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Original Article

ANTIMICROBIAL POTENTIAL OF *STREPTOMYCES CHEONANENSIS* VUK-A FROM MANGROVE ORIGIN

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ABSTRACT

Objectives: The aim of the present study was to isolate, characterize and evaluate the activity of compounds produced by *Streptomyces cheonanensis* VUK-A.

Methods: Chemical examination of the secondary metabolites of the strain *Streptomyces cheonanensis* VUK-A has led to the segregation of one bioactive compound (1) and a partially purified fraction (2). The strain was isolated from the sediment samples of mangrove ecosystem of Coringa, south coastal Andhra Pradesh, India. The chemical structure of the active compound 1 was established on the basis of spectroscopic analysis including ^{1}H NMR, ^{13}C NMR spectroscopy, FTIR and EIMS. The partially purified sub-fraction (2) subjected to Gas Chromatography-Mass spectroscopy. The antimicrobial activity of the bioactive compounds produced by the strain was expressed in terms of minimum inhibitory concentration.

Results: The compound 1 was isolated from the fermentation broth was characterized as benzoic acid (1) based on spectroscopic analysis. The partially purified sub-fraction (2) subjected to Gas Chromatography-Mass spectroscopy contained nine analogues: 1-tetradecene, tetradecane, 1-hexadecene, hexadecane, 5-octadecene, octadecane, 5-eicosene, 1-nonadecene and cyclo tetracosane. The compounds recorded moderate to significant antimicrobial activity against medicinally and agriculturally important bacteria and fungi. This is the first report of six partially purified compounds 1-tetradecene, tetradecane, hexadecane, octadecane, 5-eicosene and cyclo tetracosane from the genus *Streptomyces.*

Conclusion: The results of the present study showed that the metabolites of *Streptomyces cheonanensis* VUK-A exhibited antibacterial and antifungal activities. The study also supports that Coringa, a promising mangrove ecosystem remained to be explored for new bioactive compounds.

Keywords: *Streptomyces cheonanensis*, Mangrove Ecosystem, Natural Products, Antimicrobial activity.

INTRODUCTION

The probe for new potent compounds with novel and specific mode of action to combat against emerging infectious diseases and antibiotic resistant pathogens presumed to be major health concern challenging the entire world is to be addressed on a war footing. Secondary metabolites of the actinobacteria are endowed with a wide variety of chemical structures possessing strong biological activities and gain attention in view of their positive therapeutic effects to human health [1]. Actinobacteria are a diverse group of microorganisms that are widely distributed in terrestrial, freshwater and marine habitats [2], outnumber of studies on the terrestrial actinobacteria have been carried out when compared to the marine habitats. Mangrove swamps are important constituents of the marine ecosystem [3] and subjected to ever-changing abiotic factors such as high salinity, high temperature, extreme tides, high evaporation and high sedimentation [4]. Mangrove ecosystems remain largely unexplored and offer an excellent opportunity for isolating the potent actinomycetes with solitary properties capable of producing many useful bioactive compounds such as antibiotics, therapeutic enzymes and antitumour agents [5].

The actinobacteria of the mangrove origin can produce secondary metabolites with diverse chemical skeleton and potent biological activities have been playing an extensive role in pharmaceutical and medical industry. Thousands of purified bioactive compounds have been characterized and become a part and parcel in the development of drugs to treat a wide range of diseases in human, veterinary and agriculture sector [6]. Reports have been well documented that each actino bacterial strain has genetically potential to produce 10 to 20 secondary metabolites [7] and hence are considered not only as indispensable novel antibiotic producers but also play a promising defence role by inhibiting the other competing cells and make available more nutrients to the metabolite producing strain for survival [8].

Streptomyces generally are an acknowledged group of microbes can generate a lot of different wide spectrum bioactive compounds [9]. *Streptomyces* species account to 74% while the rare genera of actinobacteria to 26% of the total bioactive compounds isolated [10]. *Streptomyces* produce the most diverse, unprecedented, often very complicated compounds displaying exceptional antibacterial potency with usually minimal toxicity. The factors contributing to their diverse metabolic diversity are due to the extremely large genome that carries hundreds of regulatory factors to regulate the gene expression in response to specific needs [11]. *Streptomyces* has special role in the antibiotic production and yielded many therapeutic agents which include antibacterial, antifungal and anticancer drugs like Tetracycline, Amphotericin B and Asriamycin. *Streptomyces* spp. is widely recognized as industrially important microorganisms for their ability to yield different kinds of new secondary metabolites [12].

Many reports have endorsed the importance of the pharmaceutical activity of *Streptomyces* obtained from different habitats, but only a few reports are available to deal with the industrially important *Streptomyces* from mangrove ecosystem [13]. As a part of our present research for useful bioactive metabolites, we observed that the actinomycetes strain VUK-A which was isolated from unexplored mangrove sediment samples of Coringa, South coastal Andhra Pradesh, India showed broad spectrum antimicrobial activity and also investigated the active constituents of actinobacterium.

MATERIALS AND METHODS

Isolation

The actinomycetes strain, VUK-A was isolated from the Coringa mangrove ecosystem by employing soil dilution plate technique on

starch casein agar medium. The strain was identified as *Streptomyces cheonanensis* by cultural, morphological, physiological and biochemical studies along with the 16S rRNA analysis. The rRNA sequence was deposited in the NCBI Genbank with an accession number JN087502. The strain was maintained on yeast extract-malt extract dextrose (YMD) agar medium at 4 ˚ C for further study.

Purification and structural elucidation of bioactive metabolites

The twin activities *i.e.* isolation and identification of bioactive metabolites, a loopful culture of *Streptomyces cheonanensis* VUK-A was cultivated in YMD broth (seed broth) and incubated on a rotary shaker (250 rpm) at 35˚C. After 48 h incubation, the seed culture at the rate of 10% was transferred to the optimized fermentation medium consisting of lactose (1%), peptone (0.5%), K_2HPO_4 (0.05%), $FeSO_4$ (0.001%) and NaCl (3%) with pH adjusted to 7.0 [14]. The culture filtrates (20 L) obtained after cultivation of the strain for 96 h were extracted twice with ethyl acetate and concentrated in a rotavap, and freeze dried to yield a dark green residue. The weight of total crude extract was 2.5 g. The dark green crude residue (2.5g) was loaded on a silica gel column (25X 5 cm, Silica gel 100, Merck, Mumbai, India) and eluted successively with 200 ml of 100% hexane, 200 ml of linear gradient hexane: ethyl acetate (v/v, 75:25-25:75), 200 ml of 100% ethyl acetate and finally with 200 ml of 100% methanol resulting in six fractions, 5 polar residues and one non-polar residue. Among the five polar fractions, fraction IV and the single non-polar fraction (PPF) exhibited high antimicrobial activity. The fraction IV (700 mg) was selected for further studies and subjected to silica gel column chromatography (100-200 mesh), which afforded fractions 1-5. Based on TLC monitoring and NMR spectral data, the subfraction 3 was selected for further purification. The active subfraction 3 (280 mg) was subjected to further purification by silica gel column chromatography using 30% ethyl acetate and yielded compound 1 (36 mg). The purity of the compound was checked using TLC and structure of the active compound was elucidated and confirmed on the basis of FTIR, mass and NMR spectroscopic data.

The components of the partially purified fraction (PPF) (2) were analysed on Agilent GC-MS system (GC: 5890 series II; MSD 5972). The fused silica HP-5 capillary column (30 m x 0.25 mm, ID, film thickness of 0.25 µm) was directly coupled to the MS. The carrier gas was helium with a flow rate of 1.2 ml min^{-1}. Oven temperature was programmed (50˚C min^{-1}, then 50-280˚C at a rate of 5˚C min^{-1}) and subsequently, held isothermally for 20 min. The temperature of the injector port was maintained at 250 ˚C and that of the detector at 280 ˚C [15]. The peaks of components in gas chromatography were subjected to mass spectral analysis. The spectra were analysed from the available library data; NIST MS search (ver. 2.0). (Included with NIST'02 mass spectral library, Agilent p/n G1033A).

Test microorganisms

Gram-positive bacteria: *Bacillus cereus* (MTCC 430), *Streptococcus mutans* (MTCC 497), *Staphylococcus aureus* (MTCC 3160), *Staphylococcus epidermis* (MTCC 120), *Bacillus subtilis* (ATCC 6633), *Bacillus megaterium* (NCIM 2187); Gram-negative bacteria: *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 9027), *Proteus vulgaris* (MTCC 7299), *Serratia marcescens* (MTCC 118), *Xanthomonas campestris* (MTCC 2286), *Xanthomonas malvacearum* (NCIM 2954) and *Salmonella typhi* (ATCC 14028); Medically important dermatophytes: *Candida albicans* (ATCC 10231) and *Epidermophyton floccosum* (MTCC 145); Medically and agriculturally important filamentous fungi: *Aspergillus niger* (ATCC 1015), *Aspergillus flavus* (ATCC 9643), *Fusarium oxysporum* (MTCC 3075), *Fusarium solani* (MTCC 4634), *Penicillum citrinum* (MTCC 6489), *Verticillium alboatrum* and *Alternaria alternata* (MTCC 6572). The test microorganisms used in the present study were procured from ATCC, University Boulevard, Manassas, USA and MTCC, Chandigarh, NCIM, Pune, India and preserved at 4˚C.

Minimum inhibitory concentration (MIC) assay

The antimicrobial spectra of the bioactive compounds of the strain were determined in terms of minimum inhibitory concentration (MIC) against a wide variety of gram-positive and gram-negative bacteria and fungi by using the agar plate diffusion assay [16]. Triplicate sets of the plates were maintained for each concentration of the test sample. Muller-Hinton agar and Czapek-Dox agar media were prepared to grow the bacteria and fungi, respectively. The purified compounds were dissolved in dimethyl sulfoxide at concentrations ranging from 0 to 1000 µg/ml and used to assay against supra mentioned test bacteria and fungi. The inoculated plates were examined after 24-48 h of incubation at 37 ˚ C for bacteria and 48-72 h at 28 °C for fungi. The lowest concentration of the bioactive metabolites exhibiting significant antimicrobial activity against the test microorganisms was taken as the MIC of the compound.

RESULTS AND DISCUSSION

Culture filtrates obtained after 96 h fermentation were extracted with ethyl acetate and concentrated to yield a dark green residue, which in turn was subjected to silica gel column chromatography using a gradient solvent system of hexane: ethyl acetate. Among the six fractions collected the fraction IV and a non-polar partially purified fraction (PPF) exhibiting good antimicrobial activity. The fraction IV was rechromatographed on a silica gel column and yielded five fractions. Further purifications of active sub-fraction 3 by sequential chromatographic purification on silica gel yielded compound 1. The components of partially purified non-polar fraction were analysed by using GC-MS.

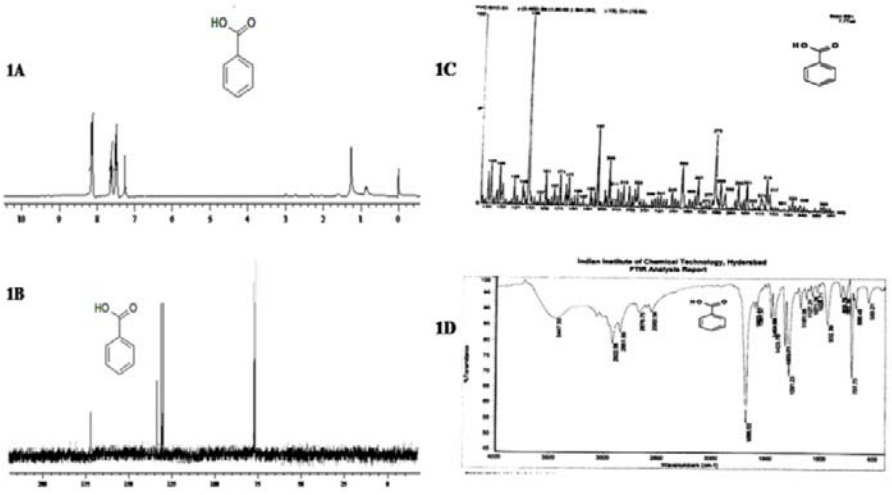

Fig. 1A: ^{1}H NMR spectrum of compound (1), 1B: ^{13}C NMR spectrum of compound (1), 1C: Mass spectrum of compound (1), 1D: FTIR spectrum of compound (1)

Compound (1) was obtained as a colourless crystalline solid, freely soluble in ethanol and partially in benzene, acetone and water. The ^{1}H-NMR spectrum of the compound showed signals at δ6.89 (m, 2H); 6.37 (m, 1H) and 6.22 (m, 2H) (fig. 1A), while ^{13}C exhibited 5 signals at δ 172.5; 133.78; 130.19 (2C); 129.32 and 128.45 (2C) (fig. 1B). EIMS analysis of the compound gave a molecular ion m/z at 145 $[M+Na]^{+}$ (fig. 1C) The IR spectrum displayed absorption band at V_{max} 1695 cm^{-1}suggested the presence of functional groups like carboxyl groups (fig. 1D). Based on the above spectral data, bioactive compound 1 was identified as Benzoic acid (fig. 2) with the molecular formula of $C_7H_6O_2$.

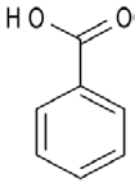

Fig. 2: Molecular structure of benzoic acid (1)

Analysis of the components of partially purified subfraction (PPF) (2) by GC (fig. 3) implied nine peaks at the retention times of 15.01 min (A), 15.13 min (B), 18.13 min (C), 18.23 min (D), 20.93 min (E), 21.01 min (F), 23.47 min (G), 25.79 min (H) and 28.01 min (I). Further examination of these peaks by MS showed molecular ions M^{+}at m/z 196, 198, 224, 226, 234, 254, 260, 247 and 370 respectively (data not shown). According to the available library data, NIST MS search (ver. 2.0) (Included with NIST'02 mass spectral library, Agilent p/n G1033 A), compounds viz., A, B, C, D, E, F, G, H and I present in partially purified fourth subfraction were tentatively identified as 1-tetradecene, tetradecane, 1-hexadecene, hexadecane, 5-octadecene, octadecane, 5-eicosene, 1-nonadecene and cyclo tetracosane respectively.

Minimum inhibitory concentration (MIC) assay

Antibacterial activities of the bioactive compounds (1 and 2) in terms of MIC are shown in table 1. The bioactive compounds exhibited antibacterial activity against a variety of gram-positive and gram-negative bacteria, for which the MIC values ranged from 16 to 256 µg/ml. Among the opportunistic and Gram-positive pathogenic bacteria, compound 1 was active against all the bacteria tested, and the best activity of this compound was recorded against *Bacillus subtilis* (16 µg/ml). The fraction 2 (PPF) presented the highest activity against *Staphylococcus aureus* and *Staphylococcus epidermis* (32 µg/ml). Of the Gram-negative bacteria, the microorganisms that presented the highest sensitivity towards compound 1 was *Serratia marcescens* (32 µg/ml) followed by *Escherichia coli* (64 µg/ml). Fraction 2 (PPF) recorded the highest activity against *Xanthomonas campestris* followed by *Escherichia coli* and *Xanthomonas malvacearum* (32µg/ml).

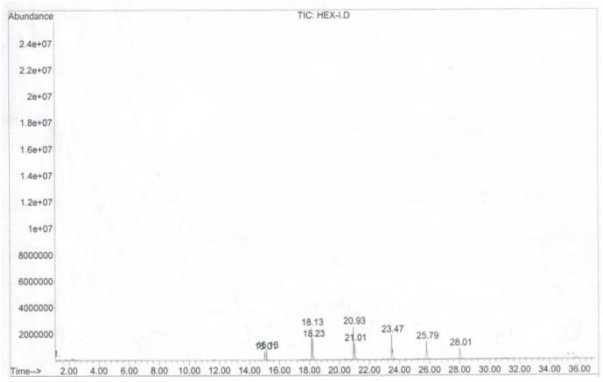

Fig. 3: GC-spectra of a partially purified fourth subfraction (PPF) of *Streptomyces cheonanensis* VUK-A. By means of mass spectral analysis, the compounds (A, B, C, D, E, F, G, H and I) with retention time 15.01, 15.13, 18.13, 18.23, 20.93, 21.01, 23.47, 25.79 and 28.01 in PPF were identified as 1-tetradecene, tetradecane, 1-hexadecene, hexadecane, 5-octadecene, octadecane, 5-eicosene, 1-nonadecene and cyclo tetracosane respectively

Tetracycline served as positive control for bacteria. Compared to standard drug tetracycline, 1 displayed high sensitivity against *Bacillus subtilis,* while 2 (PPF) recorded similar sensitivity like positive control against *Staphylococcus aureus* and *Xanthomonas campestris* (table 1). Tetracycline, in other cases, showed good antibacterial activity over the metabolites of the strain.

Table 1: Minimum inhibitory concentration (µg/ml) of the bioactive compounds produced by *Streptomyces cheonanensis* VUK-A against gram positive and gram negative bacteria

Test microorganisms	Compound-1			Compound-2			Tetracycline (Control)		
	Zone of Inhibition	±SD	P value	Zone of Inhibition	±SD	P value	Zone of Inhibition	±SD	P value
Staphylococcus aureus	64.00	±0.54	(*p<0.01*)	32.00	±0.43	(*p<0.02*)	32.00	±0.39	(*p<0.03*)
Streptococcus mutans	128.00	±0.31	(*p<0.03*)	64.00	±0.51	(*p<0.01*)	32.00	±0.68	(*p<0.03*)
S. epidermis	128.00	±0.46	(*p<0.02*)	32.00	±0.39	(*p<0.04*)	16.00	±0.32	(*p<0.03*)
X. campestris	256.00	±0.69	(*p<0.04*)	16.00	±0.63	(*p<0.05*)	16.00	±0.71	(*p<0.02*)
X. malvacearum	128.00	0.43	*p<0.01*)	32.00	±0.48	*p<0.03*)	8.00	±0.47	*p<0.02*)
Bacillus subtilis	16.00	±0.51	(*p<0.05*)	64.00	±0.21	(*p<0.02*)	32.00	±0.56	(*p<0.04*)
B. megaterium	128.00	±0.37	(*p<0.01*)	64.00	±0.48	(*p<0.02*)	16.00	±0.52	(*p<0.05*)
B. cereus	64.00	±0.72	(*p<0.02*)	64.00	±0.39	(*p<0.04*)	8.00	±0.31	(*p<0.01*)
Escherichia coli	64.00	±0.42	(*p<0.04*)	32.00	±0.63	(*p<0.02*)	8.00	±0.46	(*p<0.04*)
P. aeruginosa	32.00	±0.54	(*p<0.03*)	128.00	±0.47	(*p<0.03*)	8.00	±0.39	(*p<0.03*)
Serratia marcescens	32.00	±0.54	(*p<0.03*)	64.00	±0.39	(*p<0.04*)	32.00	±0.62	(*p<0.03*)
Proteus vulgaris	256.00	±0.51	(*p<0.05*)	128.00	±0.39	(*p<0.05*)	16.00	±0.49	(*p<0.05*)
Salmonella typhi	ND	ND	ND	128.00	±0.41	(*p<0.01*)	8.00	±0.47	(*p<0.05*)

Values are mean±SD (n=3)

The above components are subjected to one-way analysis of variance (ANOVA) tested 5% level of significance. All the values are found to be significant (*p<0.05*)**,** Compound 1: Benzoic acid**,** Compound 2: Partially purified fraction (PPF)**,** Tetracycline: control**,** ND: Not detected

Antifungal activity against dermatophytes and filamentous fungi and corresponding MIC values are recorded in table 2. The bioactive compounds showed low antifungal activity against the dermatophytes (*Candida albicans* and *Epidermophyton floccosum*)

and the tested fungi. Among the filamentous fungi tested, *Aspergillus flavus* and *Penicillium citrinum* recorded sensitivity of 64 µg/ml towards compound 1 and no activity was recorded against *Verticillium alboatrum* and *Alternaria up* to 1000 µg/ml. PPF was active against *Aspergillus flavus* at 16 µg/ml followed by *Fusarium solani* (32 µg/ml) and displayed similar sensitivity like positive control against *Fusarium solani*. In other cases, both compounds recorded lower antifungal activity than the standard fungicides such as Griseofulvin against dermatophytes and Amphotericin-B against fungi.

Table 2: Minimum inhibitory concentration (MIC) of the bioactive compounds isolated from *Streptomyces cheonanensis* VUK-A (MIC-(µg/ml)) against dermatophytes and fungi

Dermatophytes	Compound-1			Compound-2			Anti-fungal agent (Control)		
	Zone of Inhibition	±SD	P value	Zone of Inhibition	±SD	P value	Zone of Inhibition	±SD	P value
Candida albicans	64.00	±0.36	(*p<0.01*)	64.00	±0.68	(*p<0.01*)	16.00	±0.46	(*p<0.01*)
Epidermophyton floccosum	128.00	±0.47	(*p<0.02*)	64.00	±0.28	(*p<0.02*)	16.00	±0.51	(*p<0.02*)
Fungi									
Aspergillus niger	128.00	±0.36	(*p<0.05*)	64.00	±0.43	(*p<0.03*)	16.00	±0.76	(*p<0.04*)
Aspergillus flavus	64.00	±0.45	*p<0.02*)	16.00	±0.44	*p<0.04*)	8.00	±0.39	*p<0.04*)
Fusarium oxysporum	256.00	±0.57	(*p<0.03*)	128.00	±0.27	(*p<0.01*)	16.00	±0.21	(*p<0.02*)
Fusarium solani	128.00	±0.63	(*p<0.05*)	32.00	±0.37	(*p<0.01*)	32.00	±0.43	(*p<0.03*)
Penicillium citrinum	128.00	±0.37	(*p<0.05*)	64.00	±0.63	(*p<0.03*)	8.00	±0.69	(*p<0.02*)
Verticillium alboatrum	ND	ND	ND	128.00	±0.57	(*p<0.03*)	64.00	±0.59	(*p<0.05*)
Alternaria alternata	ND	ND	ND	256.00	±0.37	(*p<0.02*)	32.00	±0.31	(*p<0.01*)

Values are mean±SD (n=3)

The above components are subjected to one-way analysis of variance (ANOVA) tested 5% level of significance. All the values are found to be significant (*p<0.05*), (*p<0.05*), Compound 1: Benzoic acid, Compound 2: Partially purified fraction (PPF), ND: Not detected

*Antifungal agent: Griseofulvin (Control) against dermatophytes and Amphotericin-B (control) against fungi

The bioactive compounds of the present work extracted from *Streptomyces cheonanensis* VUK-A, showed moderate to significant antimicrobial activity against opportunistic and pathogenic bacteria and fungi. The active compound produced by the strain VUK-A was benzoic acid. Benzoic acid and its derivatives are the natural constituents of many plants and microorganisms. Benzoic acid was effective against bacteria in acid media at a level of 0.1% and in neutral media at 0.2% but inactive in alkaline media. Benzoic acid under appropriate conditions act as bactericidal and bacteriostatic agent and sodium benzoate inhibited the growth of *Vibrio parahaemolyticus* and *Listeria monocytogenes* [17, 18]. Similar results were reported for fungi and yeasts [19]. We have observed that compound **1** showed significant inhibitory activities against *Bacillus subtilis* (16 µg/ml), *Serratia marcescens* (32 µg/ml), *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* (64µg/ml). Growth and aflatoxin production by toxigenic strains of *Aspergillus* were partially or completely inhibited by benzoic acid [20] and our data also support this evidence reported. Both fungistatic and fungicidal properties have been attributed to benzoic acid according to a result of a study involving several strains of *Trichophyton* and *Microsporum* [21]; however the present study recorded antifungal activity against *Aspergillus flavus*, *Penicillum citrinum* and *Candida albicans* with an MIC of 64 µg/ml.

The partially purified subfraction (2) subjected to GC-MS contained 9 compounds. Smaoui *et al.* [22] reported 1-nonadecene from *Streptomyces* sp. TN 256 strain exhibited antibacterial activity and strong antifungal activity against *Candida albicans* [23] and 1-Tetradecene, 1-Hexadecene and cyclo tetracosane were reported from Gynura segetum's leaf extracts [24] showed potent antimicrobial activities. 5-Octadecene, 1-Hexadecene and 1-nonadecene were compounds reported from *Streptomyces* sp. TN 272, a novel terrestrial species isolated from soil and water samples of Tunisian regions [25]. A perusal of literature revealed that there was no report on the occurrence of 1-tetradecene, tetradecane, hexadecane, octadecane, 5-eicosene and cyclo tetracosane as natural products from microorganisms especially from actinomycetes, and this is the first report of their isolation from *Streptomyces cheonanensis* VUK-A. Identification of the compounds reported here is preliminary, and further structural analyses need to be performed to ascertain the identity of the compounds in the fourth subfraction. According to our antimicrobial studies the partially purified fourth sub-fraction possesses significant antibacterial as well as antifungal activities (table 1 and 2).

CONCLUSION

In the present study, the metabolites produced by *Streptomyces cheonanensis* VUK-A exhibited significant bioactivity against the test bacteria and fungi and reveals that the strain is promising producer of antimicrobial compounds. The compounds such as 1-tetradecene, tetradecane, hexadecane, octadecane, 5-eicosene and cyclo tetracosane identified by using GC-MS analysis are not yet reported from the genus *Streptomyces* and also as natural products from actinomycetes.

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CONFLICT OF INTERESTS

Declared none

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