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Antibacterial Compounds of Actinomycetes Isolated From Altitude Soils

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ABSTRACT

Background: Bacterial infection is a global threat due to antibiotic resistance. This demands the urgent need for novel antibiotics, and soil actinomycetes could be the potential candidate. The key objective of our study was to detect antibacterial compounds from selected actinomycetes species isolated from high-altitude soil samples.

Methods: Three strains *Streptomyces* sp. 12923, *Streptomyces* sp. 13102, and *Nocardia* sp. 13105 were subjected to fermentation using International Streptomyces Project (ISP) 2 medium. Crude extracts of each isolate were recovered by Rotary evaporator. Crude extracts were fractionated in HPLC and fractions were collected in a 96-well plate to evaluate the antibacterial activity of each of the 19 fractions against a test organism *E. coli* BW25113. Crude extracts of three strains were analyzed using UPLC-MS/MS for antibacterial compounds. The LC-MS/MS data were processed using Metabo Scape software and features were annotated using different libraries in the software.

Results: For *Streptomyces* sp. 12923, fractions of the crude extract with the lowest OD_{600} 0.472, 0.484 showed higher antibacterial activity against *E. coli* BW25113^{T}, while the inhibitory action against same test organism was shown better by fractions OD_{600} 0.250, 0.329, and OD_{600} 0.273, 0.326 for *Streptomyces* sp. 13102 and *Nocardia* sp. 13105, respectively. The antibacterial compounds detected included Mayamycin and Mayamycin B from *Streptomyces* sp. 12923, Nocardamine and Streptazone D from *Streptomyces* sp. 13102, and Nocardimicin B, 4-O-methylmelleolide, Spathullin B and Nannozinone B from *Nocardia* sp. 13105.

Conclusions: The identification of these compounds from high-altitude actinomycetes further strengthens the claim that actinomycetes are rich sources of bioactive compounds.

Keywords: Actinomycetes; antibacterial; metabolites; *Nocardia; Streptomyces.*

INTRODUCTION

Actinomycetes are Gram-positive filamentous bacteria producing secondary metabolites, including antibiotics, antifungals, antivirals, and anticancer agents. They have high G+C content and are found in soil, water, and plants.[1] Around two-thirds of the antibiotics are produced from actinomycetes, mainly from the genus *Streptomyces*. Some antibiotics from *Streptomyces* are Streptomycin, Tetracycline, Chloramphenicol, and Daptomycin.[2,3]

Due to the overuse and misuse of antibiotics, there is a global problem of antibiotic resistance which makes it difficult to treat infections with known antibiotics.[4] So, there is an urgent need to explore novel antibiotics from high-altitude regions characterized by low temperatures, reduced oxygen levels, and increased UV radiation. The high-altitude soils of Nepal with extreme environmental conditions due to the unique topogeography, harbor the potential actinomycetes capable of producing novel antibacterial metabolites that are not found in normal environments.[5] This study focuses on the functional strains of actinomycetes from high-altitude soil samples to identify antibacterial compounds.

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METHODS

Actinomycetes were isolated from soil samples collected from different locations and altitudes in Nepal (**Table 1**). Randomly selected isolates were subjected to screening for antibacterial activity. First, fermentation for each selected actinomycetes strains was carried out in International *Streptomyces* Project 2 (ISP 2) broth (0.4% yeast extract, 1.0% malt extract, and 0.4% dextrose; pH of 7.2) for 10 days. Crude extract from cell-free suspension was isolated using a rotary evaporator. Second, the antibacterial activity of crude extract from actinomycetes isolates was evaluated on a 96-well microtiter plate assay.[6]

Table 1. Soil sample collection details used in this study.

S.N.	Sample Code	Sampling Site	Latitude and Longitude	Altitude (m.a.s.l.)	Collection Date
1	0801	Pokhara	28°14'40.519"N 83°58'27.785"E	1142	2019
2	0854	Phulchowki	27°34'51.411"N 85°23'13.992"E	1996	2019
3	0898	Sarangkot	28°14'20.334"N 83°57'0.369"E	1348	2019

Two test bacterial strains *E. coli* BW25113^T and *Bacillus subtilis* DSM 10^T were obtained from the Department of Microbial Natural Products, Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS), Saarbrücken, Germany.

Fractionation of three strains (*Streptomyces* sp. 12923, *Streptomyces* sp. 13102, and *Nocardia* sp. 13105) was done with the help of High-Performance Liquid Chromatography (HPLC) and fractions were collected in a 96-well plate. Antimicrobial activity of 19 fractions from each of the 3 strains was tested against a Gram-negative test organism *E. coli* BW25113 following the workflow as shown in **Figure 1**. Optical density was measured on a 600 nm wavelength with 25 flashes in Tecan Infinite 200 PRO microplate reader and analysis was done using Tecan i-control software.

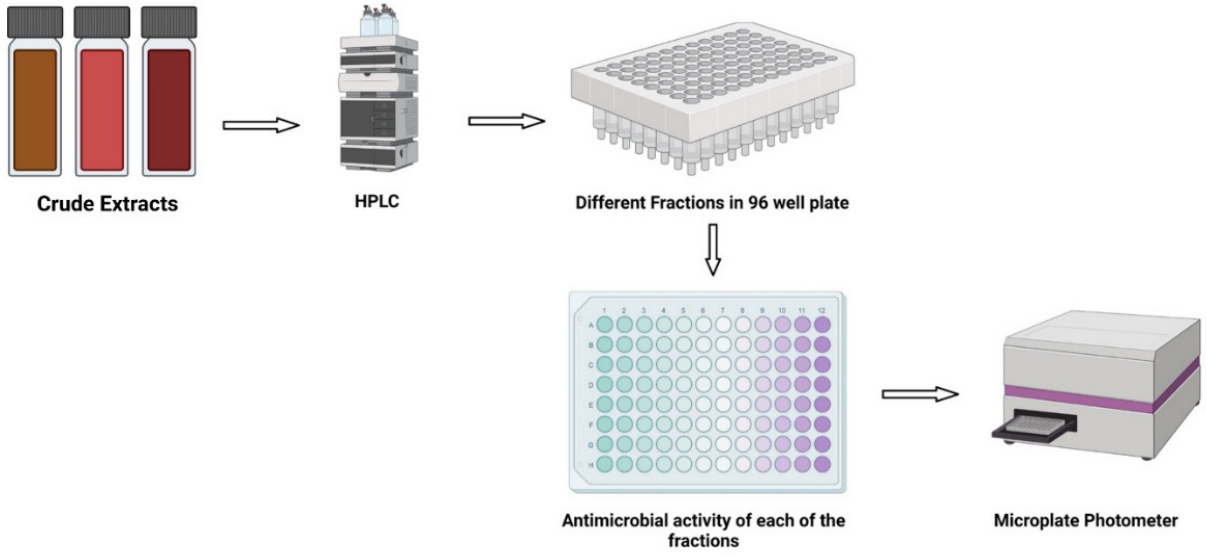

Figure 1. Workflow and methods applied in the fractionation and antimicrobial activity of the fractions.

The crude extract of *Streptomyces* sp. 12923, *Streptomyces* sp. 13102, and *Nocardia* sp. 13105, which showed antibacterial activity was diluted 1:10 with methanol and centrifuged at 21,500x g for 5 minutes at 4 °C to eliminate any remaining cell debris and other solid fragments before using the samples for Ultra-performance liquid chromatography-Tandem mass spectrometry (UPLC-MS/MS) analysis. Measurements were performed using a Dionex Ultimate 3000 RSLC (Rapid Resolution Liquid Chromatography system) system (Thermo) with a BEH C18, 100×2.1 mm, 1.7 µm column (Waters

GmbH, Germany) following the procedure described previously.[7]

All Liquid Chromatography-Mass Spectrometry (LC-MS) data from samples were processed using the T-ReX in MetaboScape (Bruker Daltonics GmbH & Co. KG) with a minimum intensity threshold of 5000 counts and a minimum peak length of 4 spectra. After processing, features were annotated with the Target List utility in MetaboScape utilizing data from different libraries.

RESULTS

Actinomycetes isolated in the present study had powdery colonies on AIA agar plates and were filamentous in shape as observed under the light microscope (**Figure 2**).

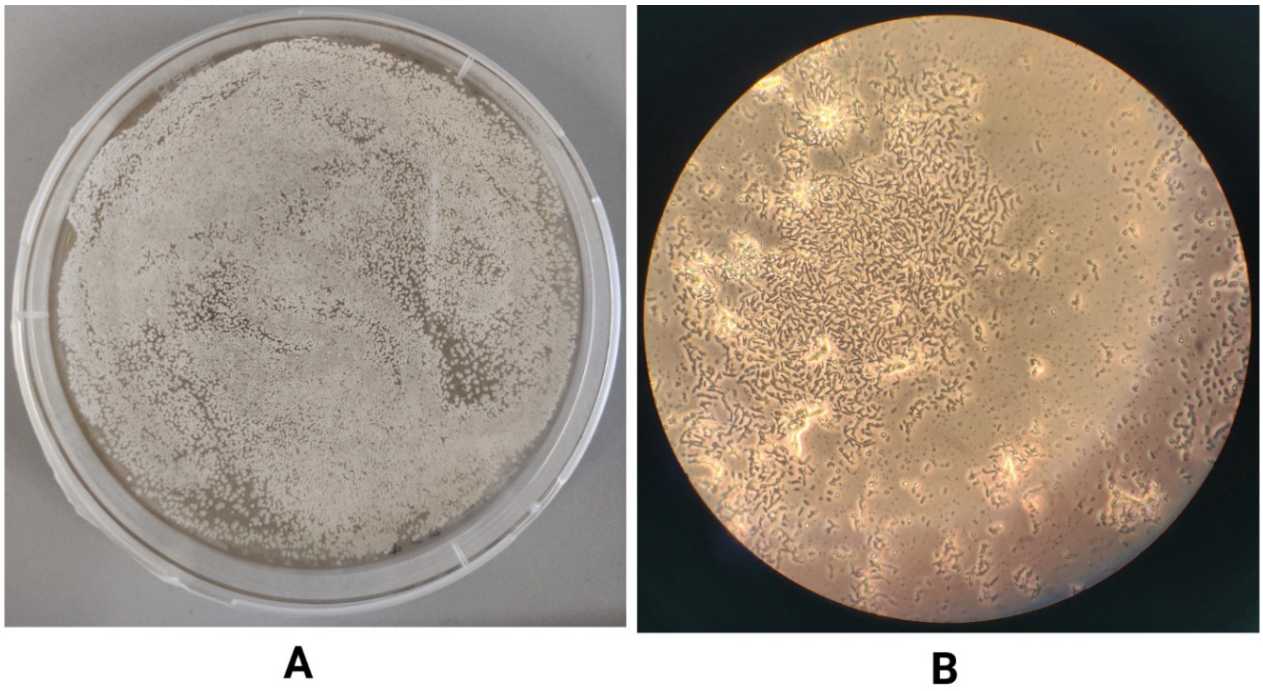

Figure 2. (A) Typical powdery colonies of actinomycetes (*Streptomyces* sp. 13102) grown on Actinomycete Isolation Agar (AIA) agar. (B) Filamentous structure of actinomycetes observed in a light microscope (100x).

Based on the potency of antibacterial activity, three isolates were selected and identified as *Streptomyces* sp. 12923, *Streptomyces* sp. 13102, and *Nocardia* sp. 13105 (**Figure 3)** which showed promising antibacterial activities against *E. coli* BW25113^T and *Bacillus subtilis* DSM 10^T.

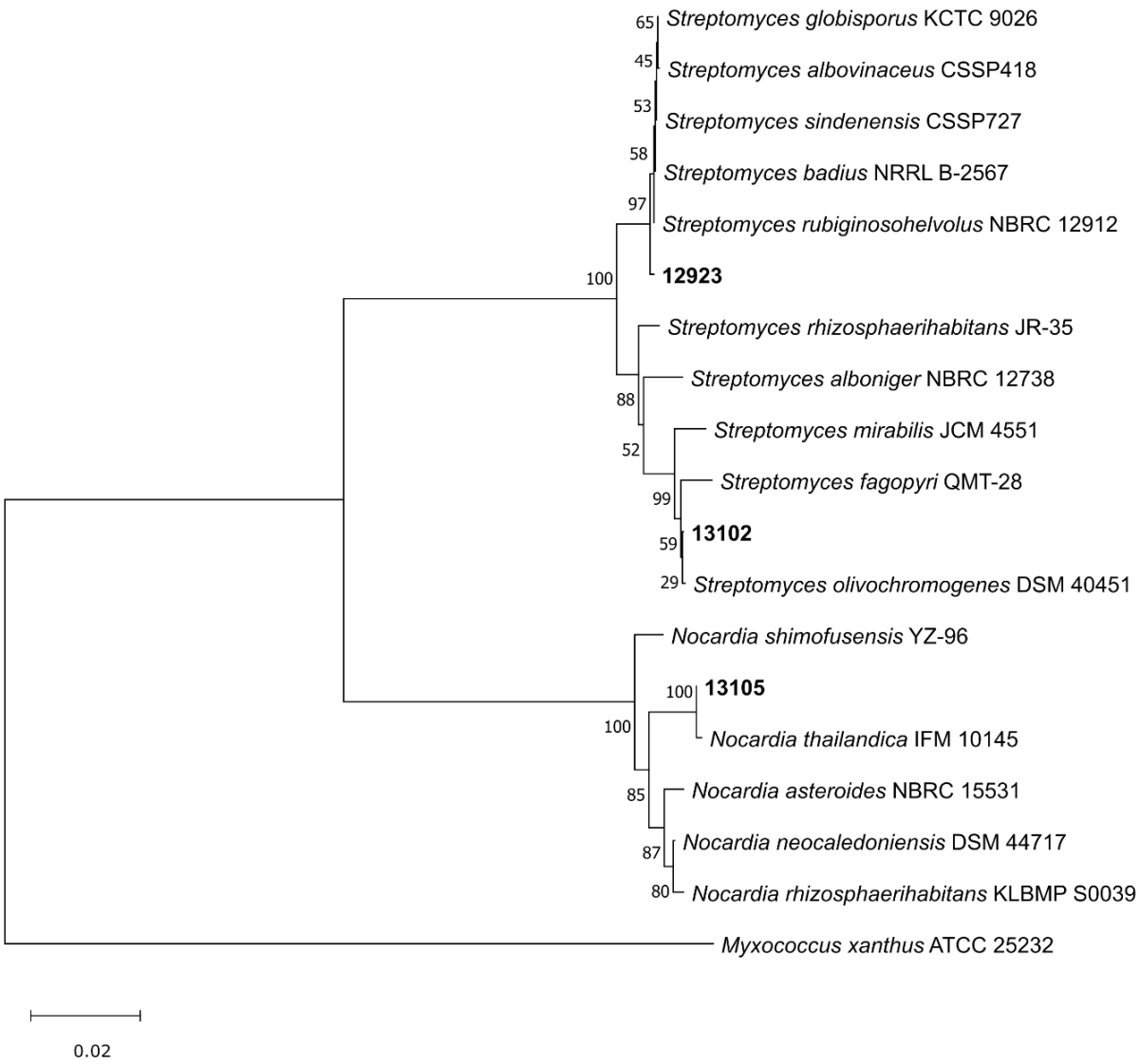

Figure 3. Phylogenetic tree showing the position of three actinomycetes strains 12923, 13102 and 13105 (shown in bold). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary history was inferred using the Neighbor-Joining method and evolutionary distances were computed using the Maximum Composite Likelihood method. Evolutionary analyses were conducted in MEGA11. *Myxococcus xanthus* ATCC 25232 was used as an outgroup.

The crude extracts from three strains inhibited the test bacterial strains in diluted concentrations (lower concentrations). So, the crude extracts for these three strains were further processed for UPLC-MS/MS analysis.

The optical density (OD_{600}) of 19 fractions along with control (solvent) and crude extract was measured by Tecan Infinite 200 PRO microplate reader and Tecan i-control software (Table 2). For *Streptomyces* sp. 12923, fractions with OD_{600} 0.472, 0.484, and 0.487 showed good antibacterial activity against *E. coli* BW25113^{T}. Similarly, for *Streptomyces* sp. 13102 and *Nocardia* sp. 13105, the best antibacterial activity was shown by fractions OD_{600} 0.250, 0.329, 0.338 and OD_{600} 0.273, 0.326, and 0.331, respectively.

Table 2. Optical density (OD_{600}) of fractions from three actinomycete strains showing antimicrobial activity# against *E. coli* BW25113^T.

	OD_{600} measurements												
	C	Crude Extract	Fractions (19 for each strain)										C
Streptomyces sp. 12923	0.546	0.447	0.538	0.562	**0.472**	0.547	0.560	0.589	**0.484**	0.534	0.540	-	0.509
	0.524		0.505	0.532	0.524	0.545	**0.487**	0.562	0.540	0.563	0.559	0.582	0.522
Streptomyces sp. 13102	0.534	0.348	0.491	0.477	0.482	**0.329**	0.400	**0.250**	**0.338**	0.376	0.465	-	0.466
	0.557		0.477	0.534	0.567	0.577	0.502	0.502	0.659	0.528	0.489	0.553	0.491
Nocardia sp. 13105	0.471	0.317	0.428	**0.331**	**0.326**	**0.273**	0.389	0.351	0.397	0.394	0.481	-	0.487
	0.510		0.428	0.456	0.400	0.431	0.510	0.467	0.569	0.602	0.578	0.535	0.495

C = Control (methanol as solvent), Crude Extract = unfractionated crude extract, Fractions = fractions obtained by HPLC at different retention times; #Best antibacterial activity is shown in bold (the lower OD_{600}), the better is antibacterial activity.

The raw mass data was analyzed using MetaboScape software where each peak was analyzed, detected, aligned, and annotated. The results were compared with the dictionary of natural products like NPAtlas and GNPS. Evaluation of UPLC-MS/MS data of the different three active extracts revealed several known compounds and compound classes which can support the antibacterial activity observed from the active extracts. The MS-MS analysis identified several compounds (Table 3) in the chromatogram.

Table 3. Antibacterial compounds identified from three actinomycetes crude extracts along with their m/z value, retention time, and formula.

Strain Name	Compound Identified	Formula	m/z value (mass-to-charge ratio)	Retention Time (RT) (in minutes)
Streptomyces sp. 12923	Mayamycin	$C_{26}H_{25}NO_7$	464.17154	7.12
Streptomyces sp. 12923	Mayamycin B	$C_{25}H_{23}NO_7$	450.15656	6.96
Streptomyces sp. 13102	Nocardamine	$C_{27}H_{48}N_6O_9$	601.35548	4.42
Streptomyces sp. 13102	Streptazone D	$C_{10}H_{13}NO$	164.10637	4.7
Nocardia sp. 13105	Nocardimicin B	$C_{39}H_{59}N_5O_{10}$	758.43196	14.36
Nocardia sp. 13105	4-O-methylmelleolide	$C_{24}H_{30}O_6$	415.21162	10.58
Nocardia sp. 13105	Spathullin B	$C_{12}H_{11}NO_2$	202.08617	7.36
Nocardia sp. 13105	Nannozinone B	$C_{16}H_{14}N_2O_2$	284.13969	4.6

DISCUSSION

The isolation of actinomycetes from high-altitude soil has yielded strains exhibiting antibacterial activity against *E. coli* BW25113^T and *Bacillus subtilis* DSM 10^T. The crude extracts from *Streptomyces* sp. 12923, *Streptomyces* sp. 13102, and *Nocardia* sp. 13105 in our study also inhibited the test organisms at lower concentrations, indicating the production of potential antibacterial compounds.

Through UPLC-MS/MS analysis of the extracts, several known and unknown compounds were identified, including Mayamycin and Mayamycin B from *Streptomyces* sp. 12923, Nocardamine and Streptazone D from *Streptomyces* sp. 13102, and Nocardimicin B, 4-O-methylmelleolide, Spathullin B, and Nannozinone B from *Nocardia* sp. 13105. These compounds have previously reported antibacterial activity which supports the observed antibacterial study of crude extracts in our study. A recent study has isolated antimicrobial compounds like Epopromycin A, Myxopyronin B, Gilvocarcin HE, and Okilactomycin A from actinomycetes isolated from Nepal that were active against *E. coli*, *Shigella sonnei*, *Staphylococcus aureus*, and *Salmonella* Typhi.[8] Another study done by[9], actinomycetes like *Streptomyces* and *Amycolatopsis* isolated from high-altitude soil samples revealed antibiotic agents such as Diketopiperazines and Hexahydropyrrolo[1,2-a]pyrazine-1,4-dione which were also active against *Shigella sonnei*, *Klebsiella pneumoniae*, *E. coli*, and *Staphylococcus*

aureus. Another study done in the Himalayan regions of Nepal showed that *Streptomyces* species are able to produce metabolites like diketopiperazine derivatives, aureolic acid derivatives such as Chromomycin A, and Lipopeptide, Actinomycin D and γ-sitosterol. These strains showed antibacterial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae*, antifungal activity against *Saccharomyces cerevisiae* and *Aspergillus niger*, and also inhibit the cervical cancer cells (HeLa) and breast cancer cells (MCF-7).[10] The LC-MS/MS analysis and GNPS-based molecular networking revealed antimicrobial compounds like Surfactin B, C, D, and Valinomycin from *Streptomyces* species isolated from soil samples collected from different areas in Nepal.[11]

Mayamycin and its derivative, Mayamycin B identified in our study possess a strong antibacterial activity in previous studies also. Mayamycin, a benz[a]anthracene derivative, showed strong inhibitory effects against various Gram-positive and Gram-negative bacteria, including clinically significant strains such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and methicillin-resistant *Staphylococcus aureus* (MRSA) with MIC value from 2.5 µM to 8.4 µM,[12] as well as potent cytotoxic activity against eight human cancer cell lines. In another study, Mayamycin B, a type II polyketide showed potent bioactivity against *Micrococcus luteus* with MIC value of 2.0 µM, whereas Mayamycin has MIC value of 8.0 µM, suggesting the N-methyl group is important to its antibacterial activity.[13]

Nocardamine, also known as Desferrioxamine E, produced by *Streptomyces* sp. 13102 is a cyclic trihydroxamate siderophore, a small molecule that binds and transports irons.[14] Although Nocardamine may not have a direct impact as an antibacterial, they have a strong iron-chelating activity which deprives bacteria of its essential nutrients and inhibits their growth.[15,16] Desferrioxamine B is currently the only available therapeutic agent for chronic iron overload and acute iron intoxication.[17] Nocardamine has shown antibacterial activity against *Proteus mirabilis* and *Proteus vulgaris*.[18,19] In another study, Nocardamin glucuronide along with nocardamin was isolated from *Streptomyces* sp. 80H647, where it showed mild antimalarial activity (IC_{50} 10 µM).[20] Nocardimicin B, a pale yellow amorphous solid is also a siderophore compound previously isolated from *Nocardia* sp. TPA0674 showed inhibitory activity against muscarinic M3 receptors.[21]

Another compound detected in our study, Streptazone D is a piperidine alkaloid isolated from *Streptomyces* species[22] which may interact with the bacterial cell membrane. While there is a limited study on the antibacterial activity of Streptazone D, it is also known to have some antibacterial properties.[23] Streptazolin, which has a similar structure to Streptazone D, showed good antimicrobial activity against *Salmonella* Typhi, *Vibrio cholerae*, and MRSA.[24]

The natural compound, 4-O-methylmelleolide, which belongs to class melleolides and analogs, was identified in our study from the strain *Nocardia* sp. 13105 which showed antibacterial activity against both Gram-positive and Gram-negative test bacteria. Similarly, 4-O-methylmelleolide has demonstrated antibacterial activity against *Bacillus cereus*, *B. subtilis*, and *E. coli*.[25] Another study showed strong antibacterial activity of 4-O-methylmelleolide against Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* but did not inhibit the growth of Gram-negative bacteria *Pseudomonas aeruginosa* and *E. coli*.[26] Furthermore, other mellein derivatives like cis-4-acetoxyoxymellein and 8-deoxy-6-hydroxy-cis-4-acetoxyoxymellein exhibited strong antibacterial activity against *E. coli* and *Bacillus megaterium*.[27]

Spathullin B, an isoquinoline alkaloid, detected in strain *Nocardia* sp. 13105 has demonstrated significant antibacterial activity against both Gram-positive and Gram-negative bacteria. Spathullin B with an MIC of 1 µg/mL showed strong antibacterial activity against Gram positive bacteria *S. aureus*[28] and Gram-negative bacteria *E. coli*, *Acinetobacter baumannii*, *Enterobacter cloacae*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*.[29] Similarly, Nannozinone B detected in our strain *Nocardia* sp. 13105 was initially isolated from myxobacteria *Nannocystis pusilla*. Nannozinone A showed activity against Gram-positive bacteria *Nocardia* sp., *Staphylococcus aureus*, and *Mycobacterium diernhoferi* while Nannozinone B was only active against *Nocardia* species. Nannozinone B also demonstrated good cytotoxicity against human ovarian carcinoma cell line SKOV-3.[30]

Identifying these compounds from high-altitude actinomycetes further strengthens the claim that actinomycetes are rich sources of bioactive compounds. Actinomycetes living in extreme conditions like high altitude presents challenges to bacteria which could evolve unique biosynthetic and metabolic pathways to produce compounds for their survival. These compounds may have novel structures with new mechanisms of action which unlocks the search for novel therapeutic agents to fight against bacterial infections and antibiotic resistance causing serious threats to the world.

CONCLUSIONS

Our findings are significant in understanding the

compounds responsible for showing antibacterial activities that underscore the value of exploring actinomycetes from unique and extreme environments, which might lead to the discovery of novel bioactive compounds. The metabolites identified in our study included Mayamycin, Nocardamine, Streptazone D, Nocardimicin B, 4-O-methylmelleolide, Spathullin B, and Nannozinone B. Though strong antibacterial activity against both Gram-positive and Gram-negative bacteria has previously been demonstrated, it is possible that this antibacterial activity in our study can be linked to these compounds. However, further research is warranted to delineate the exact role of these compounds.

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COMPETING INTERESTS

The authors declare no conflict of interest.

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