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Research Article

Exploring the Antimicrobials Production Potential of Actinobacteria Isolated from Caves at Bahadurkhel Karak, Pakistan

Shahid Nawaz, Aiman Fatima, Maira Saleem, and Imran Sajid*

Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan

Abstract: Cave actinobacteria are relatively less explored and are considered as the new targets for the discovery of novel antimicrobials, specifically against highly resistant pathogens such as extensively drug-resistant (XDR) *Salmonella*. In this study N=20 actinobacteria isolates were retrieved from the least disturbed caves situated at Bahadurkhel Karak, Pakistan (33° 16′ 52″ North, 70° 79′ 07″ East) and (33° 15′ 90″ North, 70° 94′ 72″ East). The isolates were identified using standard morphological and biochemical characterization procedures as well as by 16S rDNA sequencing. N= 16 isolates were targeted for further antimicrobial activity screening based on their preliminary morphological and biochemical identification. The methanolic extracts were prepared from the culture broth of the isolates by using Amberlite® XAD-16 resin to execute *in situ* solid phase extraction. The thin layer chromatography (TLC) showed the presence of a variety of different compounds in the crude extracts of the secondary metabolites. The extracts' antimicrobial potential was analyzed against several test strains, including *Salmonella enterica, Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae*, and *Escherichia coli*. Among the isolated strains, the isolates SNK 93 and SNK47 were found to be the most active against the tested pathogenic bacteria, showing growth inhibition zones of 20 mm and 27 mm respectively against extensively drug-resistant (XDR) *Salmonella.* Based on these results it can be concluded that cave actinobacteria are a promising source of potentially novel antimicrobials against multidrug-resistant (MDR) and XDR pathogens.

Keywords: Cave Actinobacteria, Metabolites, XDR *Salmonella*, TLC, Antibiotics, Bahadurkhel Cave

1. INTRODUCTION

Known for the high G+C ratio in their genome, actinobacteria are indeed one of the largest groups of Gram-positive bacteria. They are generally regarded as potent producers of antibiotics, antifungal, anti-cancerous, and other bioactive compounds. More than 60 % of a current lot of antibiotics are being produced by the actinobacterial species, predominantly by the genus streptomyces [1-4]. A chamber ideal to fit a human entrance could be termed as a cave. There are various ways to classify caves, however predominantly well-known types of caves are limestone and calcium-carbonated caves [5]. Microbiome data of caves is usually unexplored and underrated. The caves provide a unique stressful habitat to bacteria and other microbes, so are now attracting microbiologists for microbiome studies and for exploring the microbiota for pharmaceutically significant metabolites [6-8]. Actinobacteria are predominantly present in caves because they are prolific producers of bioactive compounds there is no surprise if novel and potent antimicrobials can be recovered from the caves actinobacteria. This can be a solution for the antibiotic resistance problem in general and XDR pathogenic strains in specific [9]. Recent literature also suggests that cave actinobacteria species have been recovered with either new compounds or new analogs of existing active compounds [10-14]. In this regard, one of the mentionable discoveries is from Italy where researchers have been able to isolate cervimycins, which showed very significant activities against MDR Staphylococci and Vancomycin-resistant Enterococci (VRE) [15].

In recent years a lethal surge in extensively drug-

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*Corresponding Author: Imran Sajid <imran.mmg@pu.edu.pk>

resistant (XDR) typhoid fever has been reported in different parts of Pakistan. Initially, the majority of the cases were reported from Sindh province in the districts of Hyderabad and Karachi [16]. Typhoid fever is caused by *Salmonella typhi*, [17]. The XDR *Salmonella* strains are resistant to a list of antibiotics that are routinely used to treat typhoid fever, including ampicillin, chloramphenicol, and trimethoprim-Sulfamethoxazole, ciprofloxacin, and ceftriaxone [18]. The situation has become alarming because the XDR *Salmonella* strains have now spread in the entire country and are causing high morbidity and mortality. Many advanced countries such as the United States, Canada, and the United Kingdom, etc, have reported the detection of XDR *Salmonella* cases and unfortunately, the majority of these cases had a travel history to Pakistan [19-23].

In this study we isolated actinobacteria strains from previously unexplored calcareous selective caves situated at Bahadurkhel district Karak, Pakistan, to screen them against a variety of pathogens including XDR *Salmonella*. The caves are located in a remote place away from the activities of native inhabitants, hence are the least disturbed caves. The idea of selecting these caves was based on the fact that these caves possess a unique stressed environment hence there are chances of getting new antimicrobials. As the caves can be harboring unique actinobacteria strains, this means that these strains could be potentially producing novel bioactive compounds including antibiotics. This study showed that actinobacteria isolates have the capacity to inhibit the growth of pathogenic strains including XDR *Salmonella*. This proves that cave actinobacteria are a unique source and need to be explored continuously for pharmaceutically significant compounds.

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil samples (N=10) were collected aseptically from the ground, ceiling, and walls of caves. The caves are located in Bahadurkhel district Karak (33° 16′ 52″ North, 70° 79′ 07″ East) and (33° 15′ 90″ North, 70° 94′ 72″ East). The precise location of the sampling site is shown in Fig 1a, b. The caves are named locally as Kam Dadghor (KDG) and Star Dadghor (SDG) respectively. The soil was dug for about 10-20 cm to avoid surface contaminants. The samples were aseptically transferred to the lab in sterile bags (Fig 2a). Extra care was taken to avoid light exposure by wrapping the sample boxes in black layerings.

2.2 Isolation of Cave Actinobacteria

A variety of selective media was used for the isolation in order to make sure to retrieve the maximum possible actinobacteria strains present in the sample. The Starch Casein Agar (SCA), Humic acid Vitamin Agar (HVA), Glucose Yeast Extract, Malt Extract (GYM), Oatmeal Agar (OM) and A medium were used to obtain diverse actinobacteria strains [24-26]. Among these media, starch Casein agar was used for both selective and pure culture isolation. The starch casein agar was simplified and modified in this study, as shown in supplementary data (Table S1). The modification and simplification involved the removal of trace salts to expose actinobacterial strains to a stressful environment which can potentially trigger their secondary metabolites production. Following isolation protocol was followed; 1 g of the soil sample was diluted in 10 mL of autoclaved normal saline which was then serially diluted up to 10-4 dilutions. 100 µL from 10^{-4} dilution was then spread onto SCA medium for initial isolation, after initial isolation, the rest of the media were used for obtaining the pure cultures. The putative actinobacteria isolates were streaked and maintained on the respective media.

2.3 Culture and Morphological Characterization

The putative actinobacteria strains were identified following a polyphasic taxonomic approach. First, the strains were randomly picked based on their morphology from a crowded plate, the criteria for picking up the actinobacteria strain was their apparent morphology and embedded growth in the medium and the colonies were examined for any sporulation if present [27]. Table 1 summarizes all the details of the characteristics which were considered and observed. Later all the isolates were characterized microscopically via gram staining and for biochemical characteristics (Fig 2b).

2.4 Biochemical Characterization

The isolates were also analyzed biochemically and were screened for melanin formation, organic acid formation and esculin hydrolysis. In the case of the organic acid production test, purple coloration was considered as positive, for melanin, and brown to black color was rendered as positive and for esculin hydrolysis, black coloration was noted as a positive result.

2.5 Preparation of Crude Extracts

To get secondary metabolites, the isolates were inoculated into 10 mL of SC broth and kept at 29 ºC at 200 rpm for three days. After this enrichment step, 2 mL from this pre-culture was added to 50 mL of SC broth. Again this 50 mL culture was placed in a shaking incubator at 29 ºC and 200 rpm for 8 days. About 1 % of Amberlite® XAD-16 N resin was then added to the fermented broth. The mixture was then sonicated for 20 m at 30 kHz and agitated for another 24 h under the same conditions and cellular mass was removed through centrifugation and methanol was used for eluting the active compounds from XAD resin, which was then evaporated on a rotary evaporator at 45 ºC and weighed as shown in supplementary data (Table S2). The final product (crude extracts) obtained was kept at -20 ℃ for further usage and analysis.

2.6 Thin Layer Chromatography (TLC)

The metabolomic extracts were dissolved in Methanol, 5 mg of each extract was dissolved in 1 mL of HPLC grade methanol to obtain a solution of 5 mg/mL and the dissolved extracts were run on (20 × 20 cm Merck-Silica with Aluminum base TLC plates, thickness (20 µm) with binder Polymeric fluorescent indicator) [29]. Various mobile phases with different ratios were used to achieve maximum separation, based upon these trials, the optimum solvent system was found to be a 3:1 chloroform-methanol solvent system.

2.7 Antimicrobial Assay

The inhibition zones for the extracts were measured by the disc diffusion method against the following selected test strains. The first suspensions for the pathogenic test strains were prepared in Muller Hinton Broth (MHB) which included *Salmonella* enterica, Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, and E. coli. After the bacterial suspensions were incubated at 37 °C for 24 hours. The optical density for the tested strains was adjusted at 0.5 (108 CFU/mL) Mcfarland standards. Crude Extracts of about 30 mg were dissolved in 250 µL of methanol, and 25 µL which is equal to 3 mg of the extracts were loaded onto the sterile discs (6 mm), which were placed in the pre-inoculated plates with test strains [30]. The test strains were spread onto the MH agar with the help

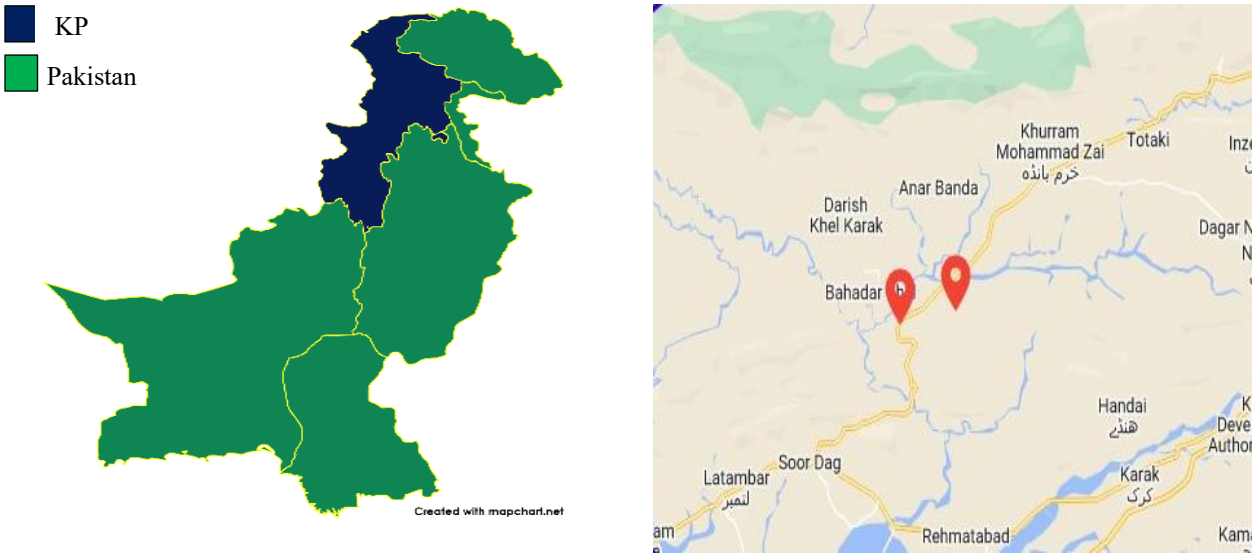

Fig. 1. (a) Overall indication of the caves from a generalized map, (b) Zoomed Map of Bahadurkhel showing specific sampling sites

Table 1. Morphological characteristics of Actinobacteria isolates from caves

Isolates	Colony Morphology	Substrate Mycelium	Aerial Mycelium	Pigments
SNK47	Embedded	Yellow	greyish yellow	white
SNK35	Rough	-	white	white
SNK59	Rough	Grey	white	white
SNK 220	Embedded	Grey	grey	white
SNK04	powdery	White	white	white
SNK03	Rough	Grey	white	white
SNK 73	Powdery	Yellow	green	green
SNK11	Embedded	White	white	white
SNK 250	Embedded	-	white	white
SNK 95	Embedded	Pink	pink	pinkish white
SNK 242	Embedded	-	white	white
SNK246	embedded	-	white	white
SNK106	embedded	White	Grey	white
SNK25	embedded	Grey	Grey	white
SNK 80	embedded	Grey	white	Grey
SNK 93	embedded	-	white	Grey

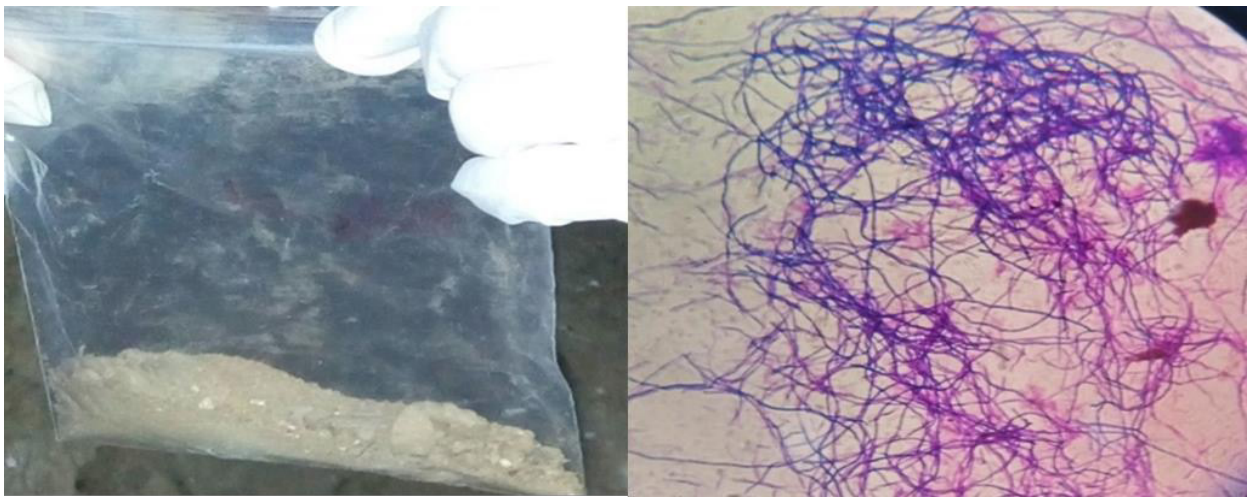

Fig. 2. (a) A soil sample collected from the SDG cave of Bahadurkhel Karak, (b) Gram staining of strain SNK 47

of sterile cotton swabs to prepare a uniform lawn of the test bacteria. The 25 µL of methanol was also added in an empty well as a negative control.

2.8 Extraction of Genomic DNA

The genomic DNA was extracted using Thermo Scientific Gene JET Genomic DNA purification Kit #K0721 (Lithuania) in accordance with the manufacturer's instructions. Briefly, the samples were lysed with proteinase k in lysis buffer and RNA was removed using RNase A, after that the lysate was mixed with ethanol and by using purification columns and washing solutions all the impurities were removed before the genomic DNA was eluted in elution buffer and the DNA was stored at -20 °C for further use.

2.9 Polymerase Chain Reaction for The Amplification of 16S rDNA

The polymerase chain Reaction for the extracted genomic DNA using universal primers 27F 5′- AGAGTTTGATCCTGGCTCAG-3' and 1492R 5′-CTACGGCTACCTTGTTACGA-3′ was performed maintaining the following cyclic

conditions. Denaturation at 95 ºC for 5 mins followed by 30x cycles of denaturation at 94 ºC for 30 sec, annealing at 61 ºC for 30 sec, and extension at 72 ºC for 30 sec. The final extension step was set at 72 ℃ for 5 mins followed by an infinite holding time at 4 ºC. The purified PCR products were sequenced using Automated Sanger chain termination sequencing.

3. RESULTS AND DISCUSSION

3.1 Morphological Identification through Gram Staining and Biochemical Characterization

All the strains from the three culturing media were streaked onto 5 isolation media, including Starch Casein Agar, A medium and glucose yeast extract malt extract media. Among all the starch Casein agar was found to be the most suitable isolation media. We modified and simplified the starch casein agar media. The recipe for the modified media is given in supplementary data (Table S1). For morphological identification, the isolates were characterized based on the texture of the colony and their pattern of growth on the agar surface, as generally, actinobacteria colonies show embedded growth into the agar medium (Fig. 3). Other than these aspects pigment formation is also a very important classification and identification tool to isolate actinobacterial strains, in this study, it was found that Green and yellow were the predominant pigments produced by the caves actinobacteria isolates.SNK 47 and SNK 93 were strongly positive for melanin formation and organic acid formation, SNK03 and SNK 04 were found to be producing a good amount of organic acid. Overall results of the tests are summarized in Table 2.

3.2 Thin Layer Chromatography Profile of the Extracts

The compounds in each of the sample got separated based on their relative polarity. The selected isolates were chosen based on their TLC chromatogram for metabolomics profiling. The chromatogram was analyzed under UV at 254 nm and 366 nm using CAMAG® Uv Cabinet 4 Switzerland. Alternatively, the extracts were also observed by staining with Anisaldehyde (Fig 4). SNK 47 and SNK 93 showed a distinct pattern of bands suggesting the presence of a variety of metabolites and compounds, based upon these results the extracts were tested against a variety of pathogens including XDR *Salmonella*.

3.3 Antimicrobial Activity of the Metabolites against Test Strains

Among all the strains, the isolates SNK 47 and SNK 93 were active against all the tested strains.

Table 2. Summary of biochemical characteristics of cave actinobacteria

Isolates	Biochemical Tests		
	Melanin Formation	Esculin Hydrolysis	Organic Acid Formation
SNK47	+	+	+
SNK 93	+	+	+
SNK 35	+	+	-
SNK 03	+	+	+
SNK 220	+	+	+
SNK 73	+	+	-
SNK 04	+	+	+
SNK 11	+	+	-
SNK 250	-	+	-
SNK 242	+	+	+
SNK 246	-	+	+
SNK 25	+	+	+
SNK 106	+	+	+
SNK 95	+	+	+
SNK 59	-	+	+

Keys: + = positive for the test, - = negative for the test

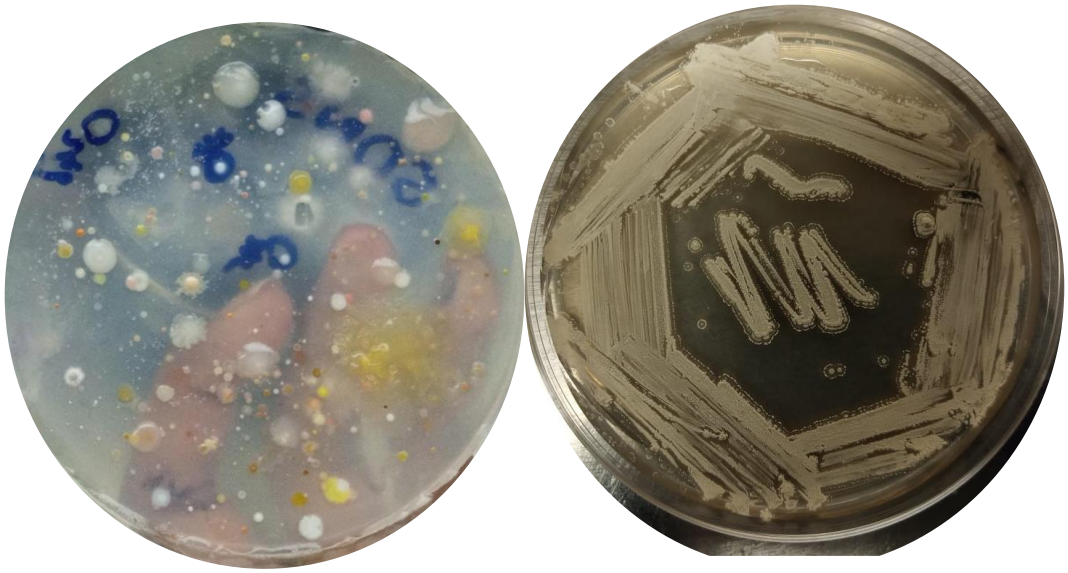

Fig. 3. **(a)** A crowded plate, showing putative actinobacterial isolates, **(b)** pure culture of SNK93 on starch casein agar

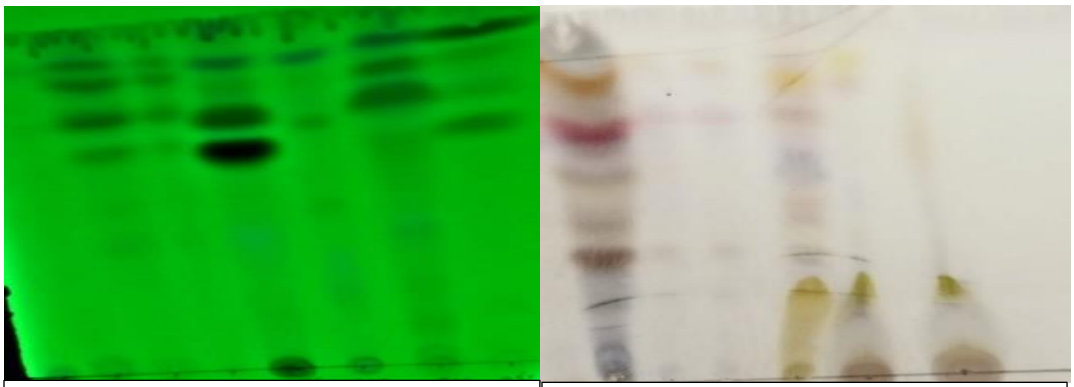

Fig. 4. (a) TLC chromatogram at UV 364 nm, showing separation of compounds based on relative charge and polarity **(b)** staining with Anisaldehyde/H_2SO_4

However, the activity of SNK 47 was found to be maximum against XDR *Salmonella* (Table 3). The XDR *Salmonella* was isolated from meat samples. The zones of inhibition measured for the SNK 47 against *Salmonella*, *Staphylococcus aureus*, *E. coli*, *Klebsiella pneumonia* and *Bacillus subtilis* were 27 mm 12 mm, 11 mm 10 mm and 10 mm respectively. While for SNK93 the zones of inhibition were measured as 20, 0, 0, 10 and 09 mm respectively(Fig 5a). After initial scrutiny of all the crude extracts against XDR *Salmonella*, it was found that only SNK 93 and SNK 47 were active against XDR *Salmonella*. That is why both of the extracts were then used in replicates against XDR *Salmonella* to ascertain the results. Both of the extracts from the said isolates produced comparable results in replicates (Fig 5b).

The isolates SNK 04 and SNK 35 showed some activity against *Bacillus subtilis*. The zone of inhibition for these isolates were measured at 10, and 12 mm respectively. In general SNK 47 was found to be the most active strain as it showed 100 % activity against all the test strains showing that it possesses the remarkable potential for antibiotics production which can be used against different pathogens including XDR *Salmonella*. However, SNK 93 was found to be active against

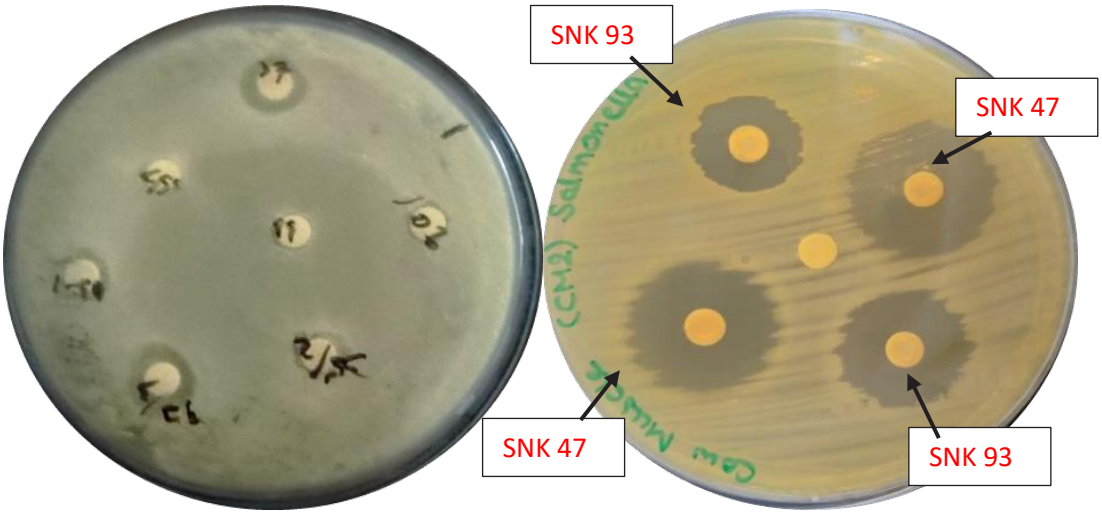

Fig. 5. **(a)** Antimicrobial activity of the methanolic crude extracts against Bacillus subtilis, activities of SNK 47, 93, 04, 106 and 35. **(b)** Activities of the crude extracts of SNK 47 and SNK 93 against XDR *Salmonella*. Both extracts were tested in replicates against XDR *Salmonella*.

Table 3. Zone of inhibition recorded in Millimeters against Test strains

Isolates	**zone of inhibition recorded in mm against test strains**				
	Salmonella	***Staphylococcus aureus***	***Escherichia coli***	***Klebsiella pneumoniae***	***Bacillus subtilis***
SNK47	27	12	11	14	15
SNK 93	20	0	0	10	9
SNK 35	0	7	0	7	10
SNK 03	0	7	0	0	9
SNK 220	0	0	0	0	0
SNK 73	0	0	0	9	12
SNK 04	0	0	0	0	15
SNK 11	0	0	9	0	14
SNK 250	0	7	8	0	8
SNK 242	0	8	13	0	11
SNK 246	0	10	11	0	10
SNK 25	0	8	10	7	13
SNK 322	0	0	10	0	9
SNK 95	0	8	0	7	9
SNK 59	0	0	9	8	10

three test strains showing an overall 60 % activity against all the test strains. These results suggest that SNK 47 and SNK 93 can further be exploited for antibiotics production.

3.4 16S rRNA gene Sequencing and Phylogenetic Analysis

The sequencing data was analyzed through Finch TV 1.4. The nucleotide sequence was then BLAST analyzed to determine the similarity against the existing data of *actinobacteria* in NCBI [31]. The results for SNK 47 showed that the strain is 99 % similar to that of *Glutamibacter uratoxydans*, For the SNK93 the similarity index was found to be 99 % against *Actinoplanes* sp. SE50. Both of these isolates are categorized as rare actinomycetes. The neighbor-joining phylogenetic tree for both SNK 47 and SNK 93 is given in figures 6 and 7. The evolutionary history was inferred using the Neighbor-Joining method, the evolutionary distances were computed using the Maximum Composite Likelihood method and in the units of the number of base substitutions per site. The analysis

for SNK 93 involved 6 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 593 positions in the final dataset. The analysis for SNK47 Involved 10 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 322 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

Due to their unique habitat, Caves provide one of the best competing environments to microbes where they can produce a variety of compounds and secondary metabolites to survive [32-34]. Cave actinobacteria in this study have been primarily targeted to assess Their potential against a variety of pathogens including XDR *Salmonella*. A variety of innovative methods were executed to obtain the optimum number of actinobacterial isolates. One of these methods includes simplification and modification of the Starch Casein agar Media given in Supplementary data (Table S1).

The trace salts were removed from the media and pH was adjusted to 7.8 so that the cave microbes could experience a stressful environment and hence this may activate their secondary metabolites for survival. The media showed perfect growth for cave actinobacteria. Antibiotic resistance is a serious concern in recent days, as WHO estimates that there will be no treatment options in the future if the rate of resistance among the bacteria continues to be the same [35, 36]. The problem is that the rate of resistance in bacteria is much more than the discovery of novel antibiotics. One possibility is to reduce the use of antibiotics and find alternatives but there is no significant progress in this regard. Medical world will rely on antibiotics in the future as well. Generally acceptable solution in this regard is to find a new antibiotic or new version (analogs) of existing antibiotics which can inhibit infectious bacterial growth [37, 38]. One promising and classic source in this regard is actinobacterial species which are producing more than 60 % antibiotics. However, actinobacteria from the most common sources such as soil have been highly exploited and there is less hope of finding any novel antibiotic agents from these sources. Recent studies have shown that Caves are one of the most unexplored habitats having unique environmental stress which can trigger the production of novel and potent antibiotics [39]. In this study among the 15 isolates, 4 isolates showed promising activity against a variety of pathogens including XDR *Salmonella*. Specifically, the isolates SNK 47 and SNK 93 were found to be highly active against XDR *Salmonella* and the rest of the pathogens. This shows that caves can be a good source of finding new and active metabolites against MDR and XDR pathogens. Sequencing analysis showed that the SNK 47 and SNK 93 are 99 % of that of *Glutamibacter uratoxydans* and *Actinoplanes* sp. SE50. To the best of our knowledge, this could be the first report of isolating *Glutamibacter uratoxydans* from the cave environment. *Actinoplanes spp*, however previously

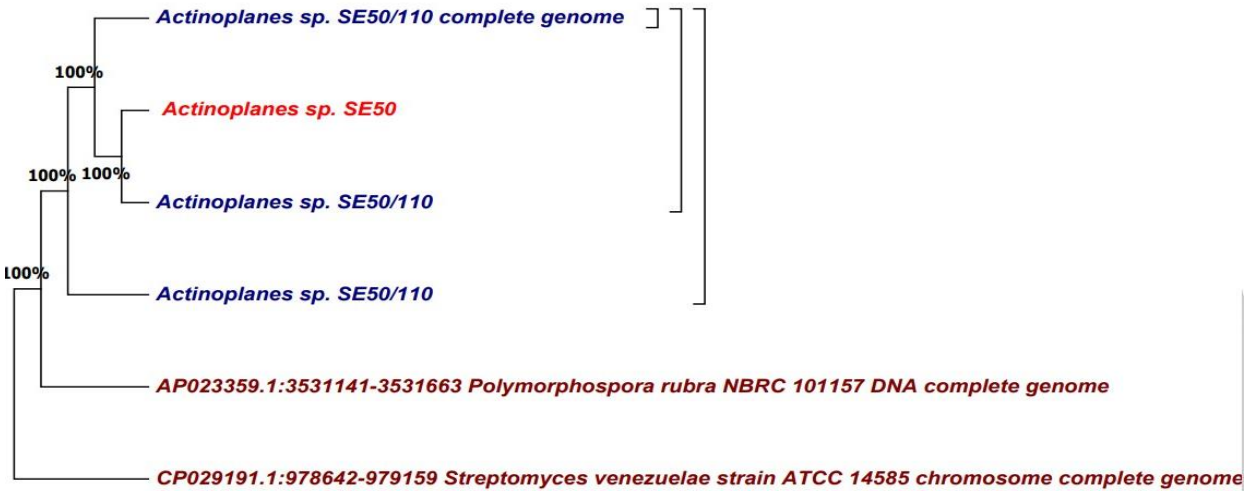

Fig. 6. Phylogenetic tree showing the relationship of SNK 93 to the *Actinoplthe anes* (Highlighted in red). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches.

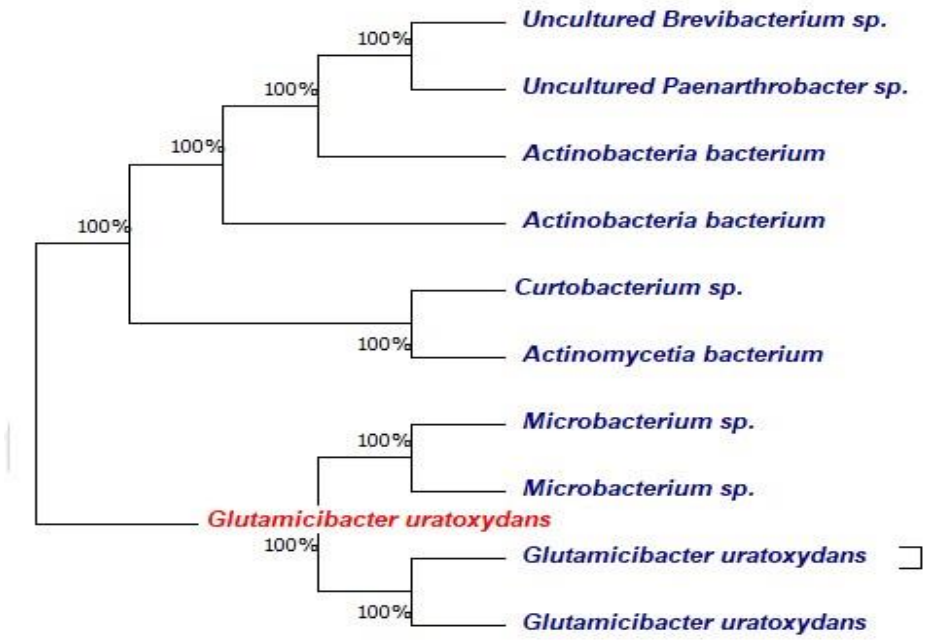

Fig. 7. Phylogenetic relationship of SNK 47 with *Glutamibacter* spp (highlighted in red). The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown. This analysis involved 10 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 322 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

have been isolated from caves [40]. This study will further be extended to identify these metabolites and also determine the structures of these active compounds. Classical approach in this regard is large-scale cultivation, purification of compounds through HPLC and structure elucidation through LC-MS and NMR methodology. The study can be used as a benchmark study in exploring other caves in the region for a large-scale hunt of potent bioactive compounds and novel antibiotics. Other alternative goals that can be achieved by exploiting caves are the discovery of some novel actinobacterial genera as well as their subsequent metabolite profiling which may result in the discovery of many other novel compounds such as antitumor, antifungal and agro active compounds other than antibiotics.

4. CONCLUSION

The actinobacteria isolates from caves showed promising activities against pathogens including XDR *Salmonella*, which supports the idea that caves can be a good source of finding novel and potent antimicrobial agents. The strains SNK47 and SNK 93 are the most active isolates. Targeting these Isolates for Whole genome sequencing, Molecular networking coupled with traditional compound isolation and purification protocols may lead us toward the discovery of novel antibiotics and other bioactive compounds from these strains. There is a need to extensively analyze caves and large-scale studies may help find some of the very promising antibiotics and other bioactive compounds.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

7. DECLARATION

This study did not use any animal models or human subjects.

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SUPPLEMENTARY DATA

Table S1. Modified Starch Casein Agar

Media Components	G/L
starch	10
casein	1
calcium carbonate	2
Agar	13

Table S2. Weight of crude extracts

Isolate	Weight (mg)
SNK47	42
SNK 93	31
SNK 35	29.9
SNK 03	40.4
SNK 220	50
SNK 73	33.5
SNK 04	34.2
SNK 95	25.7
SNK 250	47
SNK 242	33
SNK 11	39
SNK 246	33
SNK 25	45
SNK 106	37
SNK 59	48