Pak. j. life soc. Sci. (2022), 20(1): 128-138 E-ISSN: 2221-7630;P-ISSN: 1727-4915

Pakistan Journal of Life and Social Sciences

www.pjlss.edu.pk

https://doi.org/10.57239/PJLSS-2022-20.1.0013

RESEARCH ARTICLE

Acidophilic Actinobacteria: Isolation, Taxonomic Characterization and Bioactivity against Multidrug Resistant Pathogens

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ARTICLE INFO

Received: March 15, 2022

Accepted: May 20, 2022

Keywords

Acidophile

Actinobacteria

Multi-drug resistance

Antibacterial activity

Cytotoxicity

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ABSTRACT

Actinobacteria are known as potent bioactive microbes capable of producing large number of therapeutic compounds. They are playing a significant role in fight against Multidrug-Resistant (MDR) pathogens. In this study, we isolated 29 actinobacterial strains from acidic soil of various industrial sites in Lahore. The isolated strains were subjected to various morphological and biochemical characterization followed by antibacterial and cytotoxicity screening. The methanolic extracts of strains, obtained by small-scale laboratory fermentation, were screened for activity against clinically isolated MDR bacterial pathogens including *Klebsiella pneumoniae, Escherichia coli, Bacillus spp.*, methicillin-resistant *S. aureus* (MRSA) *Acinetobacter spp.,* and *Pseudomonas aeruginosa*. The Grampositive test pathogens were inhibited by 53% of the actinobacterial extracts and Gram-negative test strains were inhibited by 92% of the extracts. The extracts of two actinobacteria strains; N-100 and N-87, exhibited antibacterial activity against all the test pathogens. The test strain *E. coli* was inhibited by 55% of extracts which was the highest inhibition percentage among all test strains. The Diphenyl-tetrazolium bromide (MTT) assay was performed for cytotoxicity analysis and it was observed that the strain SS-4 exhibited highest cytotoxicity with up to 77% mortality at 80µg/ml concentration. Overall, the extracts of eleven strains resulted in 40% viability against A549 Adenocarcinoma human alveolar basal epithelial cell line. The Thin Layer Chromatography (TLC) analysis was performed for chemical analysis and different bands were visualized under long and short UV. The 16S rRNA gene sequencing of two most promising strains, N-87 and N-100, revealed them to be *Streptomyces coeruleofuscus* (97.44%) and *Streptomyces iakyrus* (97.84%) with query length of 1217 and 1187 base pairs, respectively. This study indicates that acidophilic soil actinobacteria have great potential to harbour novel strains and to produce bioactive secondary metabolites against MDR pathogens.

INTRODUCTION

Microbes are being used to treat the infections even long before the time of their discovery. This fact is established on various historical discoveries including a recipe older than 1000 years from the Anglo Saxon. This recipe amazingly found to be effective against the

inhibition of MRSA (Harrison et al., 2015). In modern era, the discovery of penicillin led to the discovery of countless bioactive compounds from living sources including bacteria and fungi (Harir et al., 2018). But the improper use of the antibiotics bring disaster in the form of antibiotic resistance phenomenon (Hwang and Gums, 2016; Morehead and Scarbrough, 2018). The Centers for Disease Control and Prevention (CDC) predicted that we are on the way of great loss in future due to the antibiotic resistance phenomenon. It was estimated that up to the year 2050, almost 300 million premature deaths would be occurred merely due to this (Review on Antimicrobial Resistance, 2014).

The antibiotic resistance phenomenon is a great threat to health, as majority of bacteria have already developed antibiotic resistance (Stracy et al., 2022). The need of hour is to develop new drugs to treat multidrug-resistant pathogens (Morel et al., 2021). It is an established fact that actinobacteria is the largest group of prokaryotes inhabiting all types of environments including fresh water bodies, oceans, and rocks but predominately they are present in soil. This group of bacteria earned the greatest importance due to the remarkable ability of drugs production Jose et al., 2021). Actinobacteria are responsible for the production of more than 60% of the total known drugs. While, the genera *Streptomyces* is of prime importance in the actinomycete family, as they contribute towards the production of largest percentage of drugs than any other known group of microbes (Al-Shaibani et al., 2021; Azman et al., 2017; Matarrita-Carranza et al., 2021). Regardless of the extensive research, there is still huge potential in this group of microorganisms to produce novel and more potent antibiotics (Cheema et al., 2021; Rasool et al., 2014).

Actinomycetes are already known to exhibit significant inhibitory activity against resistant pathogenic groups of bacteria (Siddharth et al., 2020; Talpur et al., 2020), especially from harsh habitats with different conditions like acidic or basic pH and salinity. It has been reported that actinobacteria from extreme conditions have tendency to show better inhibitory activity against multidrug-resistant bacterial and fungal pathogens as compared to those who found on relatively moderate conditions (Hui et al., 2021). Kariminik et al. (2010) obtained remarkable antibacterial activity of actinobacterial extracts against Multi Drug Resistant (MDR) bacterial pathogens with minimal inhibitory concentration (MIC) of just 4 µg/ml. Lim et al. (2018) carried a study with satisfactory results and analyzed strong broad spectrum bactericidal activity of actinobacterial extracts against MDR pathogens. Actinobacteria also harbors great antitumor activity as evident from a study conducted by Gozari et al. (2019) in which they observed anti-cancer effects of actinobacterial extracts against various cell lines. They worked on MCF7 (breast carcinoma), HUVECs (human umbilical vein endothelial cells), HCT 116 (colon carcinoma) and HepG2 (hepatocellular carcinoma).

In this study, we isolated promising actinobacterial strains from acidic soil of industrial sites, performed their molecular characterization, analyzed the inhibitory activity against bacterial pathogens and cytotoxic activity against A549 human adenocarcinoma alveolar basal epithelial cell line by MTT assay. A promising response of the acidophilic actinobacteria have been observed with reference to growth inhibition of MDR pathogens.

MATERIALS AND METHODS

Sample collection

The soil samples were collected from the industrial sites of city Lahore, Pakistan. The soil having pH range from 5-6.5 was dug up to 5 to 10 cm down and then sub-surface soil was collected into sterile vials and further processing of samples was done within 24 hours in laboratory.

Isolation of actinomycete strains

The soil samples were dissolved in distilled water and serial dilutions (10^{-1}-10^{-9}) were prepared. An aliquot of about 100 µl of each dilution was spread over Actinomycetes Isolation Agar (g/l: sodium caseinate 2.0, glycerol 5.0, K_2HPO_4 0.5, sodium propionate 4.0, $MgSO_4.7H_2O$ 0.1, $FeSO_4.7H_2O$ 0.001, asparagine 0.1, agar 15, pH 5-6.5) and Starch Casein -KNO_3 Agar (g/l: glycerol 10, KNO_3 2.0, casein 0.3, NaCl 2.0, K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.05, 0.02, $FeSO_4.7H_2O$ 0.01, $CaCO_3$, agar 18, pH 5-6.5) having nystatin as antifungal agent. After incubation of 10 to 14 days, actinobacteria colonies were observed and were sub-cultured on Glucose Yeast Malt extract (GYM) agar (g/l: glucose 5, yeast extract 5, malt

extract 10, agar 18, pH 5-6.5) and the pure cultures were obtained (Shirling and Gottlieb, 1966).

Morphological, physiological and biochemical analysis

The isolated actinobacteria strains were subjected for taxonomic identification by various morphological, physiological and biochemical properties. The morphological characterization includes both microscopic and macroscopic characters. Macroscopically, multiple features were observed including colony size, colour of aerial mycelium, colour of substrate mycelium, colony texture, colony shape, colony margins and pigment production. While microscopically, colonies were stained with Gram-stain reagent and arrangement of hyphae, nature of hyphae and chains of spores were observed. The biochemical properties were determined by applying various tests including esculin hydrolysis test, sugar utilization test, melanin formation test, utilization of organic acids, formation of organic acids and oxalate utilization.

Small-scale laboratory fermentation and methanolic extract preparation

The purified actinobacteria strains were cultivated as shake flask culture in A-media (g/l: soluble starch 20.0, glucose 10.0, peptone 5.0, yeast extract 5.0, sodium chloride 4.0, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.5, $CaCO_3$ 2.0) and incubated on a rotary shaker. The inoculum was given in 50 ml actinomycetes isolation broth (pH ranges 5-6.5) into 250 ml flasks and the flasks were incubated at rotary shaker for 10 to 14 days at 26°C and 200 rpm. After the incubation, amberlite XAD-16N gel added in flasks by 4% w/v ratio and kept on shaker overnight. For harvesting the cultures, a series of steps were carried out as liquid phase was separated after centrifugation and XAD-16N gel was washed twice with deionized water. The pallet was dipped into 20 ml of methanol and was centrifuged after proper vortex mixing and sonication. The methanolic supernatant was carefully collected in clean vials. The obtained methanolic extracts were examined by Thin Layer Chromatography (TLC) and their inhibitory activity was determined against a panel of bacterial pathogens and cancer cell lines.

Chemical profiling

The chemical screening was performed to check the presence of different metabolites in crude extract by separating the metabolites in each band through chromatography technique. For this purpose, a TLC plate was taken and the methanolic extracts were spotted and allowed to dry. The 10% solution of CH_3OH/CH_2Cl_2 was added into the TLC tank the sheet was adjusted properly. The appropriate time was given for mobile phase to rise the sufficient level until the sheet was recovered from tank and air dried. Various bands were visualized under UV. Each band represent specific compound or group of compounds. These active compounds and their band regions were marked. The H_2SO_4/anisaldehyde stain applied to the sheet followed by immediate dry heat up to 90°C for 10-15 minutes. The stain elaborated a different pattern of bands. Antibiotic susceptibility assay for test strains The test organisms (provided by The Children's Hospital and Institute of Child Health, Lahore) were isolated from blood and urine samples of various patients admitted in hospital and antibiotic susceptibility assay was performed. The assay was performed against antibiotics of routine clinical usage including amikacin, amoxicillin, ampicillin, ceftazidime, cefixime, ciprofloxacin, cefpirome, colistin, ceftriaxone, cefotaxime, cefuroxime, levofloxacin, meropenem, moxifloxacin, oxacillin, polymyxin B, penicillin, sulbactam-cefoperazone, and tazobactam/piperacillin.

Biological Screening

Determination of antibacterial activity

The antibacterial activity was performed by agar well diffusion method. For this experiment, Mueller Hinton (MH) agar media (g/l: acid casein hydrolysate 17.5, starch 1.5, agar 18 and beef extract 2) was autoclaved and poured in to the glass culture plates to solidify. For assay, the fresh test organism's isolated colony was taken from N-broth culture and dissolved into deionized water. A reference turbidity value of the colony was taken by measuring Optical Density (OD) value and compared it with 0.5% McFarland standard. A single drop of 50 µl bacterial suspension was dropped onto MH agar and swabbed over the whole media plate gently and uniformly. Uniform distance wells were made on culture plate by using sterile cork borer. Each well was labelled and fifty microliters of concentrated extracts were poured into the well and the plates were left on bench top for almost 20 minutes for agar to absorb the liquid extract

before incubating at 37°C. The clear zones around the wells were measured carefully in millimeters (mm) and zone of any extract greater than 10 mm was considered biologically significant.

Cytotoxicity analysis

The MTT [(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide] assay was performed using all the extracts against A549 Adenocarcinoma human alveolar basal epithelial cell line. For in-vitro assay, cell culture plate of 96 wells was seeded with respective cancer cells by quantity of 104 approximately. After appropriate incubation for seeded cells growth, two dilutions made with Dimethyl Sulfoxide (DMSO); (50 µg/ml and 80 µg/ml) of each methanolic extract were added in separate wells. For negative control DMSO and for positive control Actinomycin D was used. MTT reagent was added in each well and was placed in anaerobic incubator for at least 4 hours. MTT reagent actually inhibits the metabolic machinery of cells thus stops the biological activity of cells. After incubation, wells were washed with detergent and then OD value was taken under UV light of 570 nm. Wells with higher alive cell count depicted more absorption hence higher OD value (Saleem et al., 2020).

16S rRNA gene sequencing

The most promising strains were subjected to 16S rRNA gene sequencing identification. The genomic DNA was extracted by using GeneJET, thermo Scientific DNA extraction kit (made by Rockford, IL, USA). From the extracted DNA, 16S rRNA gene was amplified by a set of reverse and forward primers (1492R, 5′-GGTTACCTTGTTACGACT-T-3′; 27F, 5′-AGAGTTTGATCMTGGCTCAG-3′) (Lane, 1991). After amplification, gel electrophoresis was done of PCR products and products were purified from gel by QIAquick gel-extraction kit (Qiagen, Valencia, CA, USA). The purified products were sent for sequencing using the same set of primers. The sequencing data was BLAST analyzed at NCBI data base against the 16S rRNA sequence of already reported species. The gene sequence data was submitted to GenBank and the accession numbers were obtained.

RESULTS

Morphological, physiological and biochemical characteristics

The total of 29 pure actinobacterial strains were recovered from 15 soil samples of acidic nature obtained from different sites of the industrial areas in Lahore, Pakistan. The Gram's staining revealed that all isolated strains were Gram-positive with fungus like filamentous appearance.

The strains showed distinct characteristics under microscope after staining. Three main microscopic features were observed, including; arrangement of hyphae, nature of hyphae and chains of spores. Morphologically, purified strains showed distinct features and varied size of colonies (Table 1).

All strains were ranging from 2 to 6 mm diameter of colony with entire and undulant colony margins and different colony texture. Many colonies were seen producing diffusible pigments of various colors. All the isolated strains were subjected to various biochemical and physiological tests for their taxonomic characterization. More than half (18) of the isolated strains were able to form melanin pigment, from which 5 strains including N-82, N-83, N-85, N-89 and N-100 gave strong positive result. The majority of strains (26) gave positive result with esculin hydrolysis including 16 strains exhibiting strong positive results. For oxalate utilization test 24 strains gave positive results. The positive result for utilization of organic acids for carbon source was exhibited by 23 strains, and 11 strains exhibited strong positive results including N-15, N-83, N-90, N-91, N-91A, N-96, N-99, N-100, N-102, N-106 and N-110 (Table 2). For organic acid formation test, 23 strains exhibited positive results with 13 strains including N-87, N-88, N-89, N-90, N-91A, N-92, N-93, N-94, N-96, N-99, N-104, N-106 and N-601AB exhibiting strong positive results. For the sugar utilization test, all the isolated strains were positive for glucose utilization, 21 strains positive for sucrose, 18 strains were positive for mannose, 16 strains were positive for fructose and 20 strains were positive for mannitol, galactose and arabinose utilization (Table 2).

Metabolomic profile of the selected strains

Chemical profiling of methanolic extracts was done by Thin Layer Chromatography (TLC). The compounds in extracts were separated based on their molecular mass and visualized as separate bands under UV light of 254 and 366 nm (Fig. 1). After visualizing under UV, the TLC plate was stained with anisaldehyde/H_2SO_4 reagent and different colored bands were appeared.

Table 1: Morphological characteristics of the isolated actinobacteria strains

Strains	Colony size (mm)	Colour of aerial mycelium	Colour of substrate mycelium	Colony texture	Colony margins	Colony shape	Pigmentation
N-15	2	Grey white	Grey	Soft	Entire	Circular	-
N-82	3	White	Grey	Hard	Undulant	Irregular	-
N-83	3	Light brown	Brown	Soft	Entire	Circular	-
N-84	6	Purplish grey	Grey	Soft	Undulant	Irregular	Purple
N-85	1	White	Grey	Hard	Undulant	Irregular	Greyish black
N-86	5	Cream White	Yellow	Soft	Entire	Circular	-
N-87	3	White	Blue	Hard	Undulant	Irregular	-
N-88	4	Light brown	Brown	Hard	Undulant	Irregular	-
N-89	2	Grey white	Grey	Hard	Undulant	Irregular	Brown
N-90	5	Pinkish white	Pink	Soft	Undulant	Irregular	-
N-91	3	White	Blue	Hard	Undulant	Circular	Blue
N-91A	3	White	Blue	Hard	Undulant	Irregular	-
N-92	4	Grey	Brown	Hard	Undulant	Irregular	Brown
N-93	2	Grey White	Brown	Soft	Entire	Circular	-
N-94	5	Greenish white	Green	Hard	Undulant	Irregular	-
N-95	4	White	Orange	Soft	Undulant	Circular	-
N-96	3	Grey	Brown	Soft	Undulant	Irregular	Brown
N-98	3	Cream White	Grey	Soft	Entire	Circular	-
N-99	4	White	Dark grey	Hard	Undulant	Irregular	Black
N-100	2	Cream White	Dark grey	Soft	Undulant	Irregular	Grey
N-101	1	-	Grey	Hard	Entire	Circular	Orange
N-102	2	Grey	Cream	Hard	Undulant	Circular	Grey
N-104	3	-	Grey	Hard	Undulant	Circular	Grey
N-106	5	Grey white	Black	Hard	Undulant	Irregular	-
N-110	3	White	Grey	Soft	Undulant	Circular	-
N-0173	4	Whitish brown	Yellow	Hard	Entire	Irregular	-
N-601AB	3	-	Light orange	Hard	Undulant	Circular	-
N-602AB	3	White	Orange	Soft	Undulant	Irregular	-
SS-4	2	Grey	Yellow	Soft	Entire	Irregular	-

Antibiotic susceptibility profile of the test strains

The antibiotic susceptibility assay was performed to analyze the resistance pattern of test organism towards antibiotics of routine clinical usage. It was found that two strains i.e., *K. pneumoniae* and *Acinetobacter spp.* were resistant to all antibiotics except colistin and polymyxin B; *E. coli* was resistant to amikacin, amoxicillin, ampicillin, ceftazidime, cefixime, cefotaxime, cefuroxime, levofloxacin, moxifloxacin, penicillin and sulbactum-cefoperazone; *Bacillus spp.* was resistant to amoxicillin, ampicillin, ceftazidime, cefotaxime, levofloxacin, moxifloxacin, oxacillin, penicillin and sulbactum-cefoperazone; *P. aeruginosa* was resistant to amoxicillin, ampicillin, ceftazidime, cefixime, ciprofloxacin, cefpirome, ceftriaxone, cefotaxime, cefuroxime, levofloxacin, moxifloxacin, penicillin while *S. aureus* was resistant to amikacin, amoxicillin, ampicillin, ciprofloxacin, levofloxacin, oxacillin, penicillin and tazobactam/piperacillin (Table 3).

Table 2: Biochemical characteristics of the isolated actinobacterial strains

Strains	M	E	O	U	F	Utilization of Sugars						
						Gl	Sc	Mn	Fr	Mi	Ga	Ar
N-15	+	+++	+	+++	+	+++	+	+		+	++	+
N-82	+++	++	+	+	+	+++	++		+++	++		++
N-83	+++	+++	+	+++		+++	+++	+++	+	+	++	+
N-84	++	+++	+	+		+++			+++	++		+
N-85	+++	+++	+	+	+	+++	+++			+	++	++
N-86		+++	+	+	+	+++	+	+	+	++	+	
N-87 N-88	++	+	+		"+++ +++"	"+++ +++"	+++	+++	+	++	++	"+ ++"
N-89	+++	+++	+	+	+++	+++		+			+	+++
N-90	++		+	+++	+++	+++	+++		++	+++		+
N-91	++	+++	+	+++		+++	+	+++	++		++	++
N-91A	+	+++		+++	+++	+++	+++	+	+++	++	+	
N-92	+	+	+	+	+++	+++	+	++				
N-93		+++	+	++	+++	+++	+			+++	++	++
N-94 N-95	+	"+++ +"	+		"+++ +"	"+++ +++"	+	++	++	+++	+++	+++
N-96		+++	+	+++	+++	+++	+++	+	+++	+	+	++
N-98		+	+	+		+++	++	++	++		++	
N-99	+	+++	+	+++	+++	+++				++		+
N-100	+++	+	+	+++	+	+++	+		++		+	+
N-101	++	+++	+	+	+	+++	+++	++		++	++	
N-102	++	+++	+	+++		+++		+++		++	++	++
N-104	++	+	+		+++	+++	+	+++	++	+++		
N-106	+	++	+	+++	+++	+++	+	+		++	+	+
N-110		+++	+	+++		+++	+++			+++	++	++
N--0173		+++		+	+	+++	++	++	++		++	+
N-601AB		++	+	+	+++	+++				++		
N-602AB		+	+		++	+++	+	+	+++	+++	++	++
SS-4				+	+	+++		++	++		+	

Key: +: mild positive, ++: moderate positive, +++: strong positive, M: Melanin formation test, E: Esculin Hydrolysis test, O: Oxalate Utilization test, U: Utilization of organic acid test, F: Formation of organic acid test, Gl: Glucose, Sc: Sucrose, Mn: Mannose, Fr: Fructose, Mi: Mannitol, Ga: Galactose, Ar: Arabinose.

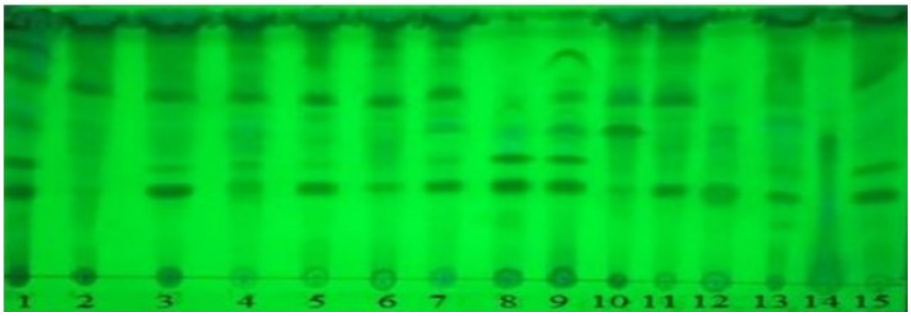

Figure 1: TLC plate under UV 254nm (1: N-15, 2: N-82, 3: N-83, 4: N-84, 5: N-85, 6: N-86, 7: N-87, 8: N-88, 9: N-89, 10: N-90, 11: N-91, 12: N-91A, 13: N-92, 14: N-93, 15: N-94)

Biological Screening

Antibacterial activity

For biological screening, methanolic extracts of actinobacteria were tested against the above-mentioned resistant pathogens by agar well diffusion method. The organism *E. coli* was found to be the most vulnerable strain, as inhibited by extracts of 16 strains with maximum clearance zone of 24mm and 22mm by strain N-85 and N-89 respectively. Methicillin-resistant *Staphylococcus aureus* (MRSA) was inhibited by extracts of 11 strains with maximum clearance zone of 21 mm and 20 mm by strain N-100 and N-92 respectively (Table 4). *K. pneumoniae* was inhibited by 14 extracts with maximum clearance zone of 18 mm by strain N-95. Two most important hospital acquired pathogens i.e., *Acinetobacter* and *Pseudomonas* were

inhibited by 12 extracts with maximum clearance zone of 25 mm by strain N-89 for *Acinetobacter* and 26 mm by strain N-95 for *Pseudomonas*. The *Bacillus spp.* was inhibited by extracts of 7 strains with maximum clearance zone of 20mm by strain N-82. In the following study, only one strain had no activity against any test organism while all other had inhibitory activity against at least one test organism. Gram positive bacterial pathogens were inhibited by extracts of 15 strains, Gram-negative pathogens were inhibited by extracts of 26 strains and 14 strains had inhibitory activity against both Gram-positive and negative test organisms. Two strains (N-87 and N-100) were found having potent inhibitory activity against all these pathogens.

Table 3: Antibiotic sensitivity pattern of human pathogenic strains

Antibiotics	*E. coli*	*K. pneumoniae*	*A. spp.*	*B. spp.*	*P. aeruginosa*	*S. aureus*
AK	R	R	R	S	-	R
AMC	R	R	R	R	R	R
AMP	R	R	R	R	R	R
CAZ	R	R	R	R	R	-
CFM	R	R	R	S	R	S
CIP	S	R	R	S	R	R
CPR	S	R	R	S	R	S
CT	S	S	S	S	S	S
CTR	S	R	R	S	R	S
CTX	R	R	R	R	R	-
CXM	R	R	R	S	R	S
LEV	R	R	R	R	R	R
MEM	S	R	R	S	S	S
MXF	R	R	R	R	R	-
OXA	-	-	-	R	-	R
PB	S	S	S	S	S	S
PCN	R	R	R	R	R	R
SCF	R	R	R	R	S	-
TZP	S	R	R	S	S	R

Key: R: Resistant, S: Sensitive, AK: Amikacin, AMC: Amoxicillin, AMP: Ampicillin, CAZ: Ceftazidime, CFM: Cefixime, CIP: Ciprofloxacin, CPR: Cefpirome, CT: Colistin, CTR: Ceftriaxone, CTX: Cefotaxime, CXM: Cefuroxime, LEV: Levofloxacin, MEM: Meropenem, MXF: Moxifloxacin, OXA: Oxacillin, PB: Polymyxin b, PCN: Penicillin, SCF: Sulbactam-Cefoperazone, TZP: Tazobactam/Piperacillin

Cytotoxicity of the methanolic extracts

In MTT assay, all actinobacterial extracts (29) were tested against A549 adenocarcinoma human alveolar basal epithelial cell line. Extracts with two different concentrations i.e., 50 µg/ml and 80 µg/ml were used and good cytotoxic activity was found. Minimum cell viability of 23% was shown by strain SS-4 and N-83 at 80 µg/ml concentration which means the strain SS-4 and N-83 had 77% cytotoxicity against A549 cell line. The extracts of four strains including N-15, N-84, N-87, N-88, exhibited up to 30% viability of cancer cells. While 12 strains in our study including N-82, N-85, N-86, N-89, N-90, N-91, N-92, N-94, N-95, N-96, N-102 and N-602AB exhibited minimum cell viability up to 50% and maximum cell viability of 76% was shown by strain N-106 which means that this strain killed only 24% of cancer cells.

16S rRNA gene sequencing

Two most promising strains, which exhibited significant antimicrobial activity including N-87 and N-100 were subjected to molecular characterization. The sequencing data was analyzed by BLAST tool at NCBI to determine the homology with already reported strains. Strain N-87 had 97.44% similarity index with specie *Streptomyces coeruleofuscus* and strain N-100 had 97.84% similarity index with specie *Streptomyces iakyrus*. After submitting the sequence data to GenBank, the accession numbers obtained for *Streptomyces coeruleofuscus* and *Streptomyces iakyrus* were ON106838 and ON106839 respectively.

Table 4: Antibacterial activity of the methanolic extracts by well diffusion method

Strain	Zone of inhibition in mm against test strains					
	E. coli	*K. pneumoniae*	*Bacillus spp.*	*S. aureus* (MRSA)	*P. aeruginosa*	*Acinetobacter* spp.
N-15	12	14	-	13	-	16
N-82	-	-	20	-	12	-
N-83	14	-	-	-	-	-
N-84	13	-	-	-	-	22
N-85	24	-	-	-	-	-
N-86	15	15	-	-	-	-
N-87	16	14	11	14	17	20
N-88	-	-	11	-	-	-
N-89	22	-	16	-	-	25
N-90	17	17	-	-	-	-
N-91	14	-	-	-	-	-
N-91A	15	14	-	-	20	-
N-92	14	16	-	20	14	12
N-93	-	-	-	10	-	-
N-94	-	-	13	19	18	20
N-95	-	18	-	10	26	-
N-96	-	-	12	-	14	-
N-98	-	14	-	-	-	12
N-99	-	15	-	-	-	12
N-100	15	16	17	21	24	19
N-101	18	-	-	-	17	-
N-102	-	14	-	12	-	-
N-104	13	16	-	16	10	-
N-106	-	-	-	-	-	-
N-110	13	-	-	-	-	16
N-0173	-	-	-	-	13	-
N-601AB	17	-	-	-	11	20
N-602AB	-	12	-	15	-	11
SS-4	-	15	-	16	-	-

DISCUSSION

From about a century, it is a well-known fact that actinomycetes family is the most significant group of bacteria in pharmaceutical field. It is widely distributed in all environments and contributes to the production of more than 70% of antibiotics, anti-fungal drugs and various other biologically active compounds.

In the following study, actinomycetes were isolated from soil samples of acidic pH from different industrial sites of Lahore. The acidic soil was selected for sampling by assuming that harsh condition like low pH can harbour actinomycetes with unique secondary metabolites which could show activity against resistant bacterial pathogens. The isolated Gram-positive strains were characterized according to various biochemical tests. In summary, melanin was produced by 62% strains, 89% of strains hydrolyzed esculin, 83% strains utilized oxalate. All the isolated actinobacteria strains in this study were positive for glucose utilization, 72% for sucrose, 62% for mannose, 55% for fructose and 68% were positive for mannitol, galactose and arabinose. The results of biochemical tests are similar to the study conducted by Cheema et al. (2021) in which they isolated actinomycetes from the soil of Himalayan region, Pakistan. The similarity in the results of both studies is probably due to the isolation of strains from soil of harsh environment.

Biological screening of methanolic extracts were tested against six resistant bacterial pathogens. The *E. coli* was inhibited by 55% strains, *Bacillus spp.* was inhibited by 24% actinobacteria strains, 37% strains inhibited *S. aureus*, and 41% strains inhibited *P. aeruginosa*. The results are similar with the study conducted by Niyasom et al. (2015) in which methanolic extracts of 32 acidophilic actinobacteria strains were tested against *Bacillus spp.*, *S. aureus, P. aeruginosa* and *E. coli*. The inhibition of all test strains by both studies confirms the potent antibacterial activity of acidophilic actinobacteria (Fig. 2). The

cytotoxicity of extracts was also tested against A549 Adenocarcinoma human alveolar basal epithelial cell line. The viability observed was up to 20% which means 80% inhibition of cancer cells (Fig. 3). The extracts tested by Chen et al. (2018) exhibited quite similar cytotoxic activity. Similarity in result can be interpreted in terms of similar compounds in extracts of both studies isolated from soil.

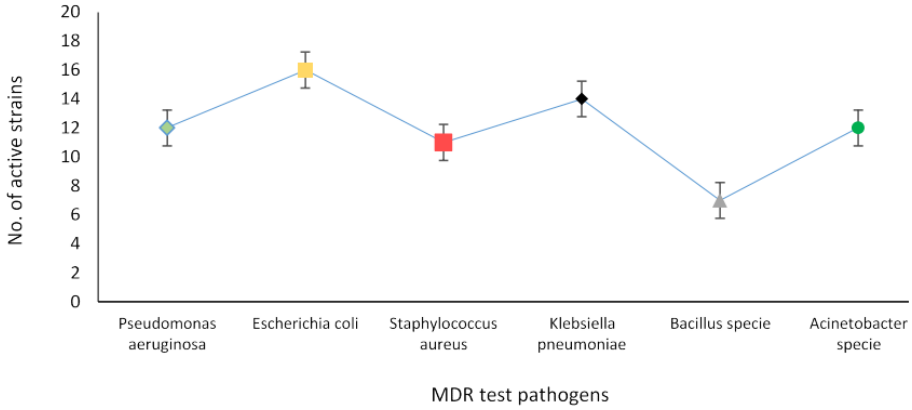

Figure 2: Number of strains having inhibitory activity against multidrug-resistant (MDR) bacteria

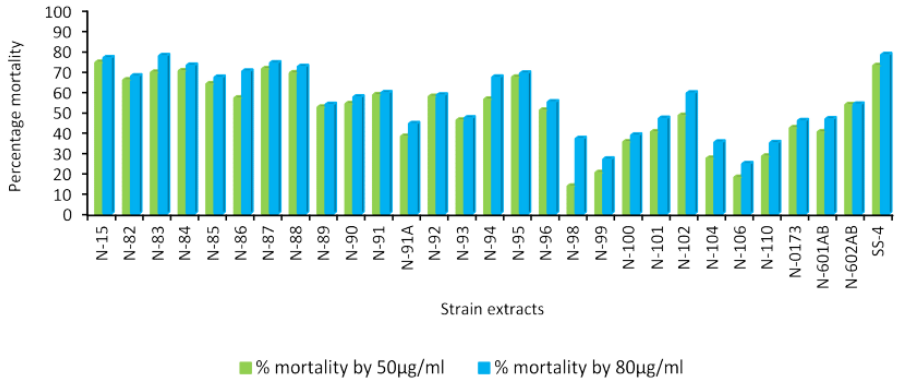

Figure 3: Percentage mortality of A549 cancer cells by extracts

Two strains including N-87 and N-100 were seen inhibiting all test pathogens. The strain N-87 had 97.44% similarity index of 1217 base pairs with specie *Streptomyces coeruleofuscus* and strain N-100 had 97.84% similarity index of 1187 base pairs with specie *Streptomyces iakyrus*. The *Streptomyces coeruleofuscus* has also being isolated by Lee et al. (2014) from the mangrove soil, which had remarkable inhibitory activity against MRSA likewise the strain isolated in our study. The same strain was also isolated by Li et al. (2014) from the unique habitat of limestone.

CONCLUSION

We investigated the bioactive potential of acidophilic actinobacteria through antibacterial activity and cytotoxicity analysis. The sequencing results of the study revealed that isolated strains could be novel strains and whole genome sequencing should be done to confirm the discovery. The isolated strains could be predicted to harbour remarkable bioactive compounds based on the inhibitory activity against the most common and resistant bacterial pathogens. The extracts also exhibited potential cytotoxicity against particular cancer cell line. Overall, the isolated strains depicted remarkable biological activities as well as diverse chemical profiling which urges towards continuous research to obtain biologically active compounds, extraction of pure compounds from crude extracts and exploration of naïve sites for rare actinobacterial species isolation. Moreover, the

isolated strains could be used in advance studies to isolate and identify the compounds exhibiting activity against MDR bacteria.

Author's contribution

MN performed research and wrote the first draft of the paper; MS, SN, SN contributed to soil sampling; SN contributed to strain isolation and purification; MS contributed in fermentation, extract preparation and chromatography; MS and IS reviewed the first draft of the paper; SN contributed in gene sequence analysis; IS contributed to experimental design and supervised the project. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by the University of the Punjab's annual research grant, which is duly acknowledged.

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