

Characterization and Antibacterial Activity of a Glycoside Antibiotic from *Streptomyces variabilis* PO-178

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Abstract

In the present study, characterization and antibacterial activity of a glycoside antibiotic from fermentation broth of bioactive *Streptomyces variabilis* PO-178 recovered previously from Western Ghat soil of Agumbe, Karnataka, India was investigated. Mass cultivation of the strain PO-178 was carried out in Starch casein nitrate broth. The fermentation broth was extracted using butanol solvent in a separation funnel. The concentrated butanol extract was subjected to chromatographic techniques viz., thin layer and silica gel column chromatography for purification of bioactive components. Out of 3 fractions (A, B and C) obtained, only one fraction (C) displayed inhibitory activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The bioactive fraction C was subjected to spectral analysis (LC-MS, IR, ¹H-NMR and ¹³C-NMR) in order to determine the structure. On the basis of result of spectral analysis, the purified fraction was predicted as a glycoside antibiotic with molecular weight 514 and molecular formula C₂₄H₃₄O₁₂. The antibiotic displayed marked inhibitory activity against *S. aureus* as revealed by wider zones of inhibition and low minimum inhibitory concentration value.

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Article Information

Article History:

Received : 28-10-2014

Revised : 25-12-2014

Accepted : 29-12-2014

Keywords:

Streptomyces variabilis PO-178

Agumbe,

Glycoside

Antibacterial activity

Spectral analysis

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INTRODUCTION

Among various secondary metabolites of microbial origin, antibiotics are considered as an important group of chemotherapeutic compounds with activity against microorganisms. These antibiotics differ in their chemical nature, mode of action, possible effect on the animal body and spectrum of activity (narrow or broad spectrum). Antibiotics are produced by bacteria, fungi and actinomycetes. The antibiotic era began with the accidental discovery of Penicillin (produced by *Penicillium notatum*) by Alexander Fleming in 1929. Later, Selman Waksman discovered Streptomycin from *Streptomyces griseus* in the year 1943. The discovery of antibiotics is considered as a turning point in medicine and their subsequent use saved countless lives and revolutionized the medicine field. However, the use of these wonder drugs has been accompanied by the rapid emergence of resistant microbes. Methicillin-resistant *Staphylococcus aureus*, multidrug-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus* spp., multidrug resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, multidrug-resistant TB are among the most important drug-resistant pathogens. A wide range of biochemical and physiological mechanisms are involved in the development of resistance against antimicrobials. Moreover, the ability of pathogens to acquire and transmit resistance has made the situation even worst. Hence,

search for new antimicrobials is a continuous process to keep pace with continually evolving pathogens (Waksman, 1952; Lenski, 1998; Yoshikawa, 2002; Levy and Marshall, 2004; Tenover, 2006; Dzidic *et al.*, 2008; Lister *et al.*, 2009; Davies and Davies, 2010).

Actinomycetes (*akitino-* ray; *mykes-* fungus) are Gram positive bacteria belonging to the order Actinomycetales, characterized by the formation of substrate and aerial mycelium, presence of spores and a high GC content of the DNA. The genus *Streptomyces* constitute the major actinomycete genera. The species of *Streptomyces* are aerobic spore formers possessing DNA rich in GC content (69-73%). *Streptomyces* species are filamentous and form extensive branching substrate and aerial mycelia. The morphological differentiation of *Streptomyces* results in the formation of hyphae that can differentiate into a chain of spores. This process is unique among Gram-positives, requiring a specialized and coordinated metabolism. *Streptomyces* are ubiquitous in nature and are most common in soil. In soil, these contribute significantly to the turnover of complex biopolymers. They are known to degrade a variety of xenobiotics. These species have produced >75% bioactive metabolites having commercial importance, notably antibiotics. The metabolites produced from Streptomycetes have found medicinal (human and veterinary) and agricultural importance. *Streptomyces*

species are shown to produce a wide array of chemically diverse bioactive agents such as antibacterial agents, antifungal agents, antiviral drugs, antiparasitic agents, plant growth promoters, antioxidants, antitumor agents, herbicides, pesticides and other pharmacologically important agents (Anderson and Wellington, 2001; Kokare *et al.*, 2004; Yadav *et al.*, 2008; Kekuda *et al.*, 2010a; Priya *et al.*, 2012; Procopio *et al.*, 2012; Manasa *et al.*, 2014). The strain *Streptomyces variabilis* PO-178 used in the present study was isolated from a rhizosphere soil of Western Ghat region of Agumbe, Shivamogga district, Karnataka, India. In our previous studies, the strain PO-178 displayed several bioactivities (Kekuda *et al.*, 2013; Kekuda and Onkarappa, 2014; Kekuda and Onkarappa, 2015). In continuation of our

previous studies, in the present study, we describe characterization and antibacterial activity of a glycoside antibiotic obtained from butanol extract *S. variabilis* PO-178.

MATERIALS AND METHODS

Isolation and Characterization of *S. variabilis* PO-178

The strain PO-178 was isolated previously from a rhizosphere soil sample of Agumbe, Western Ghat region of Shivamogga district, Karnataka, India by serial dilution-plating method (on Starch casein nitrate agar). The characterization of the isolate was done by morphological, microscopic, biochemical and 16S rDNA sequencing studies (Kekuda and Onkarappa, 2015).

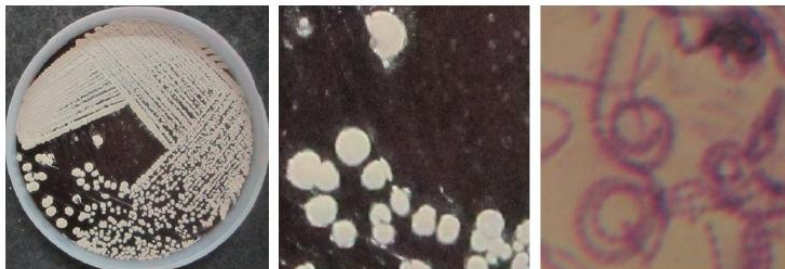


Figure 1: Culture and spore arrangement of *S. variabilis* PO-178 (Kekuda and Onkarappa, 2015)

Fermentation

The well sporulated culture of strain PO-178 was aseptically inoculated into conical flasks containing sterile Starch casein nitrate broth. The flasks were incubated aerobically at 28°C for 10 days. After incubation, culture broth was filtered through Whatman No. 1 filter paper and the culture filtrate obtained was centrifuged (10000rpm). The supernatant was extracted in a separation funnel using butanol and concentrated in rotary evaporator (Kekuda and Onkarappa, 2015).

Recovery of the Bioactive Metabolite from Butanol Extract

The brown colored crude extract obtained was subjected for isolation and purification of components by analytical methods namely Thin Layer Chromatography (TLC) and Column chromatography. The butanol extract was spotted on TLC plate and developed with solvent systems that consisted of different ratios of ethanol:water, methanol:water and ethanol:methanol:water. The chromatograms were allowed to air dry and were exposed to iodine vapours for the detection of resolved components. The recovery of bioactive principle from the butanol extract was carried out by Column chromatography. Column chromatography was performed by employing silica gel 60-120 mesh size of chromatography grade (Himedia, Mumbai) in a sintered filter column (40x4cm). The column was treated with chromic acid followed by water and then rinsed with acetone. The column was packed well with activated silica using chloroform solvent. The butanol extract of isolate PO-178 was loaded at the top of the column. The column was eluted using solvent systems. Fractions were collected at regular intervals, loaded on TLC plates, developed with solvent system and observed for spots in iodine chamber. The fractions exhibiting same Rf values were pooled and the solvent was evaporated at 40°C to get concentrated fraction (Augustine *et al.*, 2005; Rahman and Humainy, 2011). Three pure fractions (A, B and C) were obtained.

Antibacterial activity of Pure Fractions

The fractions A, B and C (0.5 and 1.0mg/ml of dimethyl sulfoxide [DMSO]) were screened for antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* by Agar well diffusion method. Streptomycin was used as reference standard (Kekuda and Onkarappa, 2015). Only fraction C (68mg yield) displayed inhibitory activity against test bacteria. Hence, fraction C was subjected to minimum inhibitory concentration (MIC) determination (Kosanic and Rankovic, 2010) and elucidation of structure.

Spectral Analysis and Structural Elucidation of Bioactive Compound

The purity and molecular weight of the bioactive fraction C (PK-06) was assessed by Liquid Chromatography and Mass Spectrum (LCMS 2010, Shimadzu, Japan). The fraction C was dissolved in methanol and 5µl of the compound was injected into a C-18 HPLC column with methanol: water (90:10) at a flow rate of 0.2ml/min. The UV-VIS detection was carried out. Mass spectral studies were carried out by atmospheric pressure chemical ionization probe (APCI) for the analysis of nonpolar compounds and Electron Spray Ionization probe (ESI) for the analysis of polar compounds. The Infra red (IR) spectra was recorded on Shimadzu IR - model. The fraction C was scanned from 400-4000cm²/range. The fraction was dissolved in DMSO and was subjected to ¹H-NMR (Nuclear Magnetic Resonance) and ¹³C-NMR in Bruker BioSpin model. The nature and structure of the bioactive fraction was elucidated by analyzing the chromatograms and spectra with pertinent literature.

RESULTS

Spectral Analysis and Structural Elucidation of Purified Bioactive Compound

The LC-MS (Figure 2) revealed that the compound is pure as the LC chromatogram showed a single prominent peak. The peak observed at Mass Speak 513 confirms the molecular weight of the compound i.e. 514 (negative mode).

User : Admin
 Sample : PK-06
 Inj. Volume : 5.000
 Data Name : C:\LCMSsolution\User\Data\PK-06-ESI-NEG1.qld
 Method Name : C:\LCMSsolution\User\Method\esi.qlm

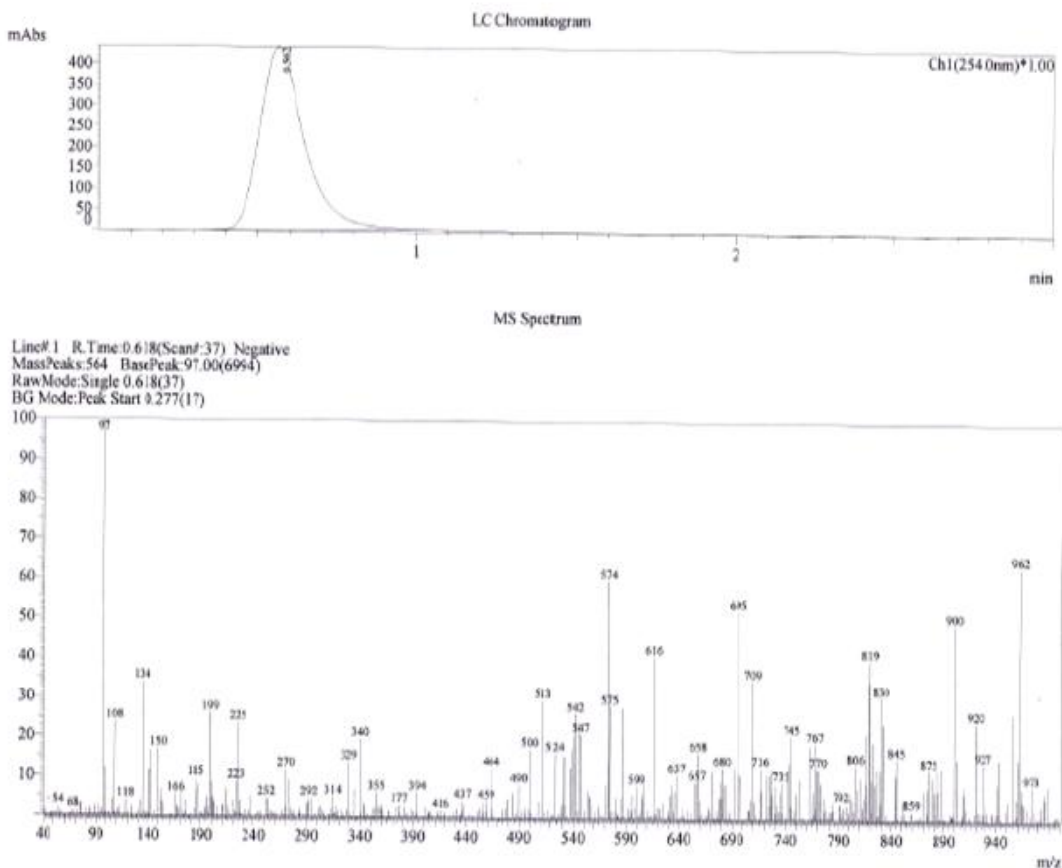


Figure 2: LC-MS of the purified compound

Figure 3 shows the IR spectra of the purified compound. Strong absorbance frequency observed at 3386 cm^{-1} and 1671 cm^{-1} indicated the presence of hydroxyl (-OH) and carbonyl (-C=O) groups respectively.

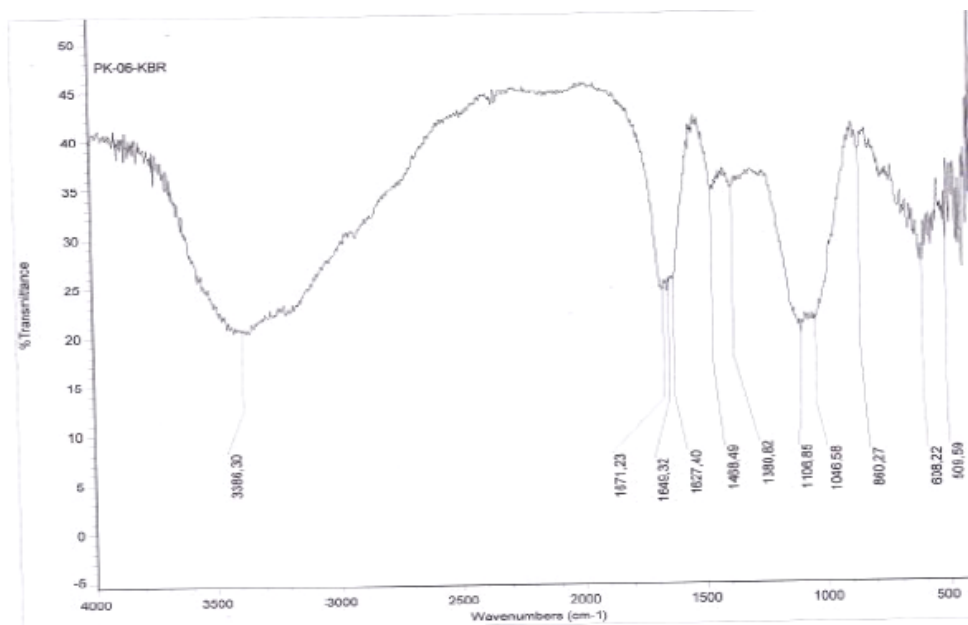


Figure 3: IR spectra of purified compound

$^1\text{H-NMR}$ spectra of purified compound is shown in Figure 4. The singlet appeared at the region of 9.6δ value is confirmed the presence of aldehyde proton. The singlet appeared at the region of 5.1δ value is for $-\text{OH}$ group present in α position to aldehyde group. The two doublets for $-\text{CH}=\text{CH}$ appeared at the region of 5.4 to 5.6δ values.

The 6 singlets for 6 $-\text{OH}$ groups appeared at the region of 4.2 to 4.9δ values. The singlet appeared at the region of 1.1δ value is for $-\text{CH}_3$ proton and the multiplets present at the region of 2.7 to 3.8 is for $-\text{CH}$ and $-\text{CH}_2$ protons and the multiplets present at the region of 2.7 to 3.8 is for $-\text{CH}$ and $-\text{CH}_2$ protons.

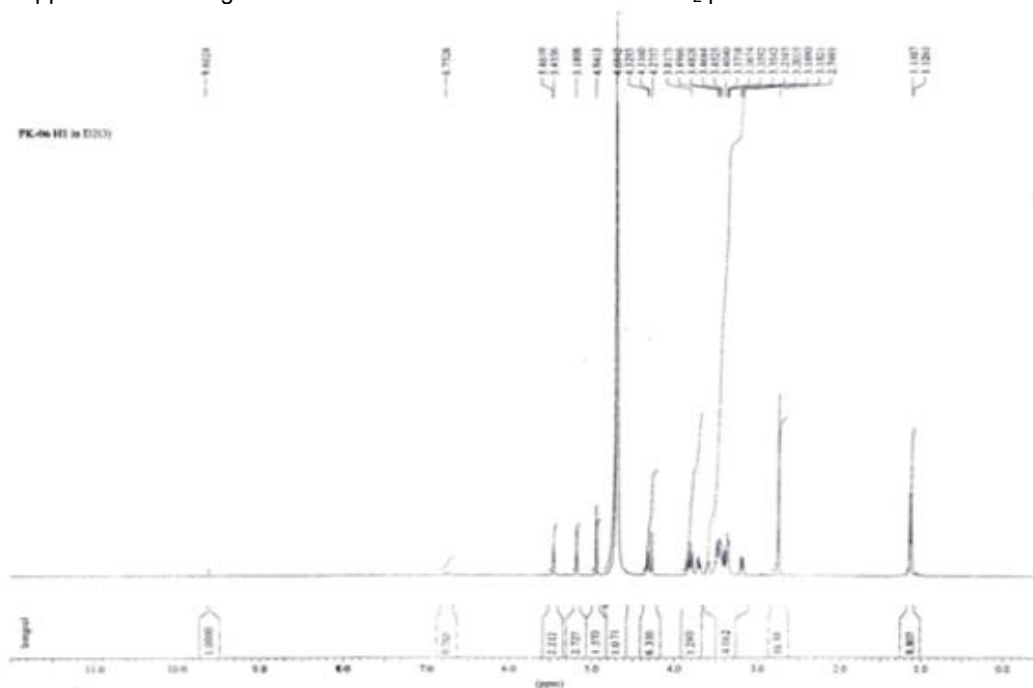


Figure 4: $^1\text{H-NMR}$ spectra of purified compound

$^{13}\text{C-NMR}$ spectra of purified compound is shown in Figure 5. The peak appeared at the region of 158δ value is confirmed the presence of aldehyde carbon. The peak appeared at 105δ value confirmed the presence of furon

ring carbon. The peak appeared at $82-94\delta$ value confirmed the presence of $-\text{C-OH}$ carbons. The peak appeared at 32δ value confirms the presence of aliphatic ($-\text{CH}$) carbons.

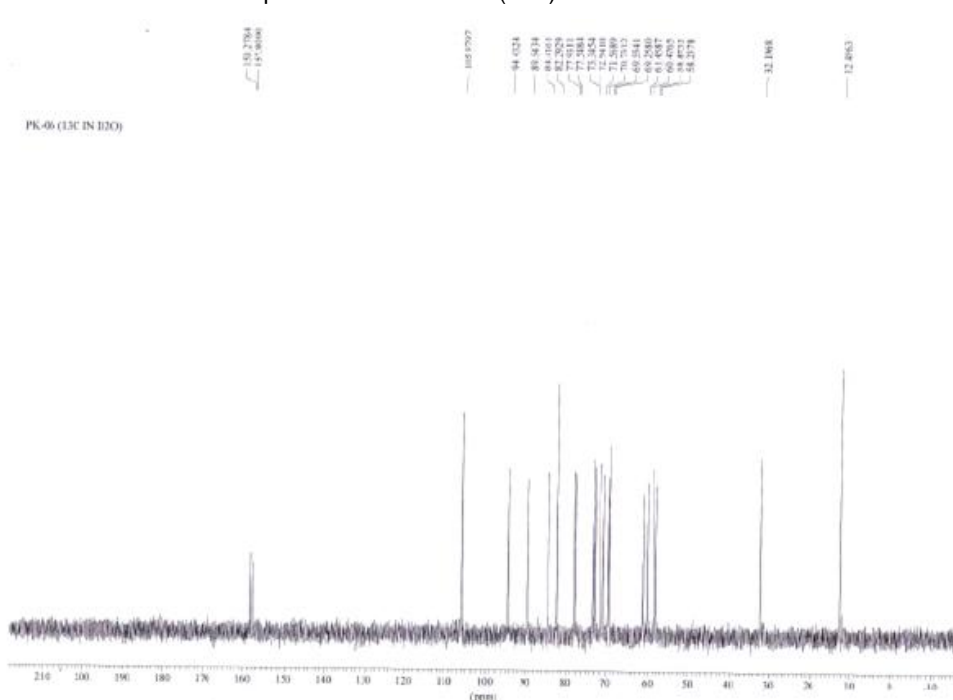


Figure 5: $^{13}\text{C-NMR}$ of purified compound

On the basis of obtained spectral data, the purified compound can be predicted as a glycoside as it contains glycoside linkage. The compound was named 5-(4-(1*E*,3*E*)-hexa-1,3-dienyl)-3,5,6-trihydroxycyclohex-3-

enyloxy)-2,3-dihydro-3-hydroxy-2-methyl-4-(tetrahydro-4,5-dihydroxy-6-hydroxymethyl)-2*H*-pyron-2-yloxy)furan-3-carbaldehyde. The molecular formula of the compound is $\text{C}_{24}\text{H}_{34}\text{O}_{12}$ (Figure 6).

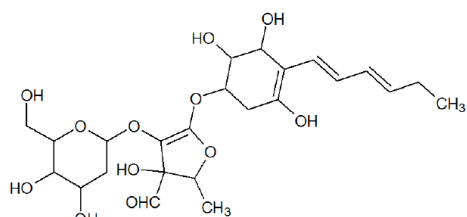


Figure 6: Structure of purified compound (Fraction C)

Antibacterial Activity of Pure Compound

The purified compound was found effective against bacteria (Table 1 and Figure 7). Both Streptomycin and the compound were effective in inhibiting the growth of *S. aureus* and *P. aeruginosa* in a dose dependent manner. Among bacteria, *S. aureus* was inhibited to higher extent. Inhibition caused by Streptomycin was marked when compared to purified compound. The MIC of compound was found to be 62.5µg/ml and 15.625µg/ml for *P. aeruginosa* and *S. aureus* respectively.

Table 1: Antibacterial activity of purified compound

Test bacteria	Zone of inhibition in cm (Mean±SD)			
	Purified compound		Streptomycin	
	0.5mg/ml	1.0mg/ml	0.5mg/ml	1.0mg/ml
<i>S. aureus</i>	2.70±0.00	2.80±0.00	3.25±0.10	3.80±0.00
<i>P.aeruginosa</i>	1.85±0.01	2.10±0.00	2.33±0.05	2.80±0.00

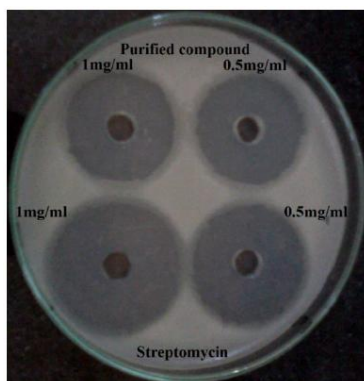


Figure 7: Inhibition of *S. aureus* by purified compound and streptomycin

DISCUSSION

Western Ghats of India represent one of the biodiversity hotspots in the world. The Western Ghats harbor diverse flora and fauna. Agumbe is one of the most scenic places on the Western Ghats of Shivamogga district, Karnataka, India. Studies on bioactivities of actinomycetes isolated from Agumbe region of Western Ghats of Karnataka have been carried out. Bioactivities such as antimicrobial, antioxidant, enzyme inhibitory, cytotoxic, anthelmintic, insecticidal, analgesic, anti-inflammatory and antipyretic activity of *Streptomyces* species isolated from soils of Agumbe, Karnataka, India have been investigated (Kekuda *et al.*, 2010b; Kekuda *et al.*, 2012; Gautham *et al.*, 2012; Gautham *et al.*, 2013; Kekuda and Onkarappa, 2015). The bioactive strain *S. variabilis* PO-178 used in this study was isolated from a soil sample of Agumbe, Karnataka, India. The extracts obtained from this strain were shown to exhibit bioactivities of such as antimicrobial, antioxidant, insecticidal, anthelmintic, enzyme inhibitory (amylase and pancreatic lipase) activity and pharmacological activities namely analgesic, anti-inflammatory, antipyretic and CNS

depressant activity (Kekuda *et al.*, 2013; Kekuda and Onkarappa, 2014; Kekuda and Onkarappa, 2015).

The identification of active compounds from fermentation medium is one of the most complicated, labour intensive, time consuming step of the screening protocols. However, the great progress in the chromatographic isolation methods and spectroscopic identification techniques are more or less satisfying the requirements of the modern screening programs. In the last decades using MS, NMR and X-Ray methods it became to solve the structure in minimum quantities of products. The structure determination of a new compound, isolated perhaps only on mg scale is a relatively easy task (Berdy, 2005). In the present study, an active compound was isolated by silica gel column chromatography from the butanol extract of the fermentation broth of *S. variabilis* PO-178. The spectral analysis was carried out by LC-MS, IR, ¹H-NMR and ¹³C-NMR. On the basis of spectral data, the structure of the bioactive agent was elucidated. The compound was found to be a glycoside having molecular weight of 514.

A large number of antibiotics are glycosides. In numerous cases, the glycosidic residues are crucial for their activity, but sometimes glycosylation only improves the pharmacokinetic parameters. Recently, the development of carbohydrate-based antibiotics has been suggested as a new approach to the problem of antibiotic resistance (Ritter and Wong, 2001). Glycoside antibiotics have shown to be effective in inhibiting microorganisms. Singh *et al.* (2000) showed antimicrobial activity of Saccharomicins, novel heptadecaglycoside antibiotics from *Saccharothrix espanaensis*. A disaccharide glycoside molecule α-L-sorbofuranose (3 → 2)-β-D-altrofuranose was isolated from the aqueous fraction of culture filtrate of *Streptomyces* sp. strain JJ45. The molecule was effective against *Xanthomonas* sp. (Kang *et al.*, 2009). In another study, Gautham (2012) isolated an antibiotic active against bacteria and molds from butanol extract of *Streptomyces fradiae* GOS01. The antibiotic was found to be xyloglucose disaccharide molecule. A new polyketide glycoside isolated from *Streptomyces* sp. QD01-2 was shown to exhibit marked antimicrobial activity (Hou *et al.*, 2012). In the present study, the glycoside antibiotic isolated from the actinomycete strain *S. variabilis* PO-178 was found to display marked antibacterial activity against Gram positive and Gram negative bacteria. The antibiotic displayed marked activity against *S. aureus* when compared to *P. aeruginosa*. The low susceptibility of *P. aeruginosa* to the antibiotic might be ascribed to the presence of outer membrane which may act as an additional barrier to the entry of antibiotic (Manasa *et al.*, 2014). In a previous study, Nakagawa *et al.* (1990) isolated a depsipeptide antibiotic Variapeptin from the culture identified as *S. variabilis*. The antibiotic was active against Gram positive bacteria and showed cytotoxicity against mammalian cell lines.

CONCLUSIONS

The present study showed the potential of Western Ghat actinomycete *S. variabilis* PO-178 to produce a glycoside antibiotic active against Gram positive and Gram negative bacteria. The antibiotic was more effective against *S. aureus* as evidenced by wider zones of inhibition and lower MIC value. Further *in vivo* studies are under progress to evaluate the safety margins for the antibiotic.

ACKNOWLEDGEMENTS

Authors thank Chairman, Department of Microbiology and Principal, Sahyadri Science College (Autonomous) for facilities and moral support given.

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