Nepal J Biotechnol. 2025 Jul; 13 (1): 1-10 Research Article DOI: https://doi.org/10.54796/njb.v13i1.355

Nepal Journal of Biotechnology
Publisher: Biotechnology Society of Nepal
Journal Homepage: https://nepjb.com/index.php/NJB
ISSN (Online): 2467-9313
ISSN (Print): 2091-1130

Antifungal Metabolites from High-Altitude Soil Actinomycetes Isolated from Nepal

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Received: 12 Jan 2025; Revised: 23 Jan 2025; Accepted: 27 Jan 2025; Published online: 31 Jul 2025

Abstract

The fungal infections are growing global threat due the resistance to antifungal agents, so demands urgent attention. Soil actinomycetes are known to produce antifungal metabolites and Nepal's soil has high potential to search antifungal actinomycetes. However, soil actinomycetes of Nepal have rarely been investigated for antifungal metabolites. In the present study, we aimed to detect antifungal compounds from selected *Streptomyces* species isolated from soil samples. We selected three strains of *Streptomyces* (*Streptomyces* sp. 13104, *Streptomyces* sp. 13081, and *Streptomyces* sp. 12933), and fermentation was carried out using International *Streptomyces* Project (ISP) 2 medium. A rotary evaporator was used to isolate the crude extract and antifungal metabolites were detected using UPLC-MS/MS. The LC-MS data were processed using the T-ReX 3D algorithm in MetaboScape 2022b, and GNPS spectral library was used to annotate the features. Feature-Based Molecular Networking (FBMN) was used to create molecular networks and visualized using Cytoscape v. 3.8.2. Evaluation of LC-MS data of the different active extracts revealed several known compounds that can plausibly exhibit antifungal activity. The large linear polyenes linearmycins A and B were detected in the crude extract from *Streptomyces* sp. 13104. Several members of the antimycin and kitamycin family were produced by *Streptomyces* sp. 13081 and annotated by GNPS library comparison and NPAtlas. Similarly, amphotericin B produced by *Streptomyces* sp. 12933 was exclusively annotated by the GNPS spectral library. The various antifungal compounds identified in our study underscore the value of exploring actinomycetes from unique and extreme environments, which may be crucial in searching the novel antimicrobial compounds.

Keywords: Actinomycetes, Metabolomics, Metabolites, Antimicrobials, *Streptomyces*

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Introduction

Actinomycetes are a broad group of gram-positive bacteria with higher G+C content in their genetic material [1] and fall under the phylum Actinomycetota [2]. They are filamentous in shape and considered a bridge between bacteria and fungi due to similar morphology and growth patterns [3]. They are primarily found in common environments like soil, water, plants and even in extreme environments like deserts, caves, mountains, marine water, high-altitude soils, and lakes [4]. The most important feature of actinomycetes is that they form branching filaments like fungal hyphae, which allows them to attach easily to substrates like soils and plants. They can produce spores that enable them to survive in harsh conditions and re-germinate when favorable conditions occur [5]. Actinomycetes play an important role in the soil ecosystem because they produce compounds capable of breaking complex organic polymers including cellulose and chitin. This way, they release some nutrients into the soil and make them available for other microorganisms. This decomposition process makes the soil more fertile and helps in the ecosystem's nutrient cycle [6,7].

Actinomycetes are well known to produce bioactive compounds including antibiotics, anticancer agents, antiviral agents, plant growth hormones, enzymes, biopesticide agents, and many more [8]. One of the best examples of actinomycetes producing antibiotics is *Streptomyces griseus,* which produces streptomycin, the first aminoglycoside antibiotic against tuberculosis [9]. Some other notable antibiotics produced by actinomycetes are tetracyclines [10], erythromycin [11], and chloramphenicol [12] used for treating bacterial infections. Apart from antibiotics, they also produce antifungal compounds like amphotericin B and nystatin [13] and anticancer agents like anthracyclines, enediynes, indolocarbazoles, isoprenoides, aureolic acids, carzinophilin, mitomycins, and peptides [14,15].

The study of small molecule metabolites helps to identify novel compounds from actinomycetes isolated from unexplored or extreme environments [16]. Advanced analytical tools like Gas chromatography-mass spectrometry (GC–MS), Liquid chromatography-mass spectrometry (LC-MS), High-performance liquid chromatography (HPLC), and Nuclear Magnetic Resonance (NMR) identify and characterize metabolites and provide the detailed structure of known and unknown compounds [17,18]. Understanding metabolites helps in biosynthetic pathway identification and molecular networking [19]. Metabolic studies of high-altitude strains help to discover the adaptation strategies and survival mechanisms of actinomycetes under extreme conditions and ecological interactions of actinomycetes through the exchange of metabolites. It helps to find unique biosynthetic routes and pathways that may produce new secondary metabolites with potential antimicrobial activities [20].

Due to the overuse and misuse of antifungal drugs, there is a worldwide problem of antifungal resistance demanding the discovery of novel compounds with unique mechanisms of action to combat these challenges [21]. Antifungal infections claim millions of lives globally, and the resistance against antifungal drugs is increasingly alarming. Therefore, searching antifungal metabolites from soil actinomycetes is an important task. One study isolated *Streptomyces felleus* that showed antifungal activity against *Candida albicans,* but the specific metabolites were not characterized [22]. In our research, we have addressed this gap by isolating antifungal actinomycetes and employing analytical techniques to identify the specific metabolites responsible for antifungal activities. Nepal, being a country of diverse geography and variable climatic conditions, is the perfect place and resource to explore the possibilities of isolating novel actinomycetes and bioactive compounds [23]. Having said that, limited studies have been done in Nepal focusing on active fungal metabolites. Previous studies have isolated *Streptomyces, Nocardia*, and *Micromonospora* from Nepal's water and soil samples without identifying specific metabolites responsible for the activity [24]. Exploring the actinomycetes from high-altitude soils to understand their full potential presents a significant opportunity that may harbor distinct metabolic pathways. Applying metabolomics to high-altitude actinomycetes from Nepal can lead to discovering previously unrecognized compounds, providing more detailed information on their biosynthetic pathways, and potentially developing new antifungal metabolites.

The present study aims to investigate the metabolite profile of antimicrobial actinomycetes isolated from high-altitude soils of Nepal to identify the potential novel bioactive compounds. Advanced tools and techniques, including LC-MS/MS, GNPS molecular networking, and NPAtlas for metabolite identification, help us to uncover novel bioactive compounds. The research findings from this study could contribute significantly to developing effective antifungal agents to combat the rising threat of fungal infections and resistance.

Materials and Methods

Isolation and Identification of Actinomycetes

Soil samples collected from different altitudes and locations in Nepal from 2018 to 2019 were used to isolate actinomycetes strains in Actinomycetes Isolation Agar (AIA). Isolated actinomycetes were identified through cultural, and morphological characteristics and confirmed by 16S rRNA gene sequencing, and subsequent phylogenetic analysis.

Crude Extract and Antifungal Screening

The actinomycetes were fermented at 180 r.p.m using shaking flasks (incubator) at 30ºC for 10 days in ISP 2 broth (0.4% yeast extract, 1.0% malt extract, and 0.4% dextrose; pH of 7.2). Crude extract from cell-free suspension was isolated using a rotary evaporator to obtain dried extracts. The dried extract was dissolved in 2.2 mL of methanol and kept in a -80°C freezer for future use.

Test fungal Strains

Two diverse groups of test fungal strains, *Wickerhamomyces anomalus* DSM 6766^T (yeast) and *Mucor hiemalis* DSM 2656^T (filamentous fungi) were obtained from the Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) Microbial Natural Products Department Germany. *W. anomalus* is an ascomycetes yeast associated with human infections, including fungemia in people with weak immunity [25]. The increasing clinical relevance of *W. anomalus* as an emerging pathogen emphasizes the importance of identifying effective antifungals against it. Similarly, *M. hiemalis* is a saprophytic filamentous fungus found in soil and decaying organic matter, sometimes causing mucormycosis and zygomycosis [26]. Testing against *M. hiemalis* also allows us to evaluate the spectrum of antifungal activity of the actinomycetes extracts.

Antifungal Screening of Actinomycetes

The antifungal activity of crude extract from actinomycetes isolates was evaluated on a 96-well microtiter plate assay against *Wickerhamomyces anomalus*

DSM 6766^{T} and *Mucor hiemalis* DSM 2656^{T} [27]. The fungal strains were grown overnight in MYC broth (w/v: 1.0% Difco Phytone peptone, 1.0% glucose, 50 mM HEPES, pH 7.0) for 24h. The fungal cultures were then adjusted to an optical density (OD600) of 0.05 for the assay. A stock solution of the crude extract was made by dissolving it in 2.2 mL of methanol, establishing this as the 100% concentration. The 96-well plates were filled with 150 µL of the fungal test strains. The first well line was filled with an additional 130 µL of fungal test strains, bringing the total volume to 280 µL. A 20 µL test crude extract was added to the first wells, along with a negative methanol control. Serial dilutions of the crude extract were performed directly on the plate using a multi-channel pipette. From the initial well (Well A) containing an undiluted extract (100%), 150 µL was transferred to the next row (Well B) and mixed with 150 µL of fungal test strains, making it a 50% (v/v) concentration. This process was repeated sequentially across the plate, halving the concentration at each step, until the eighth well (Well H), representing an 8-fold dilution (0.78%, v/v). The final 150 µL from the last row was discarded to maintain uniform volumes across all wells. Then, the plate was sealed with parafilm to prevent evaporation and incubated overnight at 30°C in a shaker incubator. Antifungal activity was assessed the next day by observing growth inhibition in the wells.

UPLC-MS and UPLC-MS/MS analysis of extracts

The crude extracts of *Streptomyces* sp. 13104, *Streptomyces* sp. 13081, and *Streptomyces* sp. 12933 extracts, showing good antifungal activity, were used for UPLC-MS/MS analysis. The measurements were done using the Dionex Ultimate 3000 RSLC system (Thermo) with the BEH C18, 100 × 2.1 mm, 1.7 µm column (Waters GmbH, Germany) following the procedure described previously [28,29], [30]. One microliter sample was separated by a linear gradient from (A) H_2O and 0.1% FA to (B) ACN and 0.1% FA with a flow rate maintained at 600 µL/min at 45°C. The gradient was started by a 60-second isocratic step at 5.0% B, followed by a rise to 95.0% B in 18 min, ending with a 2-min step at 95.0% B before the re-equilibration to the initial conditions. A DAD recorded UV spectra with a range of 200 - 600 nm. The LC flow was split to 75 µL/min using the Apollo II ESI source before entering the maXis 4G hr-ToF mass spectrometer (Bruker Daltonics GmbH & Co. KG). The split was built with capillaries (fused silica) of 75 and 100 µm I.D., and the lower dead volume T junction (Upchurch). The mass spectra were obtained from 150 - 2500 *m/z* at a scan rate of 2 Hz in a centroid mode. Every run began with a calibrant peak of the solution of basic sodium formate introduced by the filled loop of 20 µL switched into the LC flow at the start. Full scan spectra were obtained at 2 Hz, followed by acquisition of MS-MS spectra at a scanning speed of 0.68 Hz. CID energy varied linearly from 35, 45, to 60 eV, corresponding to precursor m/z from 500, 1000, to 2000 m/z. The ion cooler was fixed to ramp collision energy (80-120% of the set value), and the ion cooler RF from 700-1000 Vpp for each MS-MS scan. Every 2 seconds, the precursor list was assessed to assign the forthcoming precursors and the precursors were progressed to the exclusion list for a short time of 0.2 min after the measurement of two spectra as the width of the chromatographic peak was 0.10 to 0.15 min. The minimum precursor intensity was set to 5000.

LC-MS (Liquid Chromatography-Mass Spectrometry) Data Analysis

All the LC-MS data from samples were processed using the T-ReX 3D algorithm in MetaboScape 2022b (Bruker Daltonics GmbH & Co. KG) with a minimum intensity threshold of 25000 counts and a minimum of 3 successive scans. After processing, features were annotated with the Target List utility in MetaboScape utilizing data from the NPAtlas (v.2022_09) with matches tolerated for *m/z* differences between 1-2 ppm and mSigma between 10-50 (unitless, proprietary measurement from Bruker Daltonics, lower is better). Features were also annotated using the GNPS spectral library during molecular networking. Matches were treated as valid if at least one criterion was found to be in the tightest tolerance and evaluated later. Annotations were done in Cytoscape based on the annotation source, and the annotation's name follows the pattern NPAtlasName_GNPSName.

Molecular networking in GNPS

The molecular network was constructed following the workflow of Feature-Based Molecular Networking (FBMN) [31] on GNPS [32], following the procedures described previously [33–35]. Mass spectrometry measurements were processed initially with MetaboScape 2022 (Bruker Daltonics) and then transferred to GNPS for analysis by FBMN. The data was filtered to eliminate ions of MS/MS fragment within ±17 Da of precursor m/z. The spectra of MS/MS were cleaned by selecting only the topmost 6 fragment ions within a ±50 Da window through the spectrum. The mass tolerance of precursor ion was set at 0.03 Da, while the ion tolerance of MS/MS fragment was set at 0.05 Da. The molecular network was constructed, retaining edges with a cosine score >0.7 and >4 matched peaks. Additionally, edges between the two nodes were preserved only if every of the nodes was among the topmost ten most common nodes to the other. The largest size for a molecular family was set at 100, and the edges with the lowest scores were eliminated until the family size fell below the threshold. An analog search mode was applied to search against MS/MS spectra having a maximum precursor ion value difference of 100. The filtration of Library spectra was done similarly to the input data, and the matches of network spectra with library spectra were required to achieve a score higher than 0.7 with at least 4 matched peaks. The DEREPLICATOR tools annotated MS/MS spectra [36]. For the visualization, the resulting molecular networks, the Cytoscape v. 3.8.2 software was used [37].

Results

Antifungal actinomycetes from high-altitude

The actinomycetes identified in this study exhibited white powdery colonies on AIA agar plates and appeared as filamentous structures under a light microscope (**Figure 1**).

Among 29 isolated actinomycetes, only three isolates were chosen based on their antifungal potency and identified as *Streptomyces* species (*Streptomyces* sp. 13104, *Streptomyces* sp. 13081, and *Streptomyces* sp. 12933) through 16S rRNA gene sequencing and phylogenetic analysis (**Figure 2**) which showed promising antifungal activities against *Wickerhamomyces anomalus* DSM 6766^{T} and *Mucor hiemalis* DSM 2656^{T}. These three strains have been submitted in NCBI GenBank, and accession numbers of PQ104967, PQ104944, and PQ104929 have been obtained, respectively.

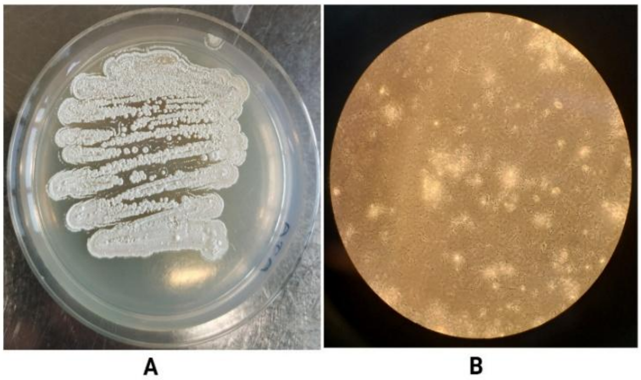

Figure 1. (A) Typical white and powdery colonies of actinomycetes (*Streptomyces* sp. 12933) grown on Actinomycete Isolation Agar (AIA) agar. (B) Filamentous structure of the actinomycetes observed under the light microscope (magnification 100X).

Crude extracts from three strains effectively inhibited the test fungal strains at diluted concentrations (lower concentrations). *Streptomyces* sp. 13104 and *Streptomyces* sp. 13081 inhibited the growth of both fungal test organisms up to well H (0.78% concentration), reflecting the presence of highly potent antifungal compounds

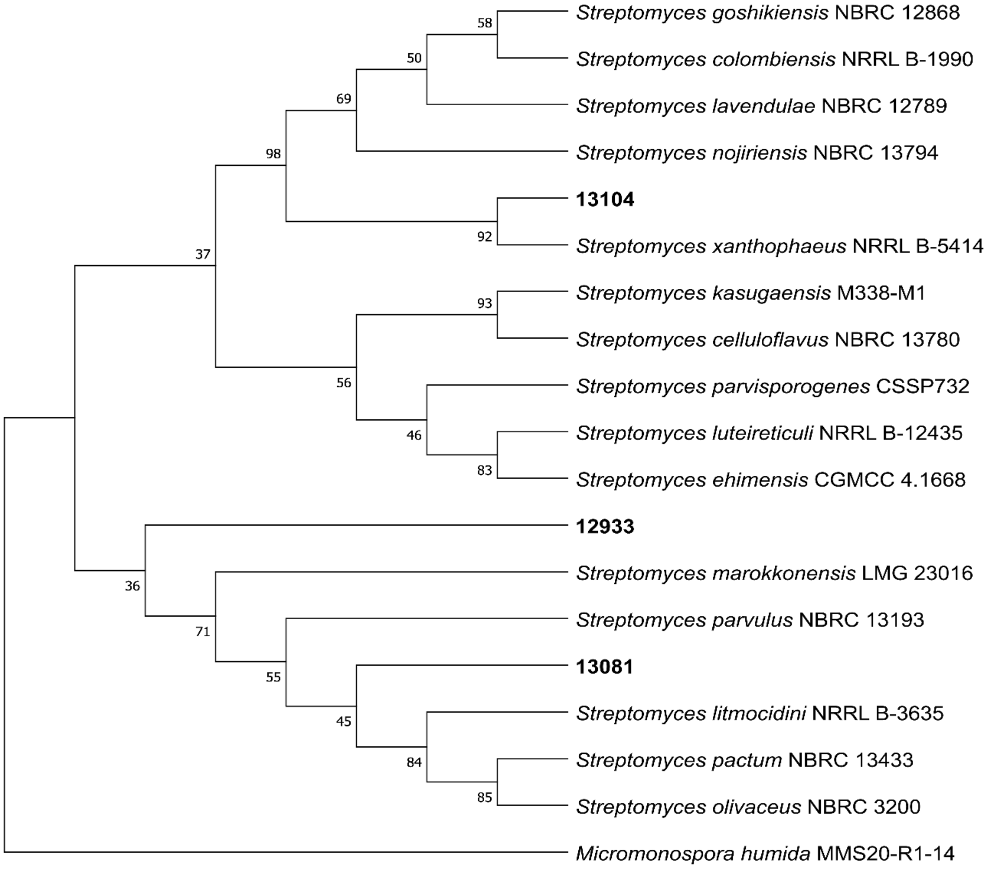

Figure 2. Bootstrap consensus phylogenetic tree (1000 replicates) showing the position of three actinomycetes strains 13104, 13081, and 12933 (shown in bold). MEGA11 was used for evolutionary analyses. Micromonospora humida MMS20-R1-14 was used as an outgroup.

capable of maintaining activity even at lower concentrations. For *Streptomyces* sp. 12933, fungal growth inhibition was observed up to well D (12.5% concentration) against *Mucor hiemalis* DSM 2656^{T}, suggesting a moderate concentration of bioactive antifungal compounds and inhibited fungal growth up to well C (25% concentration) against *Wickerhamomyces anomalus* DSM 6766^{T}, indicating a slightly lower concentration of active metabolites but still demonstrating significant antifungal activity. **Figure 3** presents the details of the antifungal activity. Consequently, the crude extracts from these three strains underwent additional processing for Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) analysis and Global Natural Product Social (GNPS) molecular networking.

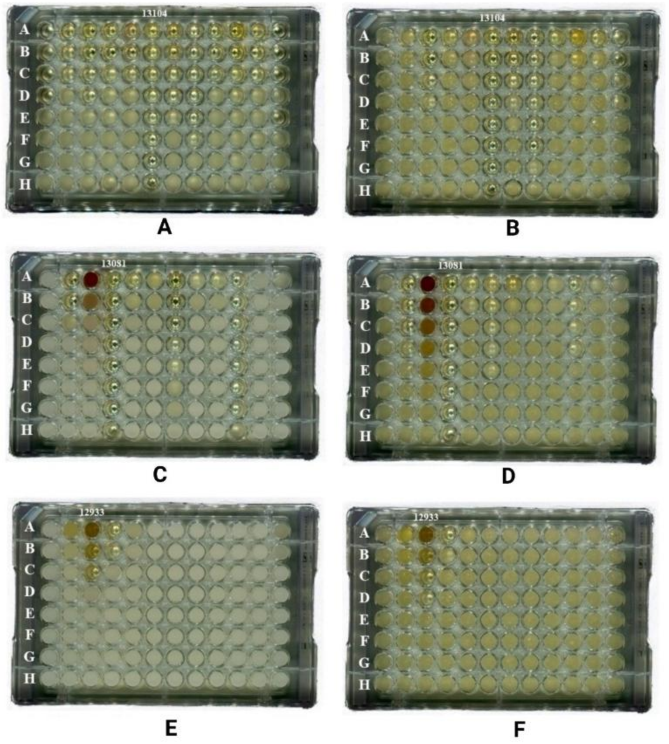

Figure 3. (A) Antifungal activity of *Streptomyces* sp. 13104 against *Wickerhamomyces anomalus* DSM 6766^{T}, (B) Antifungal activity of *Streptomyces* sp. 13104 against *Mucor hiemalis* DSM 2656^{T}, (C) Antifungal activity of *Streptomyces* sp. 13081 against *Wickerhamomyces anomalus* DSM 6766^{T}, (D) Antifungal activity of *Streptomyces* sp. 13081 against *Mucor hiemalis* DSM 2656^{T}, (E) Antifungal activity of *Streptomyces* sp. 12933 against *Wickerhamomyces anomalus* DSM 6766^{T}, (F) Antifungal activity of *Streptomyces* sp. 12933 against *Mucor hiemalis* DSM 2656^{T}. In each microtiter plate, horizontal rows of wells as designated by alphabets (white, bold) indicate the two-fold dilution of the crude extracts, from A to H maintaining the concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78% (v/v). This observation aligns with standard interpretations in broth microdilution methods, where a clear well indicate the absence of fungal growth due to the inhibitory effect of the tested extracts.

Antifungal metabolites from actinomycetes

The raw mass data was analyzed using MetaboScape software, where each significant peak was examined, spotted, aligned, and annotated. We compared these results with a natural product dictionary like NPAtlas. Evaluation of LC-MS results of the three different active extracts revealed several known compounds and compound classes, which can plausibly explain the antifungal activity observed from the active extracts. The MS-MS analysis identified several compounds in the chromatogram. From *Streptomyces* sp. 13104, Linearmycins A and B with an m/z value of 1140.71939 and 1166.73462 were detected at a retention time of 9.99 and 10.54 minutes, respectively. From *Streptomyces* sp. 13081, Antimycin and Kitamycin with an m/z value of 548.2724 and 464.2158 were detected at a retention time from 12.9 to 14.3 minutes and 8.5 to 10.2 minutes. Finally, from *Streptomyces* sp. 12933 Amphotericin B with an m/z value of 926.51077, was identified at a retention time of 8.56 minutes. The identification was based on spectral library matching and characteristic fragmentation patterns.

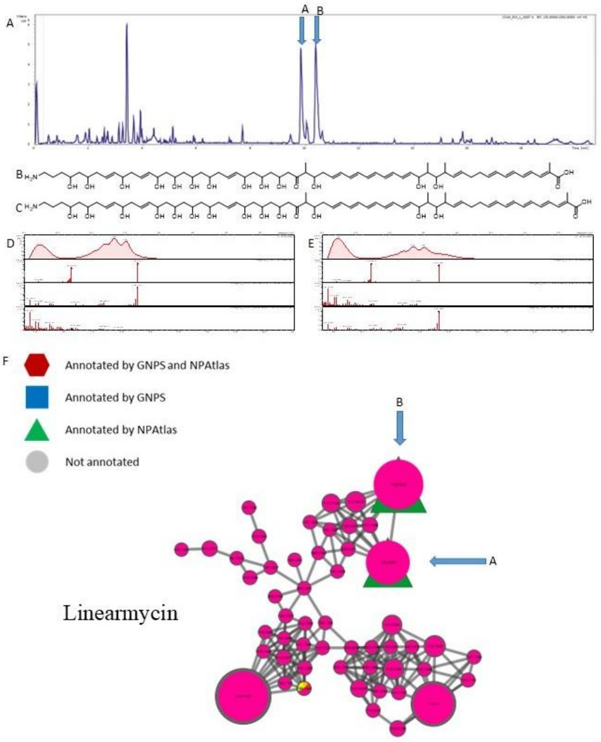

Figure 4. Linearmycin identification by LC-MS/MS. (A) LC-MS BPC trace of 13104 (*Streptomyces* sp. 13104) indicating linearmycin analogs. (B) linearmycin A structure. (C) linearmycin B structure. (D) UV and MS/MS spectrum of linearmycin A. (E) UV and MS/MS spectrum of linearmycin B. (F) Annotation legend for MS features in the linearmycin compound cluster with analogs A and B indicated. Many other featured nodes here, primarily belong to in-source fragments of linearmycin analogs which are also fragmented by MS/MS.

GNPS Molecular Networking of metabolites

The large linear polyenes linearmycins A and B were identified from NPAtlas only and can explain the antifungal activity found in sample 13104 (*Streptomyces* sp. 13104). Results of the MS and UV data compared with prior studies, confirmed the presence of these large and intriguing polyene compounds. (**Figure 4**). Several members of the antimycin and kitamycin family of compounds were annotated both by GNPS library comparison and NPAtlas de-replication (**Figure 5**) [38].

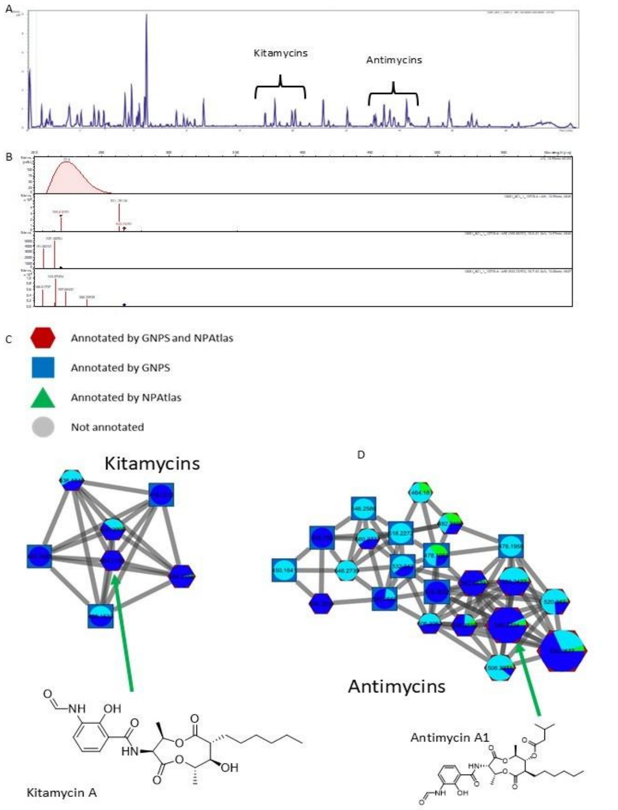

Figure 5. Antimycin and kitamycin identification by LC-MS/MS and GNPS. (A) LC-MS/MS BPC of 13081 (*Streptomyces* sp. 13081) indicating the kitamycins and antimycins identified by GNPS and NPAtlas de-replication. (B) MS/MS spectrum of antimycin A1. (C) Identification legend and compound clusters of kitamycin analogs present. (D) Compound clusters of antimycin analogs are present. The colors indicate the different extracts in which the compounds are found. Diagnostic analogs are indicated by green arrows.

These compounds can explain the potent antifungal activity observed from 13081 (*Streptomyces* sp. 13081). Amphotericin B was exclusively annotated by GNPS spectral library matching (**Figure 6**). This can account for the antifungal activity found in sample 12933 (*Streptomyces* sp. 12933). The other three nodes in this cluster showed the clear connections to the known Amphotericin and predicted to be its analogs. Other nodes in a different cluster had analogs that were not identified among the previously known compounds using the dereplication tool at GNPS and the NPAtlas platform, indicating that these may be potential new bioactive compounds. All the annotated compounds have at least one reference that can highlight their activities.

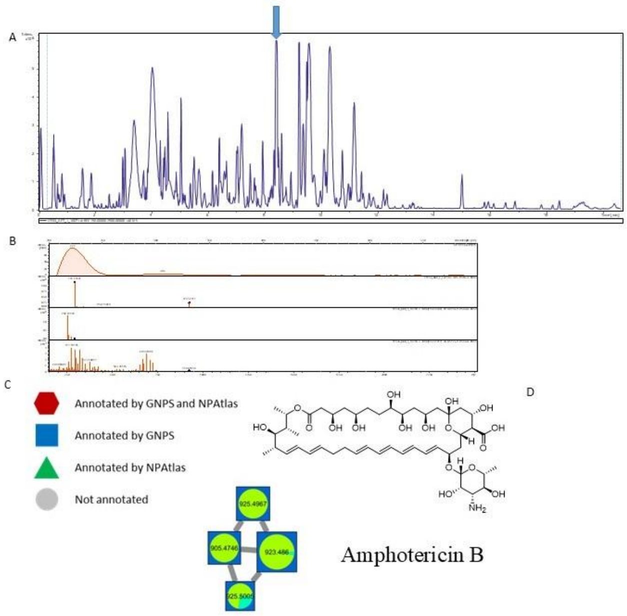

Figure 6. LC-MS/MS identification of amphotericin B via GNPS. (A) LC-MS/MS BPC of 12933 (*Streptomyces* sp. 12933) indicating amphotericin B. (B) MS/MS spectrum of amphotericin B. (C) annotation legend, cluster, and structure of amphotericin B.

Discussion

We isolated actinomycetes from high-altitude soil in Nepal, discovering strains that demonstrate antifungal properties against *Wickerhamomyces anomalus* DSM 6766^{T} *and Mucor hiemalis* DSM 2656^{T}. The strains identified as *Streptomyces* sp. 13104, *Streptomyces* sp. 13081, and *Streptomyces* sp. 12933 also showed inhibition of the test organisms even at lower or diluted concentrations, suggesting that our crude extracts contain promising antifungal compounds.

The isolates from our study exhibit antimicrobial activities, some of which are explained by known compounds from actinomycetes. The analysis of LC-MS data on our study for various active extracts indicated the presence of multiple known categories of compounds. NPAtlas identified the presence of the large linear polyenes linearmycins A and B in strain 13104 (*Streptomyces* sp. 13104) extract that explains the antifungal activity. The results from the antimicrobial activity of sample 13104 (*Streptomyces* sp. 13104) also showed antifungal activity against *Wickerhamomyces anomalus* DSM 6766^{T} and *Mucor hiemalis* DSM 2656^{T} which supports the above data from NPAtlas for the presence of linearmycins A and B. In the study done by Sakuda et al.[39], the two novel linear polyene antibiotics, named as linearmycin A1 and B2, which were isolated from *Streptomyces* sp. demonstrated antifungal activity. But a study from Hoefler et al. [40] linearmycins, produced by *Streptomyces* sp. also showed antibacterial activity against Gram-positive bacteria *Bacillus subtilis* where *Streptomyces* sp. incorporates linearmycins into the extracellular vesicles, which are capable of lysing *Bacillus subtilis* [41].

Extracts from strain 13081 showed high antifungal activity against *Wickerhamomyces anomalus* DSM 6766^T and *Mucor hiemalis* DSM 2656^T. LC-MS/MS Baseline peak chromatograms (BPC) of sample 13081 indicate kitamycins and antimycins identified by GNPS and NPAtlas de-replication. The compound kitamycin C isolated from *Streptomyces antibioticus* by Wang et al. [42] showed antifungal activities against *Candida albicans*. Similarly, in the study done by Hosotani et al. [43], several new antimycin compounds isolated from *Streptomyces* spp. showed inhibitory action against *Candida utilis.* Analogues of antimycin trigger apoptosis in cervical cancer cells which could be promising candidates for the development of HPV-related cervical cancer treatments [44].

Through GNPS spectral library matching, amphotericin B was exclusively annotated which supports the antifungal property of sample 12933. Amphotericin B is a macrolide polyene antifungal compound isolated from soil actinomycetes *Streptomyces nodosus* [45] and used for the treatment of respiratory fungal infections in humans [46] showing good therapeutic efficacy and patient tolerance. Amphotericin-related compounds were detected by analyzing LC-MS/MS data using molecular networking from *Streptomyces* sp. extracts that showed antifungal activity against *Coniochaeta* sp. [47,48]. Similarly, a macrolide amphotericin B isolated from *Streptomyces albospinus* was spotted by the high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) that showed antifungal activity against the ascomycete fungus *Phomopsis* sp. [49].

The isolation of antifungal compounds like Kitamycin, Antimycin, Linearmycins, and Amphotericin B from high-altitude actinomycetes from Nepal plays a significant role in the natural products area to combat fungal infections which also pose an increasingly serious threat to the world. Kitamycin C, along with kitamycin A and kitamycin B are used to treat the infections caused by *Candida albicans* [50]. Antimycin A, an antifungal compound, also has piscicide properties for the management of fishes in ponds, lakes, and rivers [51] and showed higher antiproliferation to two oral cancer cell lines CAL 27 and Ca9-22 with the production of higher reactive oxygen species (ROS), mitochondrial superoxide (MitoSOX) generation, and depletes mitochondrial membrane potential (MitoMP) [52]. Linearmycins have the properties of inhibiting both fungi and bacteria with the capacity to lysis cells and disrupt lipid bilayers [53]. Amphotericin B, a polyene macrolide, is one the well-studied antifungals used to treat serious fungal infections like aspergillosis, candidiasis, mucormycosis, cryptococcosis, coccidioidomycosis, and blastomycosis, due to its broad-spectrum antifungal activity [54]. This is one of the important antifungal drugs due to its low incidence of antifungal resistance. Amphotericin B is used for the treatment of primary amoebic meningoencephalitis (PAM) that is caused by an amoeba *Naegleria fowleri* [55]. Amphotericin B is also effective to treat visceral leishmaniasis which is caused by a protozoan parasite *Leishmania* [56].

Apart from antibiotic resistance, there is a substantial challenge to public health due to the increase in resistant fungal strains, which urges the scientific community to discover novel antifungal metabolites and compounds. Our identification of some unknown compounds in this study might be an opportunity for the development of new antifungal metabolites that are potentially active against resistant pathogens and contribute to the management of fungal infections [57]. During the LC-MS/MS analysis, we observed distinct molecular features of the compounds that do not correspond to the known metabolites in existing databases. The molecular networking analysis revealed nodes representing compounds with no matches in the Global Natural Products Social Molecular Networking (GNPS) database, indicating the novelty of the compounds isolated in this study [58]. Therefore, finding new antifungal compounds from actinomycetes sourced from Nepal's high-altitude soils presents great promise. Due to the emergence of multidrug-resistant fungal pathogens, developing new antifungal agents has become essential. These novel compounds can offer alternative mechanisms of action, decreasing dependence on current drugs and lessening the likelihood of resistance development [59]. These compounds can act as biocontrol agents for phytopathogenic fungi, minimizing the need for synthetic fungicides [22].

While our findings are promising, further research is necessary for the structure elucidation of the isolated compounds using NMR. Understanding the mechanism of action of these antifungal compounds helps to assess their therapeutic potential and safety. Additionally, assessing the effectiveness and safety of these compounds in animal models is crucial for advancing potential clinical applications and drug discovery.

Conclusions

Our findings are significant in understanding the compounds responsible for showing antifungal activities. These various compounds identified in our study underscore the value of exploring actinomycetes from unique and extreme environments which might lead to the discovery of novel bioactive secondary metabolites. Analytical and GNPS analyses identified some known antifungal compounds produced by the actinomycetes isolates. Having more research in natural products and modern technologies like genome mining tools, machine learning approaches, and CRISPR/Cas-based technology metabolomics are promising steps toward identifying and discovering new antimicrobial compounds.

Funding

The Ph.D. research work of Sagar Aryal was partially funded by the University Grants Commission Nepal (Award no.: PhD/74_75/S&T-12) and by the German Academic Exchange Service (Bi-nationally Supervised Doctoral Degrees, 2019/20).

Authorship contribution statement

Sagar Aryal: Conceptualization, Methodology, Investigation, Data Curation, Writing - Original Draft. **F.P. Jake Haeckl:** Writing - Review & Editing, Visualization. **Rameshwar Adhikari:** Writing - Review & Editing, Supervision. **Balmukunda Regmi:** Writing - Review & Editing, Supervision. **Dev Raj Joshi:** Conceptualization, Writing - Review & Editing, Supervision.

Competing interests

The authors declare that there is no conflict of interest.

Acknowledgments

Sagar Aryal would like to express his gratitude to the Department of Microbial Natural Products (MINS), Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) for providing the laboratory facilities and partial scholarship. We thank Prof. Dr. Rolf Müller and Dr. Ronald Garcia from HIPS for the helpful advice and guidance during this research as well as for providing the test strains.

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