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RESEARCH ARTICLE

Identification of Secondary Metabolites from Actinomycetes Isolated from the Hilly Region of Nepal

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ABSTRACT

Actinomycetes are a unique class of Gram-positive bacteria with a fungus-like filamentous mycelium. They are often called 'antibiotics factories' because they can produce many potent secondary metabolites. At present, multidrug-resistant (MDR) pathogens are the main problem for humans and animals and to overcome this problem, more potent antibiotics are urgently required. Fortunately, Nepal has great geographical and climatic diversity serving as a home for various actinomycetes. This study identified several actinomycetes using morphological and biochemical assays from seven soil samples collected from different elevations in Nepal. The primary objective of this study was to screen the potent metabolites-producing actinomycetes. For this purpose, antimicrobial assays of seven isolates were carried out against *Escherichia coli* ATCC 25922, *Salmonella typhi* ATCC 14028, *Shigella sonnei* ATCC 25931, and *Staphylococcus aureus* ATCC 43300. The sample BT36 demonstrated the highest zone of inhibition against the tested microorganisms among others. Similarly, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of all isolates were carried out. Among them, the MIC of samples BT36, PT7, and AB1 were found to be 1.952 mg/mL, 0.012 mg/mL, and 0.029 mg/mL, respectively. Ethyl acetate extracts of resulting potent isolates PT7 and AB1 were subjected to mass spectrometric analysis. A total of 22 bioactive compounds were annotated from liquid chromatography-high resolution mass spectroscopy (LC-HRMS), including epopromycin A, myxopyronin B, gilvocarcin HE, and okilactomycin A. It is concluded that this study will be useful in setting up a strategy to isolate useful secondary metabolites from soil microbes from the hilly region of Nepal.

KEYWORDS: Soil microbes, secondary metabolites, antimicrobial assays, mass spectrometry

INTRODUCTION

Soil microbes are an excellent source of therapeutically relevant compounds for isolation and screening. Various natural products have been important sources for creating and discovering new drugs from the early days (Newman & Cragg, 2020). Actinomycetales are a significant category of soil microbes. One of the biggest taxonomic groups in the bacterium domain is the phylum Actinobacteria, which includes the order Actinomycetales, containing the actinomycetes. Gram-positive bacteria in this phylum have high DNA Guanine-Cytosine (G-C) contents; these contents range from 51% in some *Corynebacterium* to over 70% in *Streptomyces* and *Frankia* (De Simeis & Serra, 2021). Even though actinomycetes are widely distributed, very few of them have been investigated and tested in the past.

A study recently carried out has demonstrated that interactions between different species of bacteria, facilitated by small-molecule natural products, produce a surprising range of makeup in bacteria that live in soil, especially in species of *Streptomyces* and *Bacillus* (Traxler & Kolter, 2015). Actinomycetes can reproduce binary fission and generate vegetative or aerial mycelia. Due to the formation of the volatile organic chemicals geosmin and 2-methylisoborneol, both *in vitro*, in cultivation, and natural environment, they have the familiar scent of moist soil (Cheng et al., 2020). *Streptomyces* species synthesize a large variety of naturally occurring chemicals with a broad range of structural variants, including glycopeptides, aminoglycosides, terpenes, tetracyclines, and macrolides. For example, *Streptomyces hygroscopicus* produces around 180 metabolites with different bioactivities (Salwan & Sharma, 2020). The *Streptomyces* genome has more than twenty gene clusters that code for specific secondary metabolites of more clinical significance, such as drugs that might slow the spread of antimicrobial resistance (Alam et al., 2022). *Streptomyces* is a well-known prolific generator of important bioactive compounds and typically makes up a significant fraction of the overall actinomycetes population in natural soil habitats. One of the major risks to modern development, food security, and global health is antibiotic-resistant bacteria. A global report on the surveillance of antimicrobial resistance of the World Health Organization (WHO) states that bacterial resistance to commonly prescribed drugs in the treatment of diseases has drastically developed in many regions of the world. Similarly, many studies have showed that actinomycetes provide 80% of the antibiotics used in medicine globally (Elbendary et al., 2018).

Most of the antimicrobials are found in actinomycetes, specifically in the *Streptomyces* genus. The produced chemicals encompass all major drug classes that are now used in clinics, such as glycopeptides, tetracyclines, macrolides, aminoglycosides, and beta-lactams (Mast & Stegmann, 2019). Only about 100 of over 5000 antibiotics that have been found in filamentous fungi, Gram-positive bacteria, and Gram-negative bacteria have been utilized in commercial medicine to treat illnesses in people, animals, and plants. However, because of their importance, natural compounds with intriguing bioactivities have recently piqued the curiosity of scientists (Aksenov et al., 2021). More than 95% of all actinomycetes found in soil are *Streptomyces*, which are often present at densities of 10^6 to 10^9 cells per gram of soil. While certain species pose serious health risks to higher plants and animals, many others do not (Ait Barka et al., 2015).

A major worldwide health problem is microorganisms that are resistant to antibiotics, known as anti-microbial resistance (AMR). Clinically relevant microorganisms with MDR strains and an alarmingly rapid pace of worldwide spread have been described in recent years (Andersson et al., 2014). The fast introduction of novel diseases and the development of MDR have increased the demand for new antimicrobial medicines more than ever (Clardy & Walsh, 2004). Actinomycetes are one

of the most significant bacterial groups due to their capacity to generate a large number of physiologically active secondary metabolites that are quite successful in preventing microbial infections (Ganesan et al., 2017).

In Nepal, very little research has been done on soil actinomycetes, and no new compounds have been isolated from soil actinomycetes. Nepal is rich in biological diversity; therefore, exploring those exploited habitats and unexplored soil can be an excellent source to produce antibiotics that can combat MDR. The study of the microbial class known as endophytic actinobacteria is still in its infancy. Endophytic actinobacteria are special in that their relationship with plants confers certain biological and chemical characteristics (Bernardi et al., 2019). Nepal is a diversely physically split nation with three distinct regions: the terai, the hills, and the mountains. Similar microflora may be observed in those soil types and altitudes, which raises the prospect of varying the distribution of actinomycetes that produce antimicrobials (Gurung et al., 2009).

Overall, to address the issues raised above, the primary objective of this study was to use LC-HRMS techniques to profile secondary metabolites obtained from actinomycetes isolated from the soil of the hilly region of Nepal, along with their antimicrobial activities. In this study, various secondary metabolites were listed, which can be further analysed and extracted for further study.

MATERIALS AND METHODS

Collection of Soil Samples

The soil samples were collected from various regions of Nepal, including drainage areas, riverbanks, woodlands, compost, and rhizospheres' soils. Different ecological places were selected for the isolation of potential actinomycetes. To isolate actinomycetes, the soil samples were taken at a depth of 10 to 15 cm where the microbial population is high. They were kept in a clear sterile zip bag and brought to the Central Department of Chemistry, Tribhuvan University, and were stored at 4°C till further use (Saadoun et al., 1999).

Isolation of Soil Actinomycetes

One gram of each soil sample was taken and serially diluted with 10 mL of sterile water. This activity was followed by a homogenous mixture, which was prepared by vortexing for 1-2 minutes. As the homogenous mixture consists of many bacteria, it must be diluted. To lower the bacterial concentration, three-fold serial dilutions were carried out. Afterward, a volume of 100 µL of the bacterial suspension was evenly spread on ISP4 (International Streptomyces Project medium 4) plates (ISP4: 10 g starch, 1 g calcium carbonate, 1 g dipotassium phosphate (K_2HPO_4), 1 g magnesium sulfate, 1 mg of ferrous sulfate, 1 mg zinc sulfate, 1 mg manganese chloride, 18 g Bacto agar, 2 g ammonium sulfate, 1 g sodium chloride, and 1000 mL autoclaved distilled water at pH 7.0 ± 0.1). Likewise, nalidixic acid (20 mg/L) and cycloheximide (50 mg/L) were added to the ISP4 medium to prevent the development of gram-negative bacteria and fungi, respectively. These plates were incubated at 28 °C for seven days (Kharel et al., 2010). Finally, after the growth of actinomycetes, they were preserved at 4°C till further use.

Biochemical Analysis of Isolates

The isolated actinomycetes colonies were examined under a microscope for pigmentation, color, substrate, and aerial mycelium. Similarly, the microscopic identification was carried out by the Gram-staining method as suggested by Bergey's Manual of Systematic Bacteriology, Second Edition, Vol, 5, The Actinobacteria, Part A. In addition, the isolated bacteria were identified using biochemical assays. A standard

protocol for biochemical characterization was followed when performing a variety of biochemical experiments (Singh et al., 2013). The oxidation of tetramethyl phenylenediamine (TMPD), catalase, and oxidase activities were examined, along with the generation of oxygen bubbles in a 3% hydrogen peroxide. On a plate with isolates that had been cultured for 6-7 days at 28 °C, Gram's iodine was flooded to check for starch hydrolysis (Bérdy, 2005). This activity was followed by the use of 1 % peptone water and phenol red, which was an indication for a test on the fermentation of carbohydrates. The physiological attribute was assessed thereafter.

Shake Flask Fermentation and Extraction of Secondary Metabolites

The dry mass method was used to analyze the bacterial growth curve. This gives information regarding the best time for harvesting secondary metabolites after fermentation. Using this technique, 1 mL of bacterial culture was taken in a 2 mL Eppendorf tube and centrifuged for 10 minutes at 4000 rotations per minute (rpm). The residue was dried and the supernatant was discarded. After it was completely dried, the dry mass was measured and noted. The identical process was carried out repeatedly over several days, with measurements made precisely every 24 hours until a steady mass was noticed (Li & Mira de Orduña, 2010). Before fermentation, the seed culture of individual isolated colonies was carried out in a Tryptic Soy Broth (TSB) medium at 28 °C for 5-7 days at 180 rpm in a shaking incubator. When there was sufficient growth of the isolates, 1 mL of bacterial suspension was taken and put into 100 mL of TSB medium for fermentation. In a shaking incubator, the fermentation was conducted at 28 °C for 5-7 days at 180 rpm.

Using the ethyl acetate extraction procedure, crude extracts were obtained. This was accomplished by adding an equivalent volume (100 mL) of ethyl acetate to the bacterial suspension and thoroughly mixing it in the separating funnel. The crude bacterial extract was obtained by transferring the clear supernatant into a dry and clean beaker and allowing it to evaporate in a hot water bath at 37 °C for 2-3 days. To get the desired extract, it was dissolved in 2 mL of ethyl acetate, moved to an Eppendorf tube, and then evaporated for 24 hours at 37 °C in a water bath. In the final step of this process, the dried extract was kept at 4 ˚C until further use.

Antimicrobial Activity of Extracts

Primary Screening

The primary screening of the isolates was performed by using the perpendicular streaking method in the Mueller Hinton Agar (MHA). The isolated bacteria were cultured at 28˚C after being streaked linearly across the plate's breadth for 5-7 days. After the growth of the isolates, the pure suspension of tested pathogens, compared with 0.5 McFarland, were streaked perpendicularly to the isolates. Then, these plates were incubated at 37 ˚C for 24 hours, and the antimicrobial assays of the isolates were carried out against test organisms (Bizuye et al., 2013).

Secondary Screening

The agar-well diffusion method was utilized for secondary screening of crude extracts. In this method, the standard culture of test organisms was swabbed over the MHA medium with the help of sterile cotton buds. To perform this test, Gram-positive pathogen *Staphylococcus aureus* ATCC 43300, and various Gram-negative pathogens, *Shigella sonnei* ATCC 25931, *Salmonella typhi* ATCC 14028, and *Escherichia coli* ATCC 25922 were tested. These tested pathogens were incubated in Mueller Hinton Broth (MHB) medium at 37 °C for 24 hours. Then, their turbidity was adjusted to that of

standard 0.5 McFarland ($1.5x10^8$ CFU/mL) for further use. After that, a sterilized cork borer was used to make the wells. Finally, the wells were filled with the positive control (1 mg/mL neomycin), negative control (50 % DMSO), and a working solution of extract dissolved in 50% DMSO. Finally, the distinct zone of inhibition was observed during the 24-hour incubation at 37 ℃ (Sapkota et al., 2020).

Determination of MIC and MBC

The MIC and MBC of the potent extract were ascertained by the Resazurin-based broth microdilution method according to the Clinical Laboratory Standard Institute (CLSI) (Andrews, 2001). To perform this activity, tested bacteria, both Gram-negative bacteria *(E. coli* ATCC 25922) and Gram-positive bacteria (*S. aureus* ATCC 43300) were grown in MHB media for 24 hours in an incubator at 37 ℃. After that, a series of 100 µL extract in duplicate (two times, the first and second wells of the first column contained the same extract) were loaded by 100 µL MHB solution in sterile 96-well microdilution plates and, then, 8-fold serial dilution was performed to get various concentrations. After matching the turbidity of MHB with 0.5 McFarland, the bacterial inoculum concentration was made of $1.5x10^8$ CFU/mL by diluting 1:100. Finally, all the wells were filled with 30 µL bacteria, except for the negative control. In this process, the common medication that was employed as a positive control was neomycin. After placing a sterile lid on the plate, it was incubated at 37 ℃ for 24 hours. Following a 24-hour incubation, 5 µL of resazurin (0.015%) was introduced into each well of the 96-well plate, and it was then incubated for 3–4 hours at 37 ℃. The wells that had bacterial growth were pink, while the wells that did not have bacterial growth were blue. MIC was found to be the one that prevents bacterial growth. The content of the wells was streaked onto nutrient agar (NA) plates, which were then incubated for more than 18 hours at 37 ºC, to measure the MBC, necessary to kill bacteria at a specified duration (Sambrook et al., 1989).

Analysis of Metabolites by LC-HRMS

The extracts PT7 and AB1 were subjected to LC–HRMS, which was performed at the CSIR-Central Drug Research Institute, Lucknow, India, at the Sophisticated Analytical Instrument Facility (SAIF). The samples were analyzed using the Agilent 6520 Accurate-Mass Q-TOF Mass Spectrometer, which was equipped with a G1311A quaternary pump, G1329A autosampler, and G1315D diode array detector (DAD). A 4 µL sample was employed for analysis. Acetonitrile (ACN), 5 mM acetate buffer, and water were added to a gradient system over 25 minutes at a flow rate of 0.5 mL/min in LC-MS separation. The starting condition was 5% ACN for 0.1 minutes, which proceeded by 30% ACN for 10 minutes, 80% ACN for 32 minutes, and then returning to the starting circumstances. The temperature of the column was maintained at 30 ℃ for the entire analysis. After passing through the DAD flow cell, the column elute was routed to the Q-TOF HRMS fitted through an electrospray interface (ESI). For the MS analysis, an ESI-positive ionization mode was employed, with mass ranges of 100–2000 Da.

RESULTS

Sample Collection and Isolation

A total of 7 soil samples were taken from 1900 m to 4919 m altitudes in various parts of Nepal. The list of soil samples and respective locations were presented in the supplementary data in Table S1. From soil samples taken from various locations, yet-to-be-explored, and unspoiled parts of Nepal, seven distinct actinobacteria were identified

after several rounds of routine screening among hundreds of bacterial colonies. The morphological traits resembled those of actinomycetes; they were rough, tough, and elevated; they were also slightly deep-grown. The bacterial isolates exhibited different colors: gray, and white, and are shown in Figure S1 (A and B). The presence of hair threads-like structures of mycelia observed in the Gram stain and similar colony characteristics to actinobacteria indicated that all strains were Gram-positive filamentous actinobacteria as shown in Figure S2. Based on the series of biochemical tests, morphological features, and macroscopic examination, the isolated colonies were found to be Gram-positive actinomycetes.

Biochemical Characterization

Several biochemical assays were performed to ascertain if the isolated bacteria were Gram-positive or Gram-negative. To identify Gram-positive bacteria, tests for oxidases, starch hydrolysis, nitrate reduction, mobility, and catalase were performed. Some of the representative colonies were selected for the tests, and the results are listed in Table 1 below.

Table 1

Showing Various Biochemical Tests for Selected Colonies (only representative results are shown)

Samples	Catalase	Oxidase	Starch hydrolysis	Nitrate reduction	Mobility
BT5	+	+	-	-	-
BT13	+	+	+	-	-
BT33	-	-	+	+	-
BT36	-	+	+	+	-
BT39	+	+	-	-	-
PT7	-	+	-	-	+
AB1	+	+	-	-	-

Note: Sign (+) for positive test results and sign (-) for negative test results

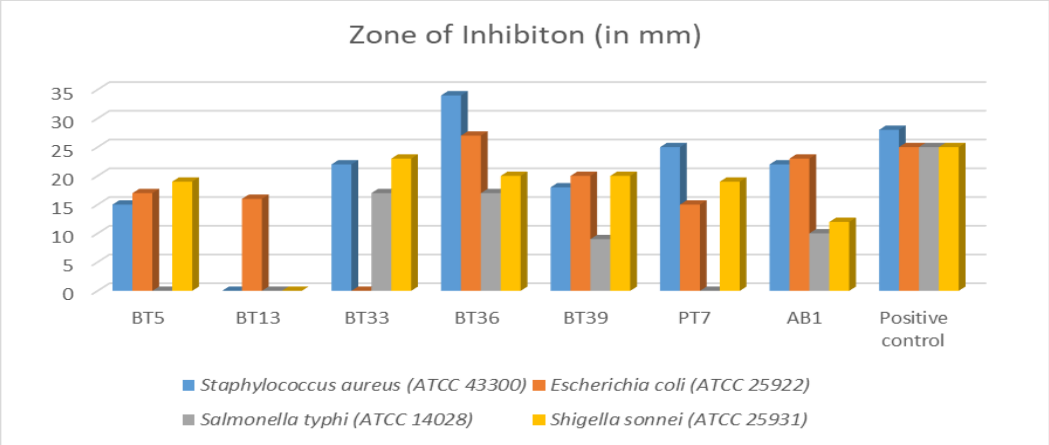
Antimicrobial Activity

All bacterial extracts showed an effective zone of inhibition against both Gram-negative and Gram-positive bacteria. The zones of inhibition of respective bacterial extracts against tested pathogenic bacteria are exhibited in Figure 1. For positive control, 1 mg/mL neomycin was used, and 50 % DMSO solution was used as a negative control. Similarly, a concentration of 20 mg/mL was used for our extracts. Supplementary data in Figure S3 demonstrated the antibacterial efficacy of potent extracts**.**

As shown in Figure 1 below, the bacterial extracts with the highest zone of inhibition against the ATCC strain of bacteria were BT36, PT7, AB1, and BT33. However, BT36 exhibited the maximum zone of inhibition against *S. aureus* among these extracts, while BT36, AB1, and BT39 showed the acceptable zone of inhibition against *S. typhi, E. coli*, and *S. sonnei*, respectively.

Figure 1

Zone of Inhibition of Bacterial Extracts against the Tested Strain Bacteria (only representative results are shown)



MIC and MBC

MIC and MBC of BT5, BT13, BT33, BT36, and BT39 were determined against the ATCC bacterial strain, Gram-positive bacteria (*S. aureus* ATCC 43300) and Gram-negative bacteria (*E. coli* ATCC 25922). MIC values of BT5, BT13, BT33, BT 36, BT39, PT7, and AB1 were exhibited 0.638, 2.472, 3.141, 1.952, 1.653, 0.012, and 0.029 mg/mL respectively and positive control neomycin showed MIC 0.625 µg/mL against *E. coli*. Similarly, the MIC values of BT5, BT13, BT33, BT36, BT39, PT7, and AB1 were found at 2.032, 1.573, 1.958, 1.202, 1.115, 1.017, and 1.213 mg/mL respectively and neomycin had MIC of 0.783 µg/mL against *S. aureus*. MBC of BT5, BT13, BT33, BT36, BT39, PT7, and AB1 was obtained 1.276, 4.945, 6.281, 3.905, 3.307, 1.213, and 1.312 mg/mL respectively and neomycin 1.253 µg/mL against *E. coli*. MBC of BT5, BT13, BT33, BT36, BT39, PT7, and AB1 were obtained at 4.064, 3.144, 3.917, 2.405, 2.231, 1.240, and 1.452 mg/mL respectively, and neomycin had MBC of 1.562 µg/mL against *S. aureus*. The results of MBC and MIC are shown in Table S4, Figure S4, and Figure S5.

LC-HRMS Annotation

The raw mass data were analyzed through MestreNova 12.0 software. Each peak was analyzed, detected, aligned, and annotated. Finally, the results were compared with literature and various online database libraries, such as PubMed, ChemSpider, Dictionary of Natural Products, etc. The secondary metabolites annotated through LC-HRMS are shown in Table 2 and Figure 2.

Table 2

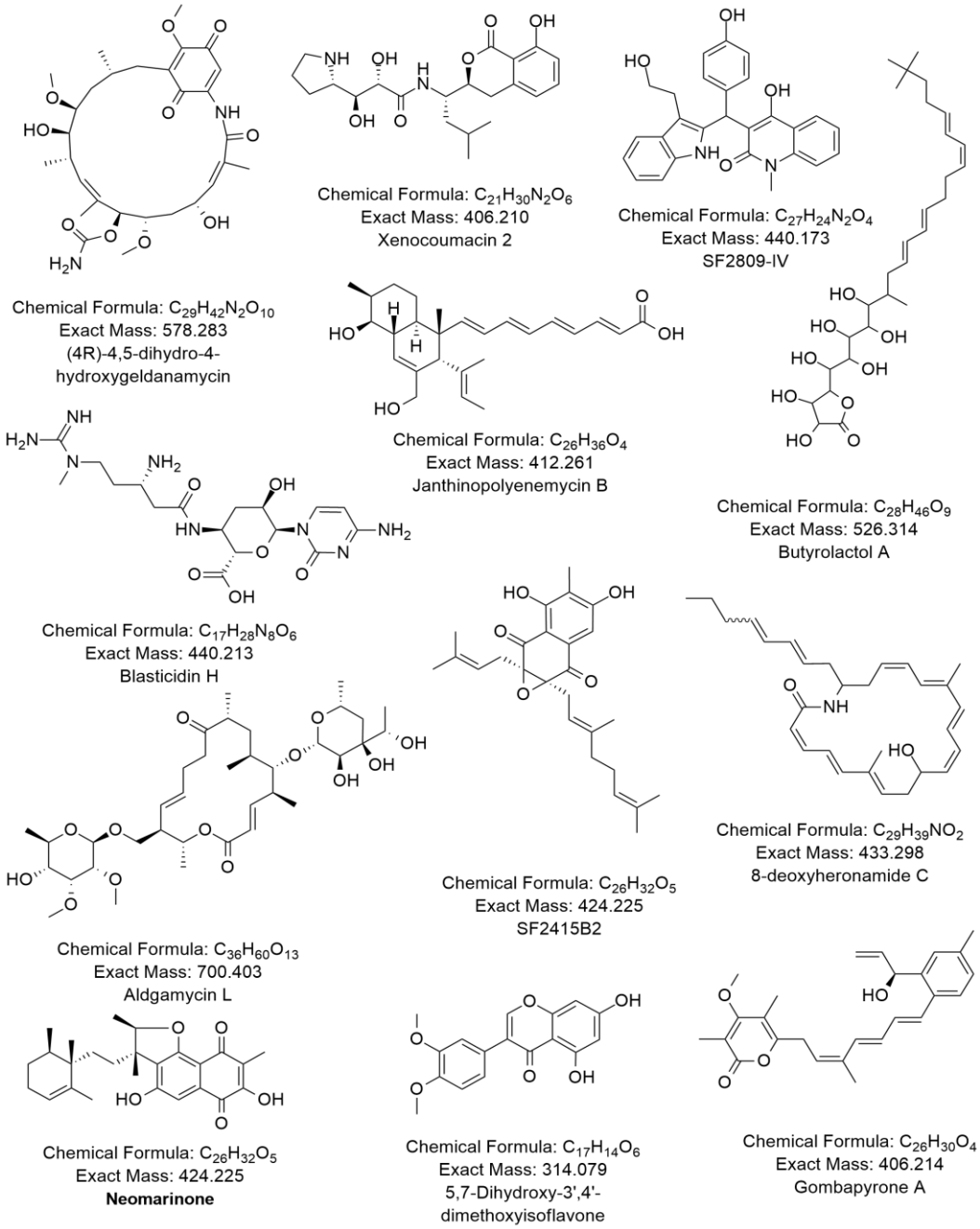
Showing the List of Annotated Compounds in PT7 and AB1 Extracts

SN	Annotated Compound	Exact Weight	Observed Mass	Adduct Type	Molecular Formula	DBE	Absolute Error	Sources	References
1	Epopromycin A	412.257	413.265	$[M+H]^+$	$C_{21}H_{36}N_2O_6$	5	4.29	PT7 and AB1	(Tsuchiya et al., 1997)
2	SF2415B2 antibiotic	424.225	425.233	$[M+H]^+$	$C_{26}H_{32}O_5$	11.0	4.70	PT7 and AB1	(Gomi et al., 1987)
3	Gilvocarcin	511.168	512.176	$[M+H]^+$	$C_{27}H_{28}$	14.0	3.72	PT7 and	(Hou et

	HE				O_{10}			AB1	al., 2012)
4	Butyrolactol A	526.314	527.324	$[M+H]^+$	$C_{28}H_{46}O_9$	6.0	4.89	PT7	(Harunari et al., 2017)
5	Epoxomicin	554.368	555.376	$[M+H]^+$	$C_{28}H_{50}N_4O_7$	7.0	2.96	PT7 and AB1	(Nihei et al., 1993)
6	Aldgamycin L	700.403	701.411	$[M+H]^+$	$C_{36}H_{60}O_{13}$	7.0	2.96	PT7 and AB1	(Wang et al., 2010)
7	Neomarinone	424.225	425.233	$[M+H]^+$	$C_{26}H_{32}O_5$	11.0	4.70	PT7 and AB1	(Dickschat et al., 2005)
8	Blasticidin H	440.213	441.221	$[M+H]^+$	$C_{17}H_{28}N_8O_6$	8.0	4.13	PT7 and AB1	(Svidritskiy et al., 2013)
9	Phoxalone	424.209	425.217	$[M+H]^+$	$C_{22}H_{32}O_8$	7	0.72	AB1 and PT7	(Guo & Tao, 2008)
10	Okilactomycin A	434.229	435.239	$[M+H]^+$	$C_{24}H_{34}O_7$	8	1.89	AB1	(Imai et al., 1987)
11	SF2809-IV antibiotic	440.173	441.179	$[M+H]^+$	$C_{27}H_{24}N_2O_4$	17	4.48	AB1 and PT7	(Tani et al., 2004)
12	(4R)-4,5-dihydro-4-hydroxy geldanamycin	578.283	579.293	$[M+H]^+$	$C_{29}H_{42}N_2O_{10}$	10	2.37	PT7 and AB1	(Li & Mira de Orduña, 2010)
13	Janthinopolyenemycin B	412.261	413.269	$[M+H]^+$	$C_{26}H_{36}O_4$	9	2.98	AB1 and PT7	(Rani et al., 2021)
14	8-deoxyheronamide C	433.298	434.306	$[M+H]^+$	$C_{29}H_{39}NO_2$	11	3.69	AB1 and PT7	(Sugiyama et al., 2014)
15	Actinoallolide C	546.356	547.364	$[M+H]^+$	$C_{32}H_{50}O_7$	12.5	1.33	AB1 and PT7	(Xu et al., 2022)
16	Glucopiericidin A	577.325	578.330	$[M+H]^+$	$C_{31}H_{47}NO_9$	9	3.80	AB1	(Shaaban et al., 2012)
17	Gombapyrone A	406.214	407.206	$[M+H]^+$	$C_{26}H_{30}O_4$	8	1.80	AB1 and PT7	(Helaly et al., 2009)
18	Xenocoumacin 2	406.210	407.218	$[M+H]^+$	$C_{21}H_{30}N_2O_6$	8	1.80	AB1	(Park et al., 2009)
19	7,3-di-(γ,γ-dimethylallyloxy)-5-hydroxy-4-methoxyflavone	436.188	437.199	$[M+H]^+$	$C_{26}H_{28}O_6$	13	2.96	AB1	(Ding et al., 2013)
20	Flavofungin	664.419	665.427	$[M+H]^+$	$C_{37}H_{60}O_{10}$	8	0.11	AB1	(URI & BEKESI, 1958)
21	Myxopyronin B	431.230	432.953	$[M+H]^+$	$C_{24}H_{33}NO_6$	11.5	3.47	AB1 and PT7	(Yakushiji et al., 2013)
22	5,7-Trihydroxy-3,4-dimethoxy isoflavone	314.079	353.042	$[M+H]^+$	$C_{17}H_{14}O_6$	5.0	2.3	AB1 and PT7	(Hosny & Rosazza, 1999)

Figure 2

Some Annotated Compounds in Extracts of Isolates PT7 and AB1 (actinomycetes)



DISCUSSION

Actinomycetes are popular for being the flexible makers of bioactive metabolites that are antibacterial, anticancer, antifungal, etc. The primary technique for isolating novel antibacterials is traditional (el Karkouri et al., 2019). Soils are most prevalent for the production of bioactive bacteria (Demain & Fang, 2000). For the current investigation, actinomycetes were isolated using seven different soil samples that were taken at different altitudes in Nepal. The isolated soil microbes were identified based on

microscopic examination, biochemical analysis, both primary as well as secondary screening, and the analytical technique LC-HRMS. All the isolates exhibited varied morphological features and under microscopic observation by Gram staining; all the isolates were found to be Gram-positive. Based on microscopic observation, the isolates were further processed for antimicrobial screening, and only a few were found to be good inhibitors of the tested bacterial strains. BT5, BT13, BT33, BT36, BT39, PT7, and AB1, showed a good zone of inhibition for Gram-positive bacteria, and isolates PT7 and AB1 were further subjected to LC-HRMS for the annotation of the possible bioactive metabolites or antibiotics.

Furthermore, these seven samples showed good antimicrobial activity against *E. coli* ATCC 25922, *S. sonnei* ATCC 25931, *S. aureus* ATCC 43300, and *S. typhi* ATCC 14028. Most of the extracts showed an average zone of inhibition against the tested Gram-positive bacteria *S. aureus* ATCC 43300, whereas these extracts seemed less effective against *E. coli* ATCC 25922. Among them, BT36 showed the most effective against *E. coli* ATCC 25922, and *S. aureus* ATCC 43300. The MIC and MBC of all extracts were tested against *S. aureus* and *E. coli* and were shown to be more effective. Because of ongoing contamination and reviving conditions, it can be challenging to isolate actinomycetes from the soil. Thus, a few were carried out for antimicrobial activity and some for different other screening processes. Some of them did not show any growth during the subculture and were discarded. Gram-positive *S. aureus* is responsible for a wide range of clinical diseases. It is still challenging to treat the illness because of the increase in MDR strains like Methicillin-resistant *S. aureus* (MRSA) (Boucher & Corey, 2008). It may be a good idea to continue working with these isolates as the current investigation has shown a notable zone of inhibition against *S. aureus* and *E. coli*, which is further confirmed by the earlier study (Shrestha et al., 2019).

Various biochemical assays were conducted to characterize the biochemical state of Gram-positive bacteria. In addition to contributing to the identification of the microbes for phenotypic characterization, physiological and biochemical parameters also point to significant traits for biotechnological applications (Chater et al., 2010). All these bacterial extracts were negative for the indole test, and positive for the catalase test. A study by Sadiqi et al. (2022) reported the similar results of biochemical analysis. Additionally, LC-HRMS was used to analyze secondary metabolites and perform molecular annotation. This revealed the existence of a variety of bioactive chemicals, including immune suppressant, antibiotic, antifungal, anticancer, and antihelminthic substances. The crude extracts of PT7 and AB1 were found to consist of various types of antibiotics, okilactomycin A, epopromycin A, and aldgamycin L, along with other important bioactive compounds. Erythromycin A is an essential antibiotic that is commonly used in clinical medicine to treat infections brought on by Gram-positive bacteria, according to a variety of literature sources. It is also the primary treatment for many lung infections, including Legionnaire's disease (Kingston, 2000). The soil samples which are from higher altitudes belong to BT36 and BT13 and those of lower altitudes are PT7 and AB1. Furthermore, the antimicrobial efficacy of molecules isolated from BT36 is higher than PT7 and AB1. Thus, this study also suggested that the isolates obtained from higher altitude regions could be a potential source of potent molecules due to the ability acquired by the soil bacteria found in such higher places to deal with harsh conditions.

Finally, this investigation has suggested to isolate actinomycetes that can produce secondary metabolites from a variety of soil samples as they were taken from Nepal's hilly regions.

CONCLUSION

Many bioactive secondary metabolites with good activity against multiple pathogens have been demonstrated to be produced by actinomycetes. In this work, seven soil samples taken from Nepal's hilly regions yielded a considerable number of actinomycetes that were isolated. The potent extracts BT5, BT13, BT33, BT36, BT39, AB1, and PT7 exhibited a significant zone of inhibition against tested pathogenic bacteria even though some of them were multi-drug resistant bacteria and were subjected to LC-HRMS analysis. From LC-HRMS analysis, 22 different secondary metabolites were annotated from PT7, and AB1 extracts, which contain various bioactive metabolites. Here, antimicrobial activities were isolated, screened, and analyzed along with bioactive compounds (secondary metabolites). To sum up, this study found that the diverse altitudes and environmental conditions in Nepal provide favorable conditions for various actinomycetes. Therefore, soils from the hilly region of Nepal have significant potential for discovering novel actinomycetes that can produce potent secondary metabolites (antibiotics).

AUTHOR CONTRIBUTIONS

Conceptualization, N.P., and B.B.T.; methodology, B.B.T., S.S., N.S. and R.P.Y.; software, B.B.T., and S.S.; validation, N.P., K.R.S., and A.D.M.; formal analysis, S.S.; writing—original draft preparation, B.B.T., S.S., and N.S.; writing—review and editing, N.P., R.C.B. and K.R.S.; supervision, N.P.; project administration, N.P.; funding acquisition, N.P. All authors have read and agreed to the published version of the manuscript.

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APPENDIX

Table S1

List of Soil Samples Collected from Different Regions of Nepal

Samples	Places	Altitude	Coordinates	Source of Soils
BT5	Kaski (Pritam Deurali)	2370 m	28.2622° N, 84.0167° E	Forest
BT13	Kaski (Badal Dada)	3420 m	28.2096° N, 83.9856° E	Forest
BT33	Ramechhap (Tame)	2173 m	28.1901° N, 83.9592° E	Near waterfall
BT36	Tilicho	4919 m	28.6833° N, 83.8567° E	Near Lake
BT39	Rolpa	3234 m	28.3816° N, 82.6483° E	Barren land
PT7	Gulmi	2000 m	28.0889° N, 83.2934° E	Agricultural Land
AB1	Gorkha	1900 m	28.2964° N, 84.8568° E	Near Waterfall

Table S2

List of Chemicals Used in This Study

S.N.	Chemicals	Company/Brands
1	Mueller Hinton Agar (MHA)	HiMedia
2	Mueller Hinton Broth (MHB)	HiMedia
3	Tryptic Soya Broth (TSB)	HiMedia
4	Nutrient Agar (NA)	HiMedia
5	Agar-Agar	HiMedia
6	Bacto Agar	HiMedia
7	Ammonium Sulphate	Qualigens Fine Chemicals
8	Zinc Sulphate	Fischer Scientific
9	Ferrous Sulphate	Fischer Scientific
10	Sodium Chloride	Marck Life Science
11	Manganese Chloride	Fischer Scientific
12	Magnesium Sulphate	Marck Life Science
13	Dipotassium Phosphate	Fischer Scientific
14	Calcium Carbonate	Fischer Scientific
15	Soluble Starch	Marck Life Science
16	Neomycin	HiMedia
17	Resazurin	Loba Chemie
18	Dimethyl sulfoxide (DMSO)	Loba Chemie
19	Ethyl acetate	Marck Life Science
20	Cycloheximide	Loba Chemie
21	Nalidixic acid	Sigma-Aldrich
22	Gram's Iodine	Thermo Fisher Scientific

Table S3

List of Instruments Used in This Study

S.N.	Instruments	Chemicals/Brands
1	Biosafety Cabinet	Sanjeev Scientific Udyog
2	Incubator	BEING Scientific
3	Refrigerator	Whirlpool and Samsung
4	Shaking Incubator	Shivaki
5	Vortex	Swirlex
6	Centrifuging Machine	Thermo Scientific
7	Water Bath	Medico
8	Autoclave	Thermo Scientific

Table S4

MIC and MBC of Soil Microbes against E. coli and S. aureus

Extracts	*E. coli*		*S. aureus*	
	MIC Value	MBC Value	MIC Value	MBC Value
BT5	0.638 mg/mL	1.276 mg/mL	2.032mg/mL	4.064 mg/mL
BT13	2.472 mg/mL	4.945 mg/mL	1.573 mg/mL	3.144 mg/mL
BT33	3.141 mg/mL	6.281 mg/mL	1.958 mg/mL	3.917 mg/mL
BT36	1.952 mg/mL	3.905 mg/mL	1.202 mg/mL	2.405 mg/mL
BT39	1.653 mg/mL	3.307 mg/mL	1.115 mg/mL	2.231 mg/mL
PT7	0.012 mg/mL	1.213 mg/mL	1.017 mg/mL	1.240 mg/mL
AB1	0.029 mg/mL	1.312 mg/mL	1.213 mg/mL	1.452 mg/mL
Neomycin	0.625 µg/mL	1.253 µg/mL	0.783 µg/mL	1.562 µg/mL

Figure S1 (A)
Isolation Plates of Actinomycetes

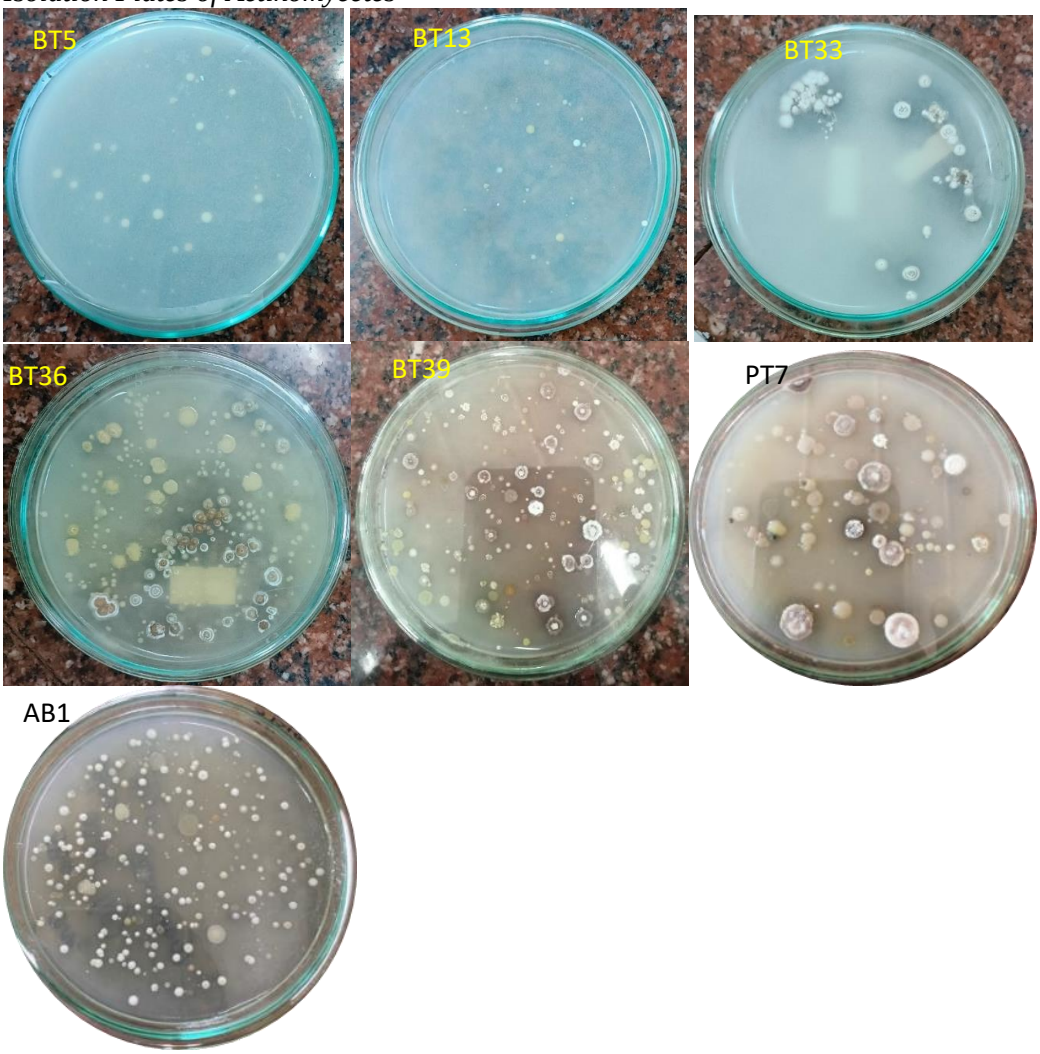


Figure S1 (B)
Substrate Mycelium of Isolated Species

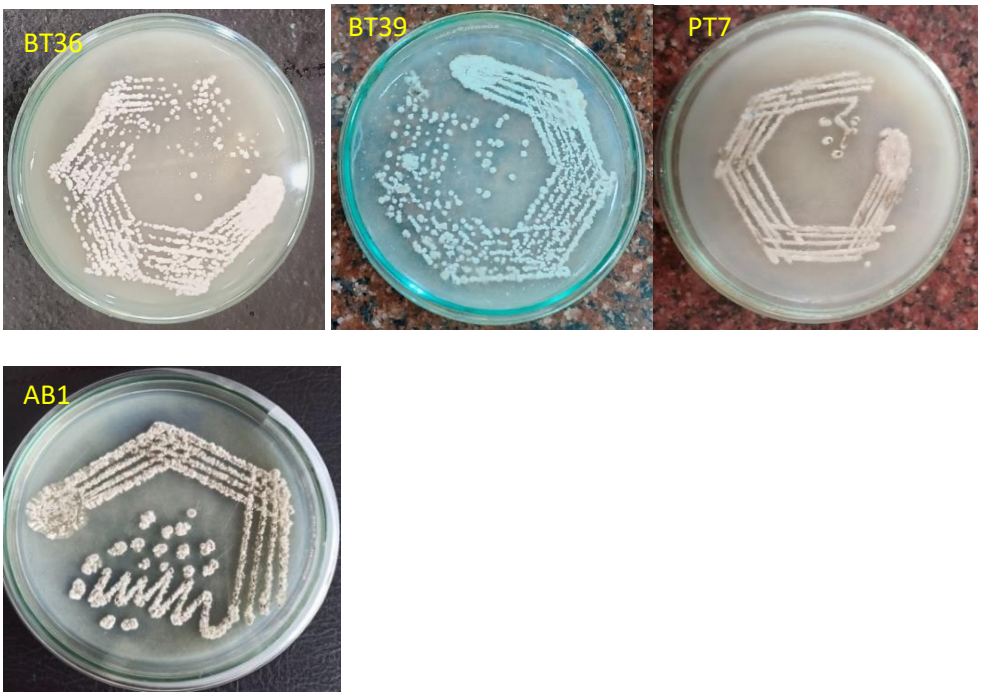


Figure S2

Filamentous Mycelia of Isolated Actinomycetes

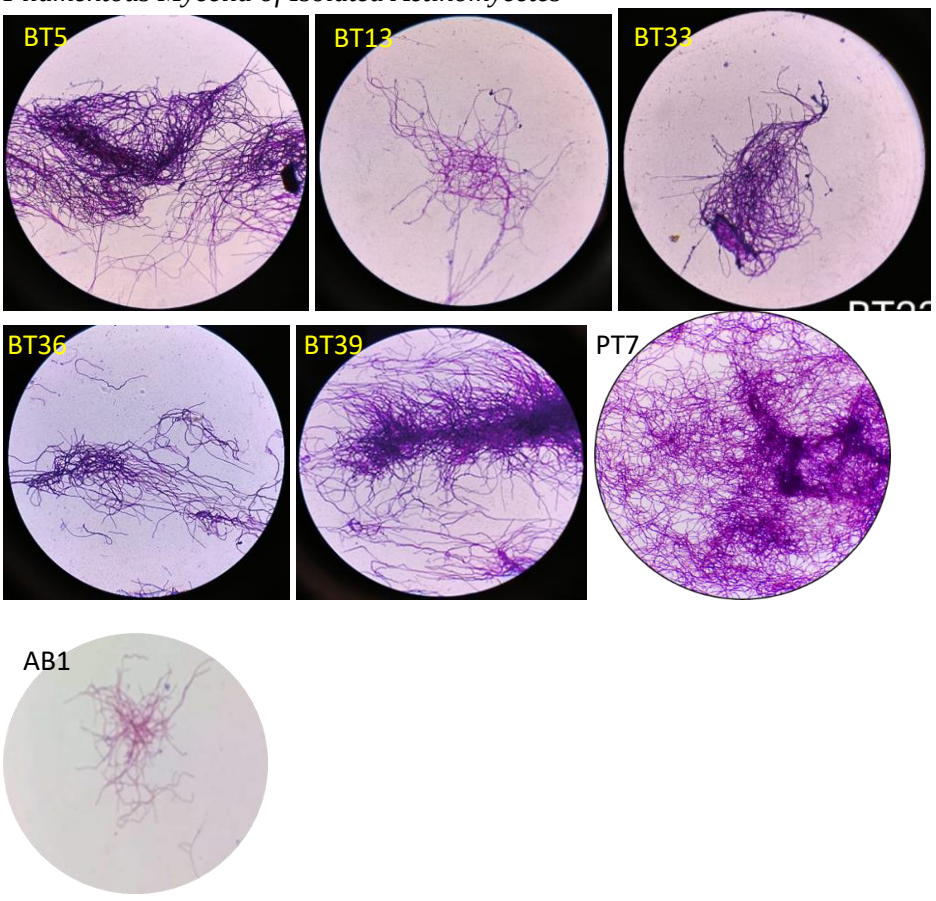
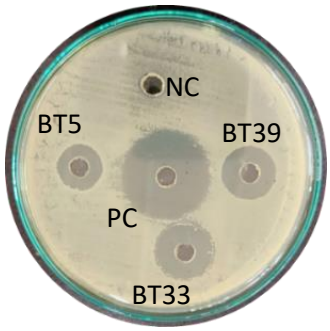
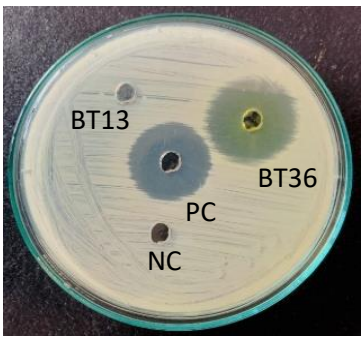


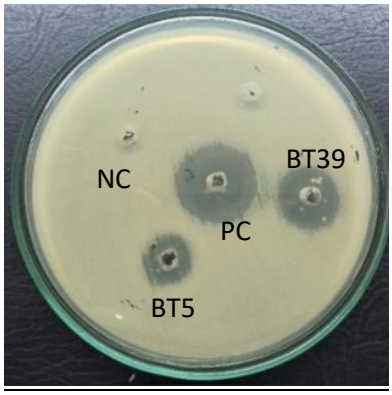
Figure S3
Zone of Inhibition Shown by Various Extracts

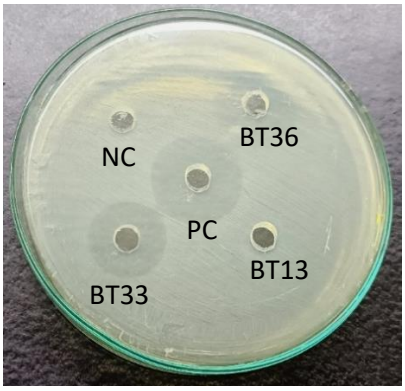
Staphylococcus aureus ATCC 43300



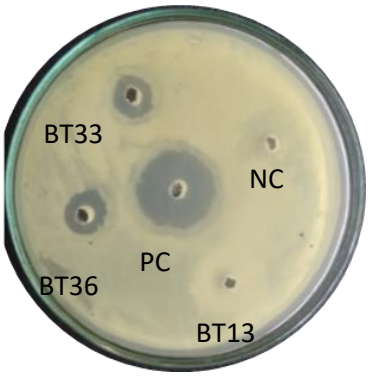


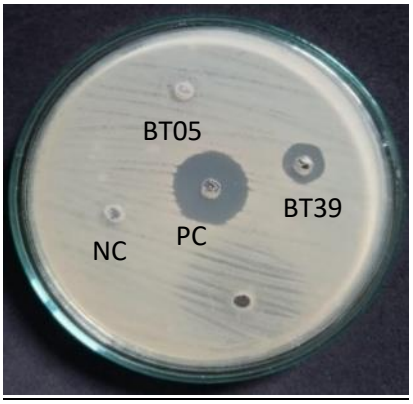
Shigella sonnei ATCC 25931



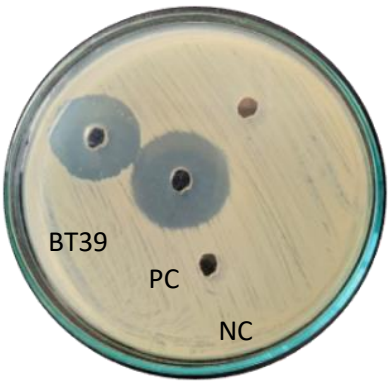


Salmonella typhi ATCC 14028





Escherichia coli ATCC 25922



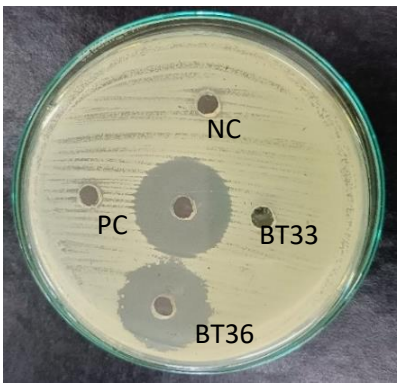
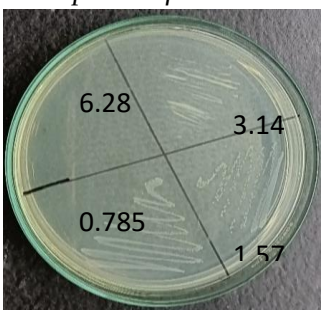
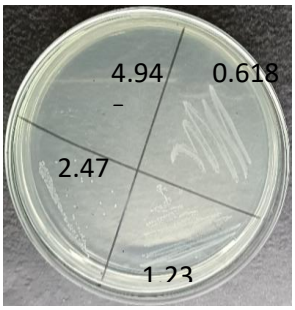


Figure S4

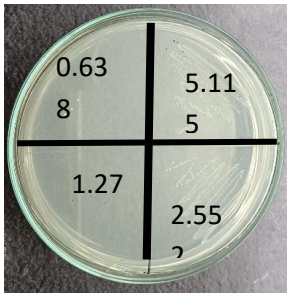
MBC plates of the extracts



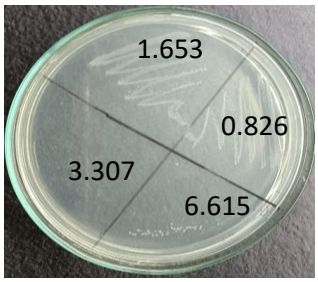
MBC of BT33 (6.281 mg/mL) in *E.coli*



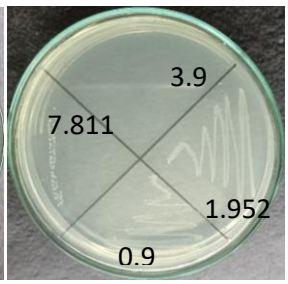
MBC of BT13 (4.945 mg/mL) in *E. coli*



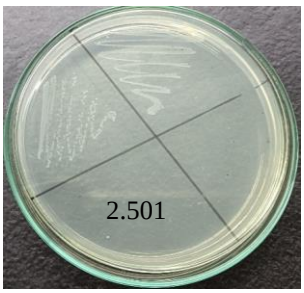
MBC of BT5 (1.276mg/mL) in *E. coli*



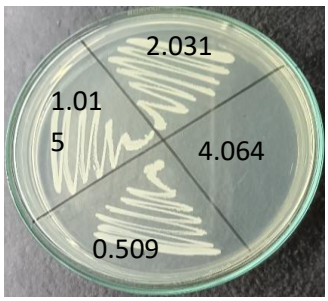
MBC of BT39 (3.307 mg/mL) in *E. coli*



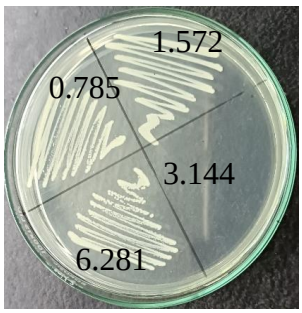
MBC of BT36 (3.905 mg/mL) in *E. coli*



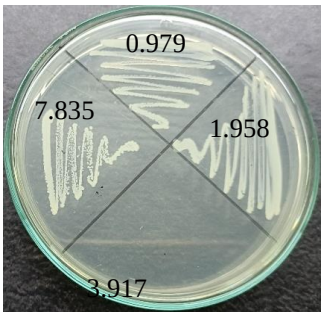
MBC of Neomycin (1.253 µg/mL) in *E. coli*



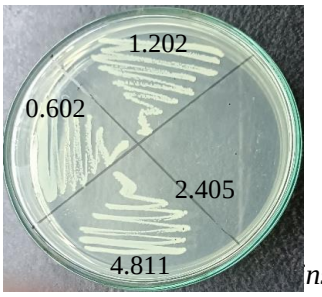
MBC of BT5 (4.064 g/mL) in *S. aureus*



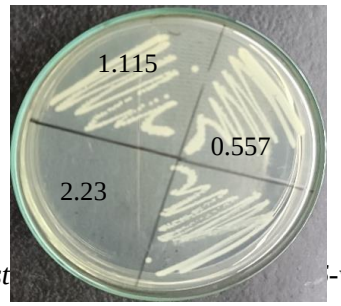
MBC of BT13 (3.144 mg/mL) in *S. aureus*



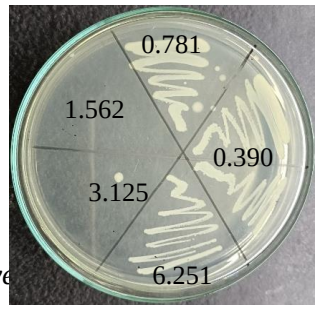
MBC of BT33 (3.917 mg/mL) in *S. aureus*



MBC of BT36 (2.405 mg/mL) in *S. aureus*



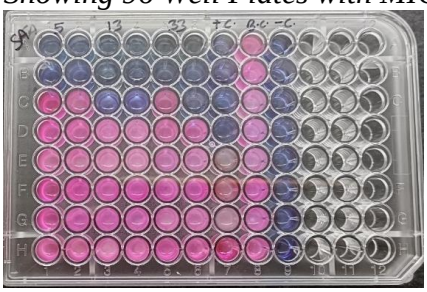
MBC of BT39 (2.231 mg/mL) in *S. aureus*



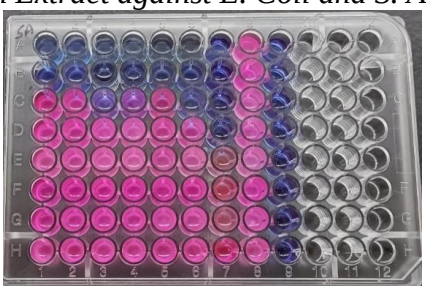
MBC of Neomycin (1.562 µg/mL) in *S. aureus*

Figure S5

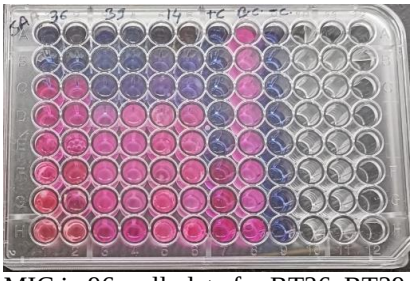
Showing 96 Well Plates with MIC of Each Extract against E. Coli and S. Aureus



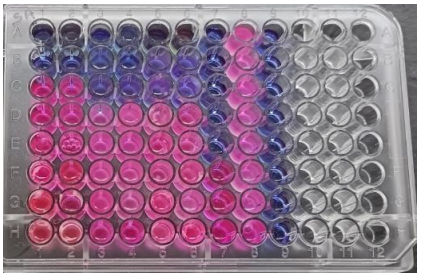
MIC in 96 well plate for BT05, BT13, BT33 and neomycin against *S.aureus* (Lid close)



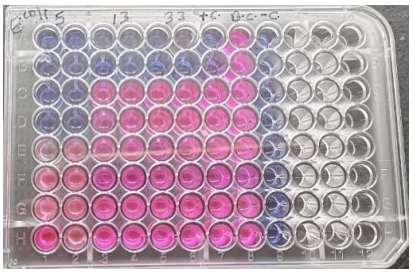
MIC 96 well plate for BT05, BT13, BT33 and neomycin against *S.aureus* (Lid open)



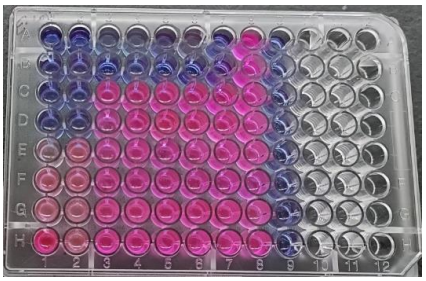
MIC in 96 well plate for BT36, BT39 and neomycin against *S.aureus* (Lid close)



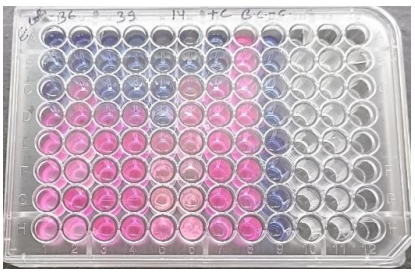
MIC 96 Well plate for BT36, BT39 and neomycin against *S.aureus* (Lid open)



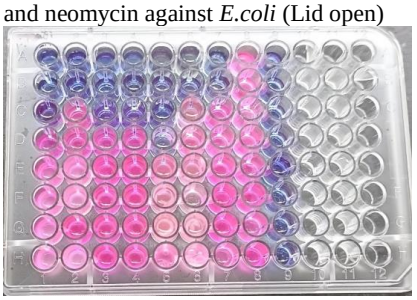
MIC in 96 Well plate for BT05, BT13, BT33 and neomycin against *E.coli* (Lid close)



MIC in 96 Well plate for BT05, BT13, BT33 and neomycin against *E.coli* (Lid open)



MIC in 96 well plate for BT36, BT39 and neomycin against *E.coli* (Lid close)



MIC in 96 well plate for BT36, BT39 and neomycin against *E.coli* (Lid open)

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