was 40 min, InjA auto was 260°C at 2 µL volume and split 10:1. Helium was used as the carrier gas with a flow of 25 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).

Nuclear magnetic resonance spectroscopy

The ^{1}H and ^{13}C NMR spectra were recorded for the isolated purified compound (UNITY-500, Varian, Switzerland, 500 MHz) using C_2D_6OS as the solvent. The structure of the anti-MRSA compound extracted from *Streptomyces* sp. VITBRK3 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) [18].

MIC_{50} value

MIC_{50} value of the pure compound was determined by broth dilution method recommended by National Committee of Clinical Laboratory Standards (NCCLS) guidelines. Vancomycinstock solution was prepared and taken as positive control. The pure compound was prepared by dissolving in DMSO to make a concentration of range 0.03–50 µg/mL [19]. The anti-MRSAactivity of the compound was analyzed using bacterial strains *Staphylococcus aureus*(ATCC 29213) and *Staphylococcus aureus* (ATCC 25923).

RESULTS AND DISCUSSION

Isolation, identification and characterization of *Streptomyces* sp. **VITBRK3**

The screening of actinomycetes isolated from soil sediment sample collected atMarakkanam coast, Tamil Nadu, India for anti-MRSA activity yielded a potential isolate named *Streptomyces* sp. VITBRK3. The spore chain morphology of the 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).Based on the molecular taxonomy and phylogeny the isolate was identified to be belonged to the genus *Streptomyces* and designated as *Streptomyces* sp.VITBRK3 (Fig. 2). The 16S rDNA nucleotide sequence was submitted to GenBank, NCBI with the accession ID (GU223624).

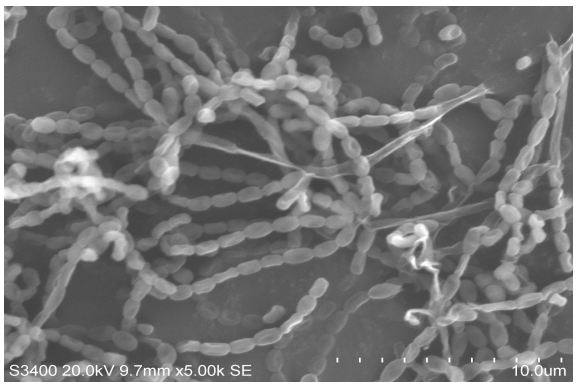


Fig. 1. SEM image of *Streptomyces* sp. VITBRK3

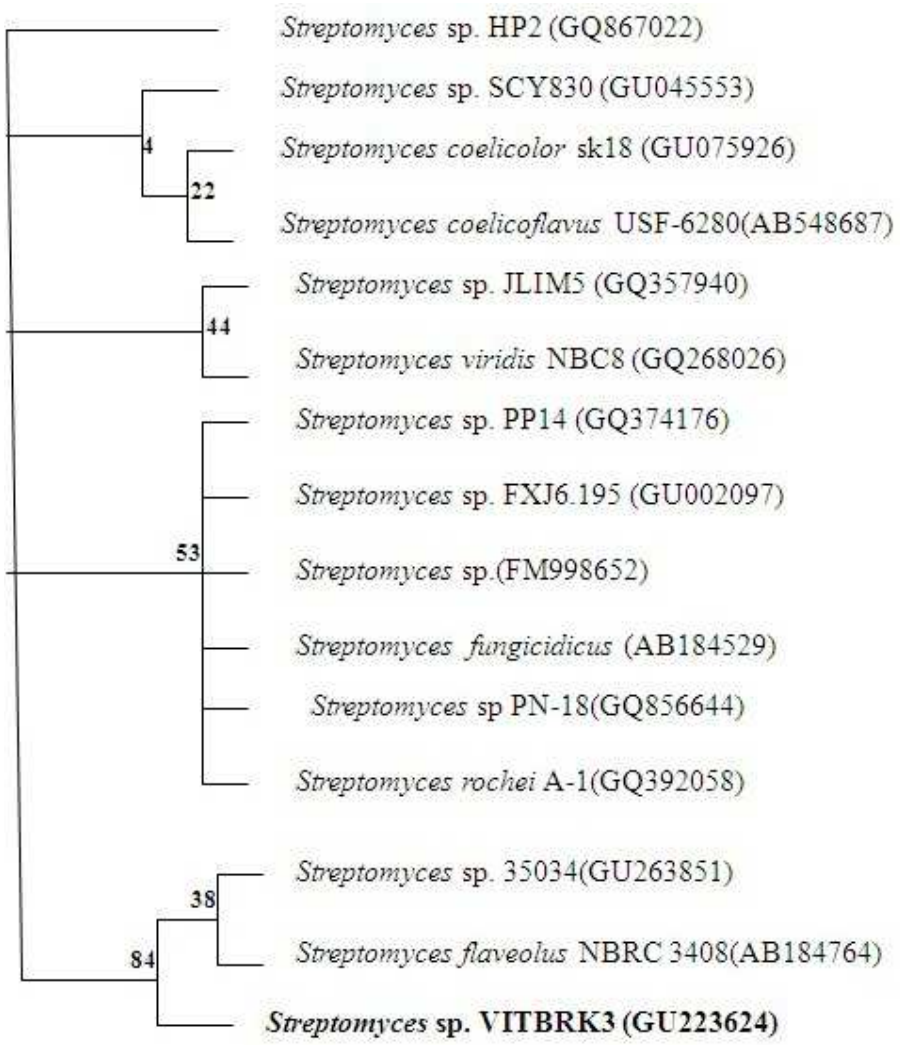


Fig. 2. The taxonomic position of the *Streptomyces* sp. VITBRK3 shown using neighbour-joining method based on Tree view version software. Bootstrap values are presented at the nodes of the tree

Purification and structure elucidation

The isolate was cultured in a shaker flasks using the production medium and incubated at 30º C, for five days under constant shaking (150 rpm). The EA extract (1.2 g) was injected to column chromatography and eluted stepwise with chloroform-methanol as the eluent. Thirty fractions were collected and screened for anti-MRSA activity. The active fraction was purified by preparative HPLC.All fractions were collected and fractions showed anti-MRSA activity against *Staphylococcus aureus*(ATCC 29213) and *Staphylococcus aureus* (ATCC 25923) were identified. There were three fractions, one major fraction (III) with the retention time of 5.4 min found to have anti-MRSA activity. Two other fractions (I and II), with retention time of 10.05 and 16.1 min, respectively, were also identified but they did not show any activity against tested MRSA pathogens. The chromatogram pattern of preparative HPLC is presented in Fig.3. 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, ^{1}H and $^{13}CNMR$ and GCMS.

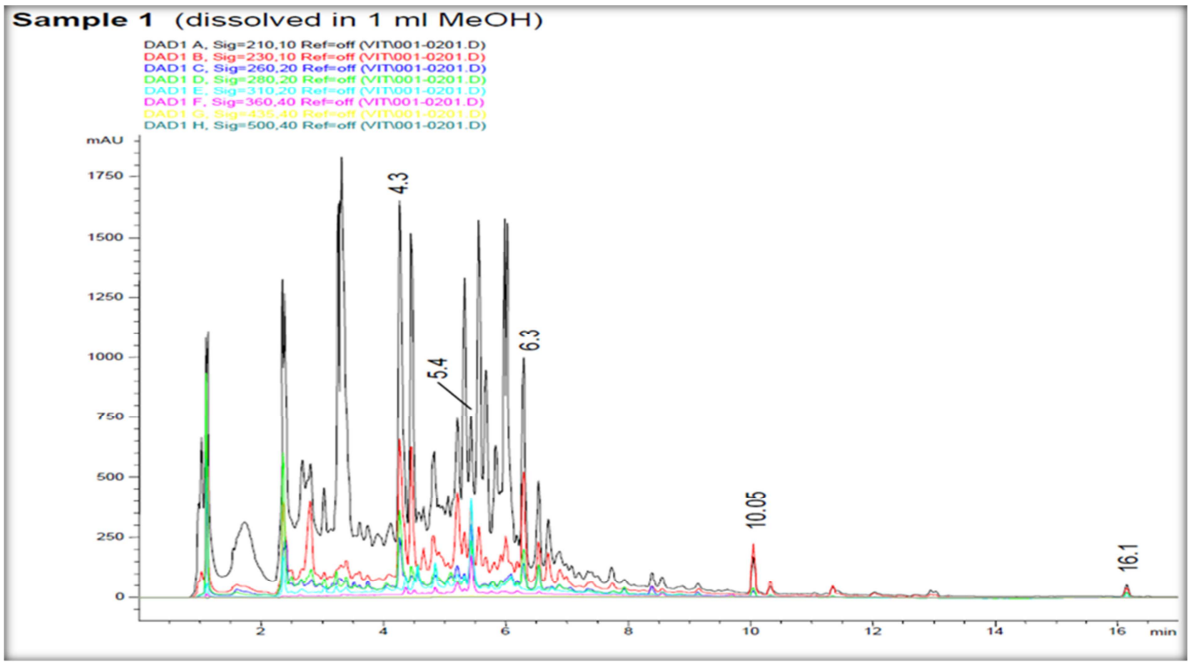


Fig. 3. The HPLC-DAD chromatogram of ethyl acetate extract of *Streptomyces* sp.VITBRK3. a) 2,4-dichloro-5-sulfamoyl benzoic acid, b) Unknown metabolite and c) Unknown metabolite

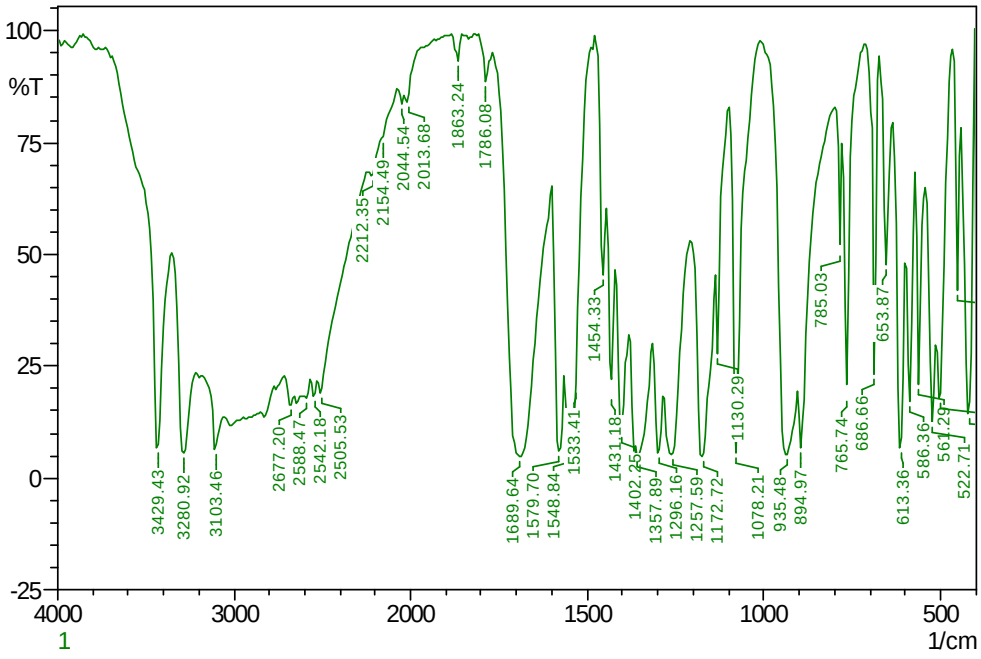


Fig. 4. FTIR spectrum of the purified compound from *Streptomyces* sp. VITBRK3

Fourier transform infrared spectroscopy

FTIR spectra of the purified compound showed peaks corresponding to standard library spectra (Fig. 4). The peak observed at 3429.43cm^{-1} corresponds to NH stretching frequency.The peak observed at 3280.92 cm^{-1} corresponds to OH stretching frequency. A broad peak at 1786.08 cm^{-1} indicates C=O stretching and a peak at 1579.70 cm-^{1}

corresponds to aromatic C=C stretching. A peak observed at 3103.48 cm^{-1} indicates CH stretching. There were two peaks at 1357.89 cm^{-1} to 765.74cm^{-1} corresponding to S=O sulfone stretch and C-Cl alkyl halide stretching.

Gas chromatography-mass spectrometry

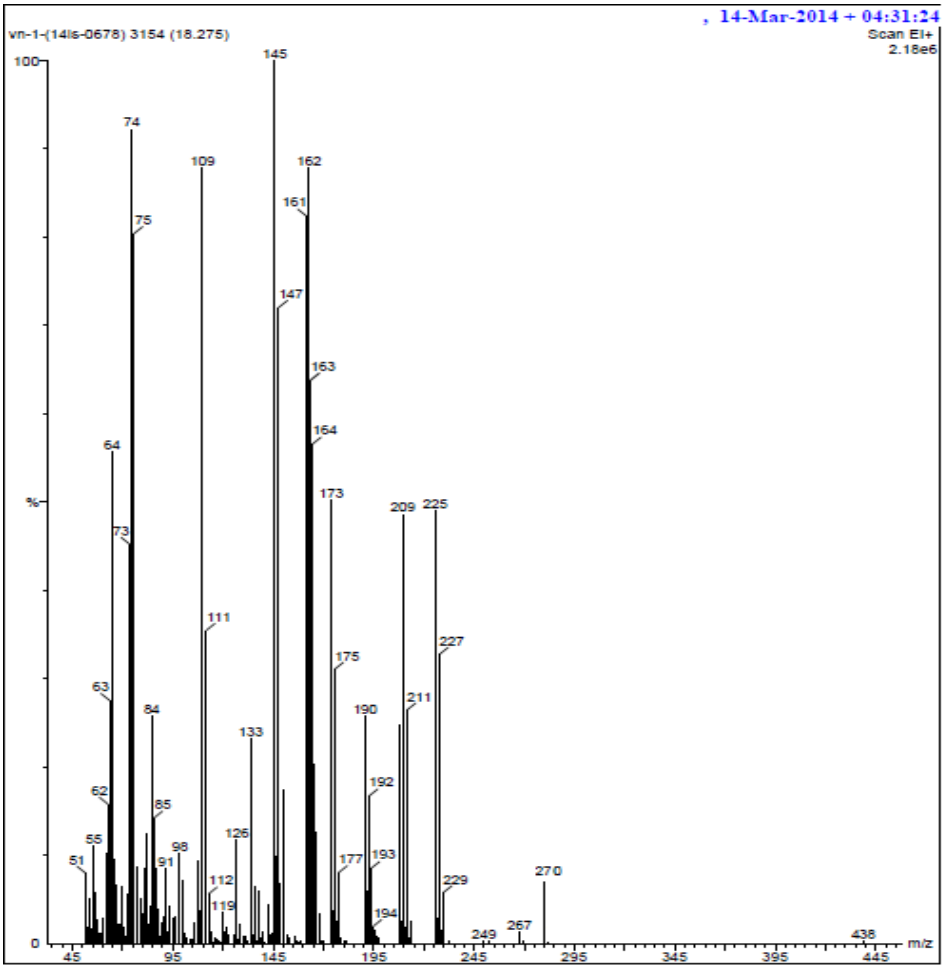


Fig. 5.GC-MS spectrum of the purified compound from *Streptomyces* sp. VITBRK3

The GC-MS spectra obtained for the extracted compound (Fig. 5) was matching with 2,4-dichloro-5-sulfamoyl benzoic acid with a molecular weight of 270 using the NIST library.

Nuclear magnetic resonance spectroscopy

The ^{1}H-NMR spectrum δ_H (500 MHz, DMSO, Me_4Si) 11 (1H,s, OH) 2 (2H,s,NH_2) 8.1 (1H, CH) 8.8 (1H, CH) is demonstrated in Fig. 6. The ^{13}C-NMR spectrum δ_C(500 MHz, DMSO) 125, 130.2, 130.6, 137, 138,139 and 164 ppm is given in Fig. 7. Based on the spectral data, the compound was identified as 2,4-dichloro-5-sulfamoyl benzoic acid (DSBA)with themolecular formula of $C_7H_5C_{l2}NO_4S$. The 2D structure of the purified compound (Fig.8) was modelled using Chem3D Ultra software (version 10).

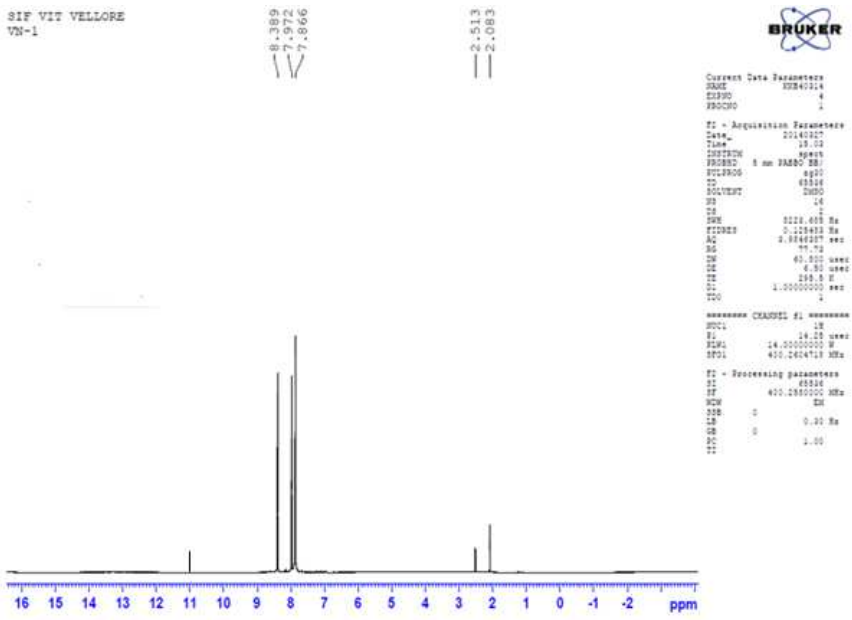
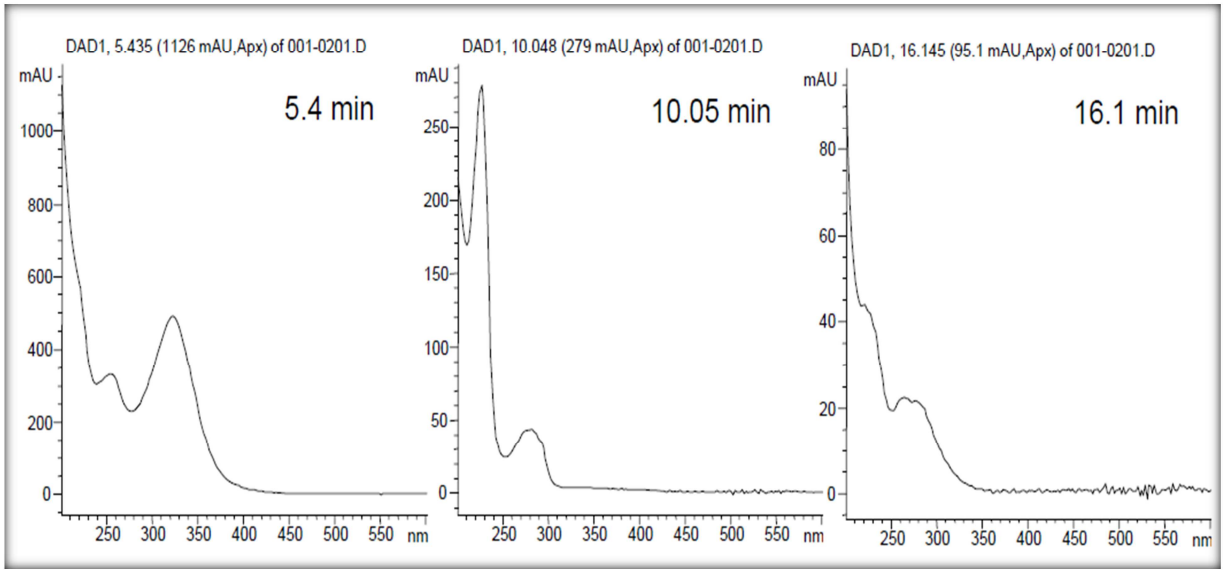


Fig. 6.^{1}H NMR spectrum for the purified compound from *Streptomyces* sp. VITBRK3



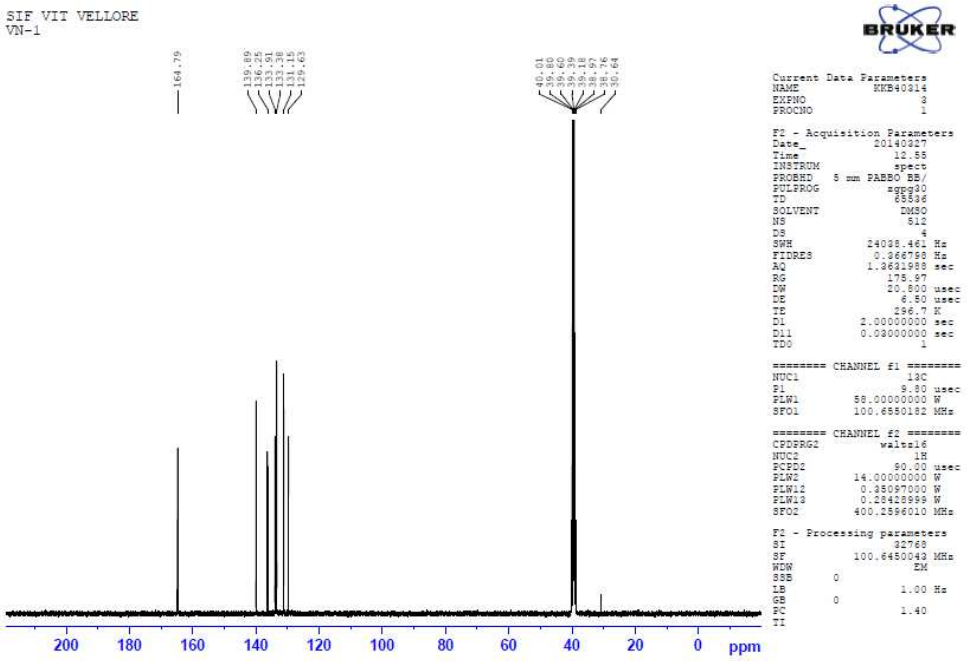


Fig. 7.^{13}C NMR spectrum of the purified compound from *Streptomyces* sp. VITBRK3

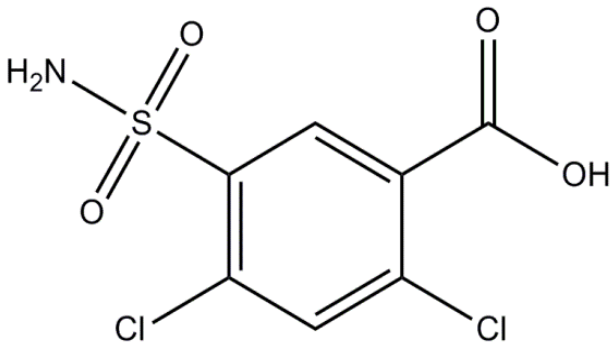


Fig. 8. Structure of the purified compound from *Streptomyces* sp. VITBRK3predicted based on based on the NMR spectrum (Chem3D Draw Ultra software-Version 10)

MIC_{50} value of the lead compound

The MIC_{50} values of anti-MRSA activity of the purified compound are given in Table 1. 2,4-dichloro-5-sulfamoyl benzoic acid showed MIC_{50} values of 4.0 ± 0.5 µg/mL, against *Staphylococcus aureus*(ATCC 29213) and 0.8 ± 0.14µg/mL*Staphylococcus aureus* (ATCC 25923).Methicillin was used as the positive control.

Table 1. MIC_{50} values of 2,4-dichloro-5-sulfamoyl benzoic acid isolated from *Streptomyces* sp.VITBRK3

Strains	Methicillin	2,4-dichloro-5-ulfamoyl benzoic acid
	MIC_{50} (µg/mL)	
Staphylococcus aureus (ATCC 29213)	6.0±0.31	4.0±0.5
Staphylococcus aureus (ATCC 25923)	2.0±0.22	1.8±0.14

In the present study we report the extraction, purification and identification of anti-MRSAcompound2,4-dichloro-5-sulfamoyl benzoic acid from *Streptomyces* sp. VITBRK3, isolated from a marine sediment sample.DSBAisolated from *Streptomyces* sp. VITBRK3 demonstrated strong antagonistic activity against methicillian resistant *Staphylococcus aureus*(ATCC 29213), *Staphylococcus aureus* (ATCC 25923). On the other hand, to our knowledge, this is the first report on isolation of 2,4-dichloro-5-sulfamoyl benzoic acid from marine *Streptomyces* sp. VITBRK3. Microbes are playing a great role in the expansion of drug development. Several biomolecules in the pharmaceutical product development are produced by microbes. Actinomycetes have produced several biomolecules as secondary metabolites and have been used as "wonder drugs" till date [20]. *Streptomyces* contribute around 80% of the antibiotics available in the market [21].Identification of novel antagonistic molecule(s) is needed for MRSA due to emerging resistance [22] Previous guidelines have recommended the use of vancomycin in the management of severe MRSA infection [23].

CONCLUSION

The significant anti-MRSA activityexhibited by the compound 4-dichloro-5-sulfamoyl benzoic acid extracted from *Streptomyces* sp. VITBRK3 further confirmed that *Streptomyces* serves as a novel source for new antibiotics and also suggest that *Streptomyces*can be explored further for newer antibiotics.

Acknowledgement
Authors are thankful to the management of VIT University for providing facilities to carry out this study.

REFERENCES

[1] D.Raghunath. *J. Biosci.*,**2008**, 33, 593.
[2] LL. Maragakis, EN. Perencevich, SE. Cosgrove. *Expert. Rev. Antiinfect. Ther.*,**2008,** 6, 751.
[3] PS. Loomba, J. Taneja, B, Mishra.*J. Glob. Infect. Dis.*,**2010**, 2, 275.
[4] MC.Enright. *Curr. Opinion. Pharmacol*.,**2003**, 5, 474.
[5] R.Singh, P. Ray, A. Das, M. Sharma. *J. Med. Microbiol*.,**2009**, 58, 1067.
[6] LA. Jevitt, AJ. Smith, PP.Williams, PM. Raney, JE. McGowan, FC.Tenover. *Microb. Drug Resist*.,**2003**, 9,389.
[7] VG. Meka, HS. Gold.*Clin. Infect. Dis.*,**2009**, 39, 1010.
[8] MT. Guskey, B.T. Tsuji. *Pharmacother.*,**2010**, 30, 80.
[9] S. Valli, SS. Sugasini,OS. Aysha,P. Nirmala, PV. Kumar, A. Reena. *Asian Pac. J. Trop. Biomed.*,**2012**, 2, 469.
[10] R. Solanki, M.Khanna, R. Lal. *Indian J. Microbiol*.,**2008**, 48, 410.
[11] K. Hong, AH. Gao, QY Xie, H. Gao, L. Zhuang, HP. Lin, et al. *Mar. Drugs*,**2009**, 7, 24.
[12] K. Sivasubramanian, S. Ravichandran, M. Vijayapriya. *Afr. J. Microbiol. Res.*,**2011,** 5: 562.
[13] Y. Okami, K. Hotta, *Actinomycetes in Biotechnology.*, (Eds: M. Goodfellow, ST. Williams and M. Mordarski), London: Academic Press, **1998.**
[14] TRP.Kekuda, KS. Shobha, R. Onkrappa R.*J. Pharm. Res.*,**2010**,1, 30.
[15] JE. Stach, LA. Maldonado, AC. Ward, M. Goodfellow, AT. Bul.*Environ. Microbiol.*,**2003**,5, 828.
[16] N. Saitou, M. Nei,*Mol. Biol. Evol*.,**1987**,4,406.
[17] RDM. Page. Comput.*App. Biosci.*,**1996**,12, 357.
[18] JV. Gopal, *J. Korean Soc. Appl. Biol. Chem***.,2013**, 56, 361.
[19] K. Saurav, K. Kannabiran, *Der Pharm Lett.*,**2013**, 5,178-184.
[20] JH. Chen, XP. Lan, Y. Liu, AQ. Jia, *Biorg. Med. Chem. Lett*.,**2012**,22, 3177.
[21] C. Schleissner, M. Perez, A. Losada, P. Rodriguez, C. Crespo, P. Zuniga, R. Fernandez, F. Reyes, F. De la Calle. *J. Nat. Prod*.,**2011**,74,1590.
[22] HW. Boucher, G. Sakoulas. *Clin. Infect.Dis.*,**2007**, 45, 601.
[23]FK. Gould, R. Brindle, PR. Chadwick, AP. Fraise, S. Hill, D. Nathwani, et al. *J.Antimicrob Chemother.*,**2009**, 63, 849.