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Taxonomic Diversity, Antimicrobial Potential and Metabolite Profiling of Aquatic Actinobacteria from Kallar Kahar Lake, Pakistan

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Authors' Contribution
SA performed the experiments, collected and analyzed the data and drafted the manuscript. WTS planned the experiments and contributed to analytical support. IS conceived, designed and supervised the study and finalized the draft.

Key words
Aquatic actinobacteria, Taxonomic analysis, Actinobacterial diversity, Kallar Kahar lake, Antimicrobial potential, Ultra performance liquid chomatographic analysis

ABSTRACT

As the discovery of novel antibiotics and other biotechnologically useful compounds has been exhausted so molecular characterization of actinomycetes from less explored habitats may be an alternate solution to this challenge. The aim of this study was to explore the actinobacterial diversity of the saline lake, situated in the salt range, Punjab, Pakistan and to screen them for antagonistic activities against multi drug resistant pathogens and to characterize the bioactive metabolites produced by them. A total of seventy strains of actinomycetes were isolated from lake samples, out of them only forty bioactive strains were selected for further characterization on the basis of their preliminary antimicrobial activities. The isolated actinobacterial strains showed an optimum growth at alkaline pH (9.0-9.5). The taxonomic analysis revealed that these isolates were closely affiliated with the genus *Streptomyces* (62.5%), *Lentzae* (6.25%), *Pseudonocardia* (6.25%), *Lechevalieria* (6.25%) and *Nocardiopsis* (12.5%). Most of the isolated strains exhibited strong inhibition against a broad range of pathogenic test organisms. The partial purification and metabolite profiling using ultra performance liquid chomatography and mass spectrometric (UPLC-MS) analysis suggested the presence of a variety of bioactive compounds including totarol, kifunensine, talaromycin and clausine F. These results indicated that Kallar Kahar Lake harbors a significant diversity of actinobacterial strains which are able to produce a diverse range of biotechnologically and pharmaceutically potent compounds. Further studies related to the structure elucidation of secondary metabolites can lead to the discovery of new and useful drug candidates.

INTRODUCTION

In recent era, the search for discovery of new drug candidates from natural sources is driven by the force to combat an incessant increase in the emergence of infectious diseases caused by multiple drug resistant super bugs. Microbial natural products are considered to be the most significant origin of novel secondary metabolites being extensively used in medicine, food industry and agriculture (Singh *et al*., 2014). Among the producers of commercially important metabolites, actinomycetes has marked an era as evident by the production of two third of the worlds antibiotics where *Streptomyces*, the most frequently isolated genus, contributed for more than 75% of these antibiotics (Bindu *et al*., 2017).

Actinomycetes are ubiquitous, spore forming filamentous bacteria inhabiting different ecosystems such as soil, plants, lakes and forests *etc*. (Passari *et al*., 2017). Various researchers have isolated millions of actinomycetes strains from terrestrial environment (Zothanpuia *et al*., 2017). Consequently, the probability of finding the new strains of actinomycetes and discovery of novel compounds has reduced due to over-exploitation of terrestrial reserves. One approach to deal with this situation is to isolate the novel streptomycetes and rare genera of actinomycetes found in unexplored habitat and screen them for the discovery of unique chemical moieties (Wang *et al*., 2017). There are several reports on the isolation of non-streptomycetes genera such as *Pseudonocardia*, *Actinomadura*, *Micromonospora* and *Nocardiopsis* from different habitats including marine sediments, forests, lakes and rivers (Tiwari and Gupta, 2014; Hozzein *et al*., 2011) at international level. In our previous reports (Aslam and Sajid, 2016), the isolation and antimicrobial activity of different *Streptomyces* strains from the Kallar Kahar Lake, Pakistan was reported however, this study

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reports the isolation of rare actinobacterial genera (along with *Streptomyces* sp.) and in-depth chemical profiling of the bioactive extracts using ultra performance liquid chomatography and mass spectrometric (UPLC-MS) analysis.

The microbial communities residing in extreme environments such as high salinity or high pH, still exhibit an outstanding diversity of natural metabolites due to the presence of large genome along with extra-transcription factors, pertaining to respond under stress conditions (Shuvankar *et al*., 2015). In addition to this, wetlands are also regarded as an important ecosystem with respect to the discovery of biotechnologically important compounds as actinobacterial diversity of these ecosystems remain unexplored yet. Some researchers reported the biodiversity of actinomycetes from marine sediments, estuaries, rivers and lakes (Wang *et al*., 2011; Guan *et al*., 2011). Despite these findings little is known about actinobacterial diversity and secondary metabolite profiling in extreme environments particularly aquatic ecosystems. Previous studies have also reported some difficulties in the extraction and identification of secondary metabolites from actinomycetes due to which a huge pool of natural metabolites remain to be undiscovered (Rong and Huang, 2012). Many scientists are now exerting their endeavors to utilize the rapid and more accurate analytical techniques including UPLC-MS analysis for the profiling of secondary metabolites produced in minute concentrations (Kim *et al*., 2012).

The Kallar Kahar Lake is a saline water lake, located in the salt range of Province Punjab, Pakistan. Raza *et al*. (2015) reported the highly alkaline (pH 9.0) nature of water in this lake, with high electrical conductivity. It was found that the concentration of sodium increases in winter season and decreases in summer due to the diluting effect of water and the maximum concentration of sodium observed with an average of 650.14 ppm (Raza *et al*., 2007). In view of the above facts, the current study was carried out to explore the taxonomic diversity of freshly isolated rare actinobacteria from Kallar Kahar Lake, and a comprehensive profiling of the secondary metabolites have been done using advanced analytical techniques to predict the nature of the bioactive compounds produced by them.

MATERIALS AND METHODS

Sample collection and selective isolation of actinobacteria

The water and mud samples were taken from eight different locations of Kallar Kahar Lake at a depth of 10-15cm and were kept them in refrigerator at 4°C until further processing. The collected samples were pretreated under wet heat at 70°C for 20 min, dry heat at 100°C for 1 h, air dry for 24 h, 1.5% phenol treatment, $CaCO_3$ treatment to minimize the growth of Gram-negative bacteria and fungal contamination (Kitouni *et al*., 2005). The samples were serially diluted and spreaded on different media plates *i.e.,* starch casein agar, glycerol casein KNO_3 agar, glycerol asparagines agar, humic acid vitamin agar and actinomycetes isolation agar. For the isolation of extremophilic actinobacteria, pH of the isolation media was maintained in the range of 7-10. The isolation plates were kept at 28°C for 10-12 days and purified isolates were transferred on glucose, yeast extract, malt extract (GYM) agar.

Taxonomic analysis of the actinobacterial strains

The purified actinobacterial strains were identified up to the genus level according to methods described by Bensulatna *et al*. (2010). The colony characteristics of the purified strains were observed using light microscope including color of substrate and aerial mycelium, diffused pigments and colony size. The physiological features such as growth pattern on different pH ranges (*i.e.,* 7-11) and temperature range (*i.e.,* 28°C, 37°C, 55°C), tolerance to various NaCl concentrations (*i.e.,* 4, 7, 11, 13 gm/100ml) were identified. Biochemical characteristics such as utilization of carbohydrate source, melanin production, urea hydrolysis and utilization of citrate and oxalate were also observed.

For genetic characterization, the genomic DNA of the selected strains was extracted using FavorPrep™ DNA isolation kit and 1.5kb fragment of 16S rDNA was amplified along with universal primers using the protocol developed by Aslam and Sajid (2016). The amplified product was analyzed using 1% agarose gel and FavorPrep™ PCR clean up kit was used to remove the extra dNTPs. The PCR conditions consisted of 94°C for 10 min and 35 cycles of 94°C for 1 min; 50°C for 1 min, 72°C for 1 min and followed by 72°C for 7 min. The 1.5kb PCR product was sequenced on an automated sequencer and the nucleotide sequence data was BLAST analyzed. Finally, the accession numbers were obtained by submitting the nucleotide sequences in NCBI Genbank. The multiple sequence alignment was performed to make a comparison between unknown nucleotide sequences and the reference sequences downloaded from Genbank database using CLUSTAL W. The phylogenetic analysis was performed by constructing a neighbor joining tree with bootstrap value of 1000 replicates using MEGA-6.

Small scale cultivation and preparation of methanolic extracts

The isolated strains were inoculated into the flasks

containing 500ml of glucose yeast extract broth and were kept at incubation on an orbital shaker at 28°C. After 5-7 days, the broth cultures were sonicated for 15 min and ethyl acetate solvent extraction was performed to separate the extracellular metabolites from cellular mass. The dried crude extract was obtained by passing the organic layer though rotary evaporator.

Screening for antimicrobial potential of aquatic actinobacteria

The antimicrobial potential of aquatic actinobacteria was determined by using the agar well diffusion assay following the methods described by Aslam and Sajid (2016). The pathogenic test organisms used for this assay were methicillin resistant *S. aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Klebsiella pneumoniae*, *E. coli*, *Chlorella vulgaris*, *Aspergillus fumigatus* and *Candida albicans*. On the basis of nutritional requirement of test organisms, different culture media *e.g.,* Luria Bertani (LB) agar for antibacterial activity, potato dextrose agar for antifungal activity, Bold basal medium for antialgal activity were used.

Metabolite profiling of the actinobacterial strains

For TLC analysis, methanolic extracts were spotted on TLC plates and then these plates were developed in TLC tank containing 10% methanol/CH_2Cl_2. After developing the plates, different color of bands were observed and marked under UV at a wavelength of 254nm and 366 nm. The anisaldehyde/H_2SO_4 reagent was used to stain the TLC plates. In case of HPLC-UV analysis, HPLC-UV (Sykum-3210) along with C18 column was used and a mixture of methanol and water in 9:1 ratio was run as a mobile phase with a flow rate of 1ml/min. A 20µl of each crude extract was loaded though microsyringe and was run for 15 min at a wavelength of 254nm. Sample peaks were compared with the reference peaks of known secondary metabolites.

Lab scale fermentation of the selected actinobacterial strains

The lab scale fermentation was performed to get an adequate amount of the purified metabolites. The cell free extracts were prepared by inoculating the selected strains in 10L broth dispensed in twenty flasks (1 liter size), each containing 500ml of glucose, yeast extract, malt extract broth and were incubated for 7 days at 28°C on an orbital shaker (M-6145, Thomas Scientific). The liquid portion was separated from mycelium by filteration and Amberlite® XAD®-2 beads were added to it and the mixture was shaked overnight. The active metabolites were recovered from XAD-2 resin by methanolic extraction and the solvent was recycled on a rotary evaporator. The crude antibiotic extract was also recovered from mycelial mass by methanolic extraction.

Purification and partial identification of the secondary metabolites

The crude extract obtained after lab scale fermentation was loaded on the silica gel column. The packed column was run with a stepwise gradient of dichloromethane/methanol in order to get the separated compounds in different fractions. The fractions containing a mixture of two or three components were further purified using preparative TLC and sephadex column. Finally, the isolated compounds were detected though ultra performance liquid chomatography and mass spectrometric (UPLC-MS) analysis. The spectral data was compared with the reference data available in Metlin secondary metabolites database (https://metlin.scripps.edu) to identify the isolated compounds.

RESULTS

Taxonomic characteristics of the aquatic actinobacterial strains

A total of seventy actinobacterial strains were recovered from water and mud samples of Kallar Kahar Lake. Most of the actinobacteria produced powdery, compact and dry colonies in different sizes ranges (1.5-3.0 mm). All of the isolated strains were observed to have different colors of aerial and substrate mycelia. In some isolates the color of aerial mycelium was white as in case of SAK-3, SAK-9, SAK-13, and SAK-17 while in others it was grey such as SAK-2, SAK-5, SAK-7, SAK-10, maroon in SAK-1 and pink in SAK-18 (Fig. 1). In case of physiological properties, all of the isolates showed optimum growth in the pH range of 9.0 to 9.5. However, some of the isolates including SAK-2, SAK-3, SAK-9, SAK-13 and SAK-15 also exhibited good growth at pH 10. In case of NaCl tolerance, 85% of the isolates were found to exhibit good growth at 4% NaCl concentration. Some of the strains exhibited little growth at 7% NaCl while no growth was observed at higher concentrations of NaCl (11%-13%). Majority of the strains (82%) showed production of melanin pigment. Considering the utilization of sugars as a carbon source, it was observed that most of actinomycetes strains showed good growth on glucose, sucrose, fructose and ribose. On the other hand, inositol, sorbitol and raffinose were not as efficiently utilized by the isolated strains. Most of the strains were able to produce urease enzyme except SAK-4, SAK-6, SAK-14, SAK-18, SAK-21, SAK-33 and SAK-37. Some of the isolates were also found to utilize the citrate and oxalate as shown in Table I.

Table I. Physiochemical characteristics of the actinobacterial strains from Kallar Kahar Lake.

Actinomycete strains	Melanin production	Utilization of sugars as a carbon source							Hydrolysis of urea	Citrate utilization	Oxalate utilization
		Gluc	Sucr	Fruc	Ribos	Inosit	Sorb	Raffin			
SAK-1	+++	++	+++	+	+++	++	+	-	++	+++	+++
SAK-2	+++	++	++	++	+	++	++	-	++	-	-
SAK-3	+++	+	++	++	+	-	-	-	+++	+++	+++
SAK-4	++	+++	+	+	++	++	++	-	-	-	-
SAK-5	++	++	++	++	++	++	-	++	++	-	++
SAK-6	++	++	+++	++	+++	-	++	-	-	-	-
SAK-7	-	++	+	+	+	++	++	-	++	++	+++
SAK-8	+++	++	+++	++	++	-	-	-	+++	-	-
SAK-9	++	++	+	+	+++	++	++	-	+++	-	+
SAK-10	+++	++	+	++	++	-	-	-	++	-	-
SAK-11	++	++	+	++	+	-	-	++	++	-	-
SAK-12	-	+++	++	-	++	++	++	-	+++	-	-
SAK-13	+++	++	+++	+	++	-	-	++	+	+++	+++
SAK-14	++	+	+++	+++	++	-	-	-	-	++	+++
SAK-15	++	+	+	+++	+	++	++	+	+	-	-
SAK-16	+++	+	+	++	++	-	-	-	+	-	-
SAK-17	-	+++	+++	-	++	++	+	++	++	+++	+++
SAK-18	+	+	+	++	+	-	+	+	-	-	+
SAK-19	++	+++	+	++	++	-	-	-	++	-	++
SAK-20	+	+	+++	+	++	-	++	-	+	++	+++

Key: Gluc, glucose; Sucr, sucrose; Fruc, fructose; Ribos, ribose; Inosit, inositol; Sorb, sorbitol; Raffin, raffinose; (+++), strong positive result; (++), moderate positive result; (+), slightly positive result; (-), negative result.

Table II. Genbank accession numbers of the aquatic actinobacterial strains and their similarity indices.

Actinomycete strains	No. of nucleotides sequenced (base pairs)	Genbank accession numbers	Similarity with closely related microbes	Percentage similarity
SAK-7	990 bp	MK559630	*Streptomyces alboflavus* (CP021748)	99.90%
SAK-8	911 bp	MK466357	*Streptomyces pseudogriseolus* (KT719755)	99.34%
SAK-9	862 bp	MK559688	*Streptomyces thermoviolaceus* (KU214677)	99.19%
SAK-12	889 bp	MK466356	*Lentzea guizhouensis* (NR_144590)	98.76%
SAK-14	405 bp	MK559737	*Streptomyces variabilis* (JN627185)	99.76%
SAK-17	938 bp	MK521048	*Lechevalieria aerocolonigenes* (EU570358)	98.51%
SAK-19	1112 bp	MK530149	*Streptomyces iakyrus* (NR_041231)	99.64%
SAK-20	834 bp	MK559662	*Streptomyces californicus* (JQ068811)	99.88%
SAK-22	1005 bp	MK526906	*Streptomyces collinus* (MK526906)	98.80%
SAK-25	892 bp	MK559612	*Nocardiopsis dassonvillei* (MH843133)	99.55%
SAK-28	854 bp	MK526909	*Psedonocardiatropica* (MK318577)	99.65%
SAK-30	418 bp	MK530152	*Streptomyces violaceoruber* (MG752946)	99.28%
SAK-32	701 bp	MK530137	*Streptomyces fradiae* (MK715477)	99.57%
SAK-34	927 bp	MK530162	*Actinobacteria bacterium* (MG263520)	99.25%
SAK-37	602 bp	MK530147	*Streptomyces mutabilis* (LM644086)	100%
SAK-39	790 bp	MK559613	*Nocardiopsis aegyptia* (NR_025589)	99.75%

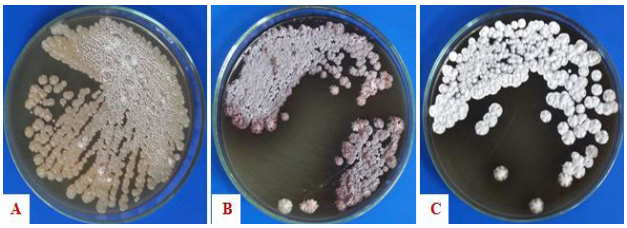

Fig. 1. Pure cultures of aquatic actinobacterial strains on glycerol, yeat extract, malt extract (GYM) agar. (A=SAk-13, B=SAK-1, C=SAK-21).

The genetic analysis of the isolated strains using ribosomal RNA gene sequencing and BLAST program indicated the presence of strains belonging to the different genera of actinomycetes (Table II). Among the selected isolates, 10 strains exhibited 98-100% genetic similarity with the different type strains of genus *Streptomyces* already reported in Genbank. Some of the isolates belonged to actinobacterial genera including SAK-12 which exhibited 98.76% similarity with *Lentzea guizhouensis*, SAK-17 showed 98.51% similarity with *Lechevalieria aerocolonigenes* and SAK-28 showed 99.65% similarity with *Pseudonocardia tropica*. On the other hand, two strains were related to the genus *Nocardiopsis* such as SAK-25 and SAK-39. A neighbor joining tree of 16 selected strains is shown in Figure 2 where the associated taxa clustered together in the bootstrap test. The phylogenetic tree indicated that all of the isolated actinobacterial strains were sharing common ancestor and having close taxonomic relationships with each other.

Antimicrobial potential of the selected actinobacterial strains

Majority of the isolated actinobacteria exhibited promising antimicrobial activity against a diverse collection of pathogens. The strains SAK-9, SAK-12, SAK-17, SAK-25, SAK-28 and SAK-39 were found to be the most active strains. In case of methicillin resistant *Staphylococcus aureus*, the most promising activity was exhibited by SAK-9 (27mm), SAK- 12 (26mm), SAK-15 (22mm) and SAK-39 (23mm). The extracts of strains SAK-5, SAK-12, SAK-17, SAK-27, SAK-28, SAK-37 and SAK-39 exhibited remarkable activity against *Acinetobacter baumanni*. For *Pseudomonas aeruginosa*, the maximum inhibitory zones were exhibited by SAK-15 (18mm), SAK-19 (18mm), SAK-9 (19mm) and SAK-25 (20mm). In case of *Klebsiella pneumoniae*, the most promising antimicrobial potential was exhibited by SAK-15, SAK-16, SAK-27, and SAK-28. While the isolates SAK-8, SAK-17, SAK-19, SAK-25 and SAK-27 also exhibited considerable antibacterial potential for E. coli. For *Salmonella typhi*, the most striking zones of inhibition were produced by SAK-7, SAK-12, SAK-15, SAK-17 and SAK-39. On the other hand, some of the isolates including SAK-7, SAK-9, SAK-15 and SAK-25 also exhibited strong antialgal activity against *Chlorella vulgaris*. In case of antifungal activity, the most interesting clearance zones were produced by strains SAK-7, SAK-9, SAK-16, SAK-25, SAK-35 and SAK-39 against *Candida albicans* and *Aspergillus fumigatus* (Table III).

Secondary metabolites profile of the methanolic extracts of aquatic actinobacteria

An impressive chemical diversity of metabolites was observed when crude extracts were analyzed by thin layer chomatography and HPLC-UV. The developed TLC plates showed various intense bands under long (366nm)

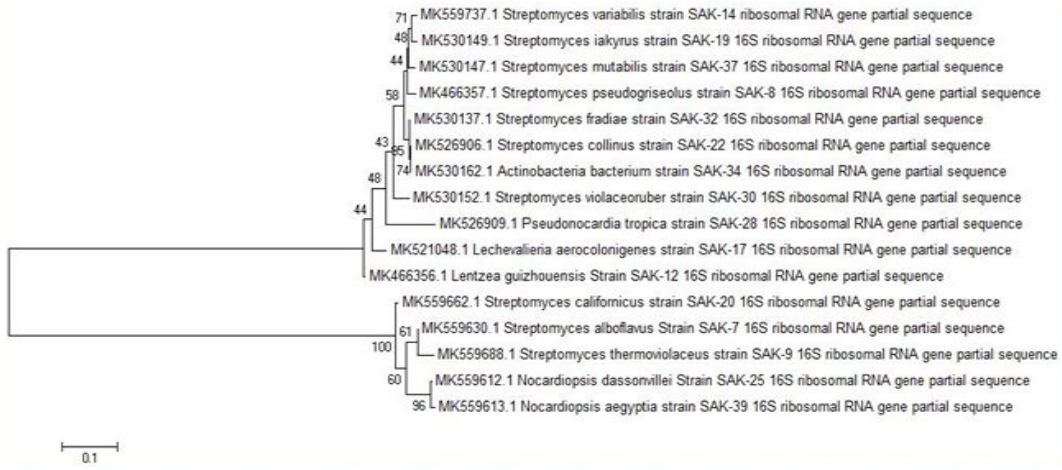

Fig. 2. Phylogenetic relationship of the aquatic actinobacteria with closely related taxa based on 16S rRNA gene sequence where evolutionary history was inferred using the neighbor joining method.

Table III. Antimicrobial profile of actinobacterial strains active against pathogenic organisms.

Actino-bacterial strains	Antimicrobial activity against multi drug resistant pathogens (zones of inhibition in mm)								
	M	A	P	KP	E	ST	CV	CA	AF
SAK-1	18	14	13	16	19	13	12	14	14
SAK-2	15	13	12	14	15	14	17	19	14
SAK-3	17	13	-	-	16	14	13	12	13
SAK-4	14	12	-	13	15	-	13	-	-
SAK-5	16	18	15	14	16	13	15	12	19
SAK-6	12	14	13	-	12	-	-	12	-
SAK-7	23	13	17	15	13	17	19	20	22
SAK-8	18	14	14	13	21	14	19	15	12
SAK-9	27	16	19	14	21	16	20	17	19
SAK-10	17	13	14	-	19	14	15	13	-
SAK-12	26	19	14	17	18	21	18	15	18
SAK-15	22	14	18	18	16	19	19	-	16
SAK-16	19	13	14	23	15	13	17	19	16
SAK-17	21	17	17	-	21	18	-	-	16
SAK-19	17	-	18	15	19	-	15	13	13
SAK-25	22	17	20	-	20	16	19	17	19
SAK-27	17	19	15	19	22	14	18	13	18
SAK-28	26	18	-	21	13	-	13	23	14
SAK-35	19	15	13	-	16	15	15	18	17
SAK-37	19	21	20	13	13	-	-	14	-
SAK-39	23	24	14	17	19	21	-	20	17

Key: M, *Methicillin resistant Staph aureus*; A, *Acinetobacter baumannii*; P, *Pseudomonas aeruginosa*; KP, *Klebsiella pneumoniae*; E, *E. coli*; ST, *Salmonella typhi;* CV, *Chlorella vulgaris;* CA, *Candida albicans;* AF, *Aspergillus fumigatus.*

and short (254nm) UV. Some of the isolates produced different colored bands in a unique pattern after treatment with anisaldehyde/H_2SO_4 reagent. The isolate SAK-8 produced purple bands, SAK-12 gave maroon and purple bands, SAK-27 formed brown, maroon bands and SAK-34 produced light green and yellow bands (Fig. 3). In case of HPLC-UV analysis, crude extract of each isolate showed multiple number of peaks at different retention times (t_R) (Fig. 4). The chomatogram of SAK-25 indicated thee major peaks at t_R 2.264, 3.004, and 3.340 min with peak area of 1666.566, 2244.050 and 9310.692 mV.s respectively. The HPLC-UV chomatogram of SAK-9 had five major peaks, among which the most prominent peak with a peak area of 4066.946 mV.s appeared at 3.624 min. In case of SAK-28 and SAK-32, chomatograms demonstrated five and four major peaks under different peak areas respectively. These observations indicated the presence of a diverse range of metabolites with different concentrations in the extracts.

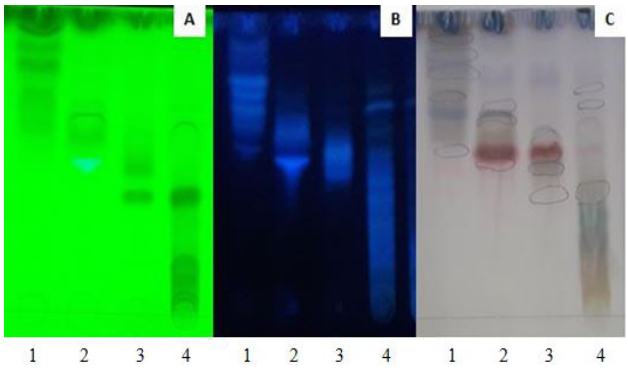

Fig. 3. Metabolic fingerprints of actinobacterial strains on TLC plates (**A**) under UV at 254 nm (**B**) under UV at 366nm (**C**) after treatment with anisaldehyde/H_2SO_4.

Fermentation, purification and partial identification of the bioactive metabolites

In order to get the sufficient quantity of purified compounds, the most active actinobacterial strains SAK-9 and SAK-12 were cultivated up to 10 liters and the crude extract from each of the strain was passed though different column chomatographic separations. In case of strain SAK-9, thee partially purified fractions were obtained; those were further subjected to separation on TLC plates and sephadex column. Later, ultra performance liquid chomatography mass spectrometric analysis (UPLC-MS) of the partially purified components confirmed a remarkable diversity of the metabolites (Table IV, Fig. 5). In fraction-1, a major peak appeared at retention time (t_R) 7.58 min with a molecular mass of 286.23 Da which showed closest match with Totarol and Cyanthiwigin A in Metlin database. In case of fraction-2, four prominent peaks appeared at t_R 4.48, 4.71, 4.83 and 6.57 min suggesting the presence of Davidigenin, Calophyllin B, (±)-Tembamide and (E, E)-Boviquinone 3 respectively (Fig. 5A). The total ion chomatogram of fraction-3 showed thee major peaks at t_R of 4.31 min and 5.06 min for which the suggested compounds were reported to be Kifunensine, Talaromycin A and B respectively.

The ultra performance liquid chomatography with mass spectrometric (UPLC-MS) analysis of the partially purified components from SAK-12 exhibited interesting profile of the chemical metabolites as shown in Table IV and Figure 5B. In case of fraction-1, two most interesting peaks appeared at t_R 6.21 and 7.53 min for which the most probable compounds were suggested as Songorine (357.23 Da) and Cularicine (311.12 Da). The fraction-2 demonstrated a peak at t_R 6.20 min with a molecular mass of 336.10 Da suggesting the presence of Isoderrone. The chomatogram of fraction-3 displayed various significant peaks at t_R 4.17, 5.15, 5.87, 6.35 and 7.26 min indicating

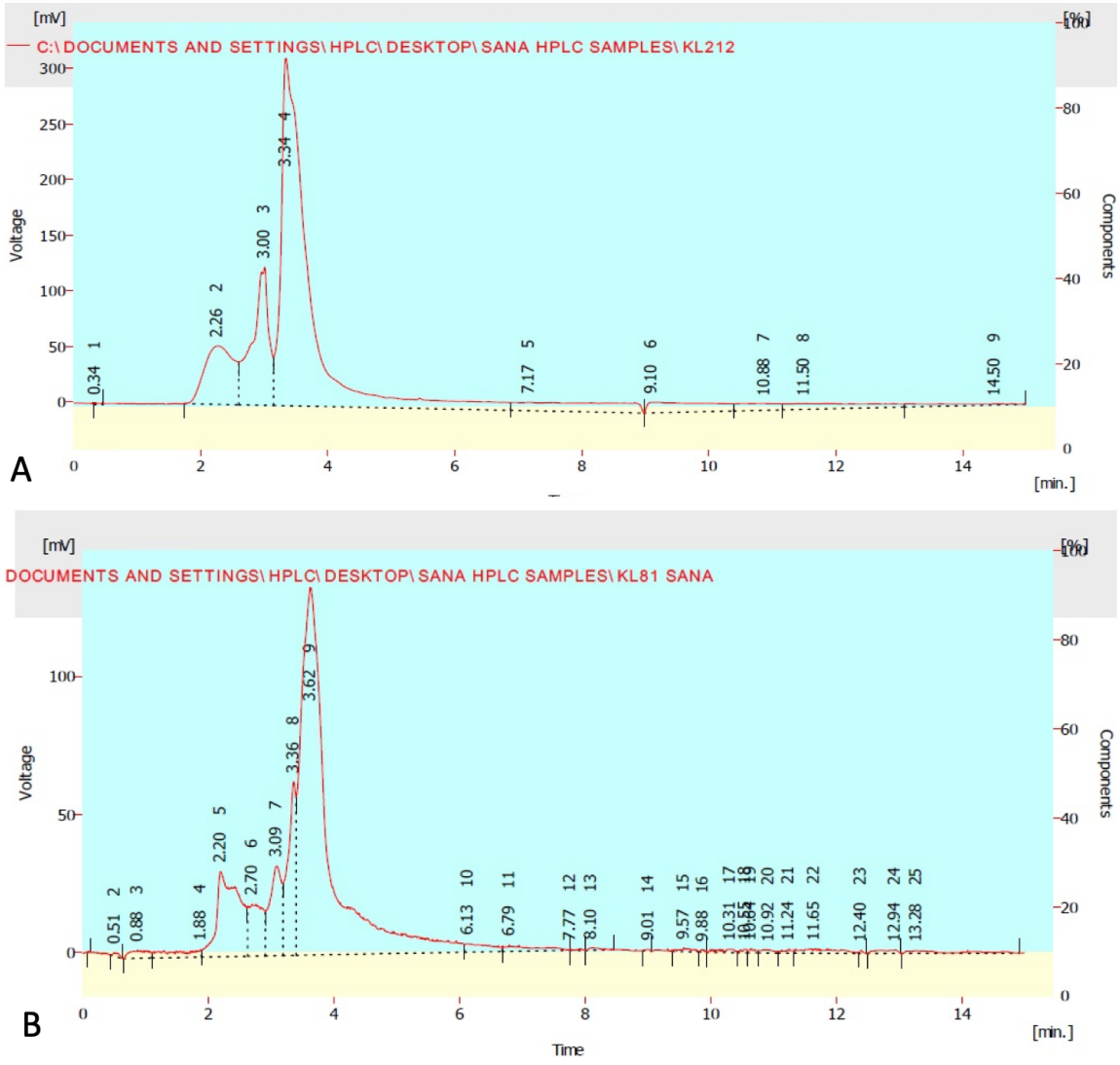

Fig. 4. HPLC-UV chromatograms of aquatic actinobacterial strains SAK-25 (A) and SAK-9 (B) showing three and five prominent peaks at different retention times respectively.

the presence of compounds with different molecular masses such as Kumujancine, Serratine, Clausine F, Boldine and Horsfiline respectively. The fraction-4 also exhibited a unique peak at t_R 7.84 min, with a molecular mass of 288.244 Da for which the closest compounds were found to be Dictyolene, Pachydictyol A and Sarcophytol A.

DISCUSSION

The rapid development of antibiotic resistance among pathogens underlines the need to search for new antibiotics. Actinomycetes have been recognized as priceless producers of a wide diversity of bioactive compounds many of which possess useful applications in medicine and industry (Barka *et al*., 2016). The development of unique antibiotics requires the screening and identification of new strains of streptomycetes and rare genera of actinomycetes from unexplored habitats. Some researchers reported the isolation and screening of rare actinobacteria from different lakes (Jose and Jebakumar, 2013; Benhadj *et al*., 2018), however there are no comprehensive reports on the isolation of rare genera of actinomycetes from the lakes of Pakistan. In our previous studies (Aslam and Sajid, 2016), the isolation and antimicrobial potential of only *Streptomyces* strains from Kallar Kahar Lake (Pakistan) was reported. In Pakistan, the actinomycetes flora of lakes is almost uninvestigated with reference to biodiversity

and in-depth screening of biotechnologically important metabolites so this study enabled us to isolate the diverse actinobacterial strains from lakes, and to screen them for various bioactive compounds as a means of assessing their bio-discovery potential.

In the results reported here, seventy actinobacterial strains were isolated from Kallar Kahar Lake and were preliminary screened against test pathogens where forty strains were found to be the most competent. The selected isolates were characterized by morphological, physiological, and biochemical analysis which suggested the presence of strains belonging to different genera of actinomycetes (Table I, Fig. 1). The 16S rRNA gene sequencing and phylogenetic analysis further confirmed the presence of diverse and rare actinobacterial genera and species in Kallar Kahar Lake where most frequently isolated strains were belonged to the genus *Streptomyces* however other species from rare genera including *Pseudonocardia*, *Lentzae*, *Lechevalieria* and *Nocardiopsis* were also found (Table II, Fig. 2). The predominance of streptomycetes strains has also been observed in other saline environments (Satheeja and Jebakumar, 2011).

In antimicrobial activity screening, all selected isolates exhibited remarkable inhibitory activity against methicillin resistant *S. aureus*, 85% of isolates showed activity against *Acinetobacter baumannii*, 60% against *Pseudomonas aeruginosa*, 87.5% against *E. coli*, 62.5% *Salmonella typhi*, 80% against *Candida albicans* and 72.5% against *Chlorella vulgaris*. These findings were in accordance to the observations of Zothanpuia *et al*. (2017).

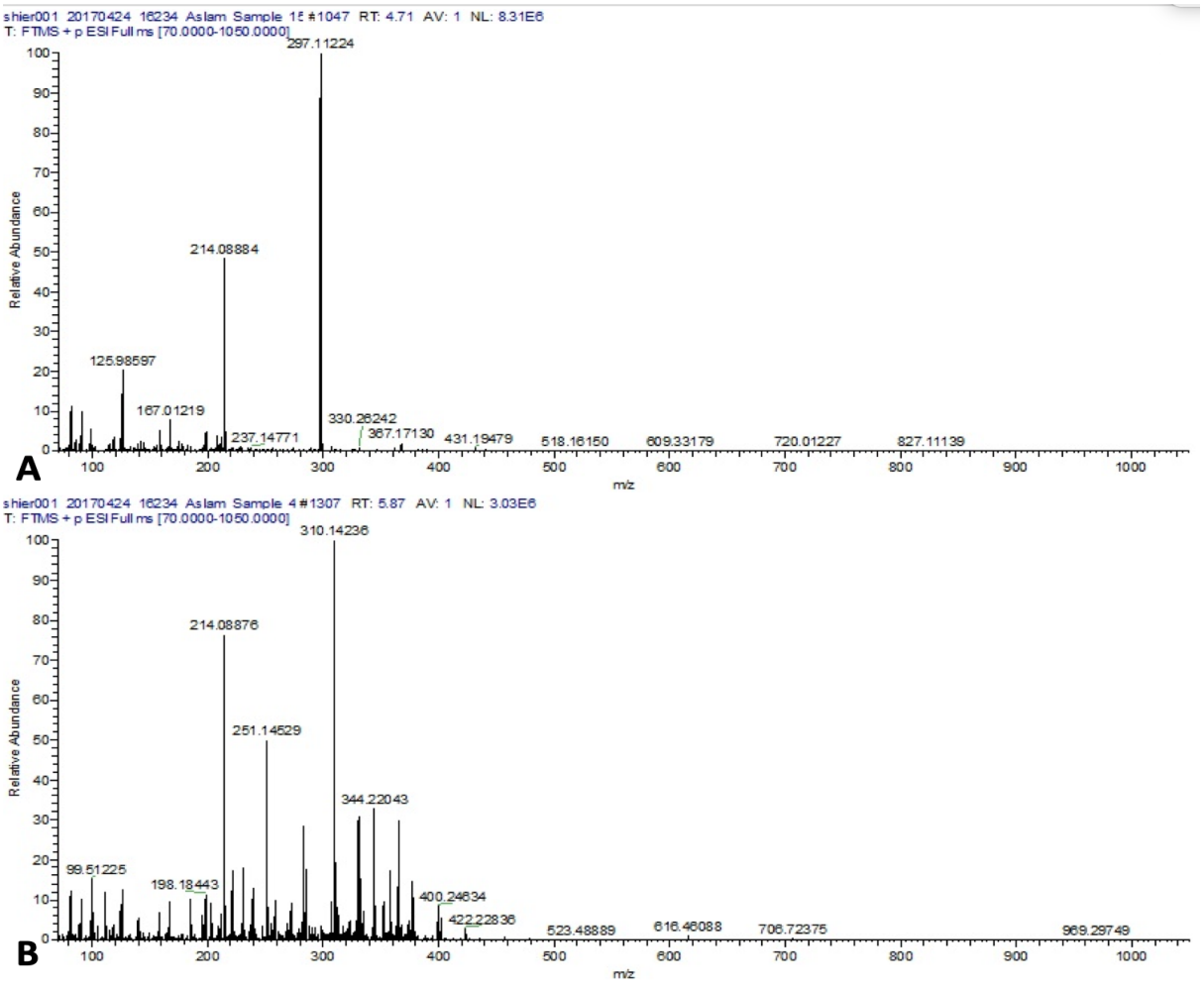

Fig. 5. UPLC-MS spectrum of different fractions obtained from chromatographic separation where (A) showed a total ion chromatogram of fraction-2 (SAK-9) at retention time of 4.71 min and (B) chromatogram of fraction-3 (SAK-12) at retention time of 5.87 min.

Table IV. UPLC-MS analysis of purified fractions obtained from selected actinobacterial strains and suggested metabolites in Metlin database.

Purified fractions containing different compounds	Retention time t_r (min)	ES1-MS: (m/z)	Molecular weights (Daltons)	Suggested metabolites from Metlin database	Antibacterial activity of fractions
SAK-9 (Fraction-1)	7.58	287.23$[M+H]^+$	286.23	Totarol, Cyanthiwigin A	22mm inhibition zone against MRSA, 19mm inhibition zone against *E.coli*, 21mm clearance zone against *Salmonella typhi*
SAK-9 (Fraction-2)	4.48	259.09 $[M+H]^+$	258.09	Davidigenin, Xanthoxyletin	24mm inhibition zone against MRSA, 17mm inhibition zone against *E.coli*, 17mm clearance zone against *Salmonella typhi*
	4.71	297.11$[M+H]^+$	296.11	Calophyllin B	
	4.83	294.10 $[M+Na]^+$	271.10	(±)-Tembamide,	
	6.57	367.19 $[M+Na]^+$	344.19	(E,E)-Boviquinone 3	
SAK-9 (Fraction-3)	4.31	233.07 $[M+H]^+$	232.07	Kifunensine	21mm inhibition zone against MRSA, 23mm inhibition zone against *E.coli*, 19mm clearance zone against *Salmonella typhi*
	5.06	253.142 $[M+Na]^+$	230.152	Talaromycin A, Talaromycin B	
SAK-12 (Fraction-1)	6.21	358.23 $[M+H]^+$	357.23	Songorine	20mm inhibition zone against MRSA, 22mm inhibition zone against *E.coli*, 20mm clearance zone against *Salmonella typhi*
	7.53	312.12 $[M+H]^+$	311.12	Cularicine	
SAK-12 (Fraction-2)	6.20	337.10 $[M+H]^+$	336.10	Isoderrone	19mm inhibition zone against MRSA, 18mm inhibition zone against *E.coli*, 19mm clearance zone against *Salmonella typhi*
SAK-12 (Fraction-3)	4.17	227.080 $[M+H]^+$	226.073	Kumujancine	22mm clearance zone against MRSA, 20mm inhibition zone against *E.coli*, 19mm clearance zone against *Salmonella typhi*
	5.15	302.173 $[M+Na]^+$	279.183	Serratine	
	5.87	310.142 $[M+H]^+$	309.135	Clausine F	
	6.35	328.156 $[M+H]^+$	327.149	Boldine	
	7.26	233.13 $[M+H]^+$	232.123	Horsfiline	
SAK-12 (Fraction-4)	7.84	289.251 $[M+H]^+$	288.244	Dictyolene, Pachydictyol A, Sarcophytol A	23mm inhibition zone against MRSA, 20mm inhibition zone against *E.coli*, 17mm clearance zone against *Salmonella typhi*

The chemical profiling using TLC and HPLC contribute to detect the metabolite fingerprints of the crude extracts. After staining with anisaldehyde/H_2SO_4 reagent, the isolated strains developed maroon, brown, purple, yellow and light green bands on TLC plates indicating the presence of different structural classes of compounds such as peptides, quinolones, allyl alcohol, amines and esters (Fig. 3). These findings were in concordance to those reported by Fatmia *et al*. (2019). HPLC-UV chomatograms also indicated the comparative metabolic diversity of the actinobacterial strains where a various number of peaks appeared at different retention times indicating the presence of a variety of compounds in different concentrations (Fig. 4). These results were comparable to those reported by Aftab *et al*. (2015).

The large scale cultivation of two strains including SAK-9 and SAK-12 resulted in the extraction and purification of different partially purified compounds. In case of SAK-9, UPLC-MS analysis of fractions and subsequent comparison of the masses in the Metlin database indicated the presence of diverse metabolites (Table IV, Fig. 5A) including Totarol, Cyanthiwigin A, Davidigenin, Xanthoxyletin, Calophyllin B, Tembamide, Boviquinone-3, Kifunesine, and Talaromycin A and B. Various researchers reported the isolation and pharmacological effects of these compounds (Hong and Ying, 2015; Klein-Junior *et al*., 2017; Tangjitjaroenkun *et al*., 2012). Tacon *et al*. (2012) reported the biological evaluation of Totarol as potential anti-malarial agent. The production of kifunesine, a potent inhibitor of glycoprotein processing mannosidase-1, has been reported from *Streptomyces sp.* by Yu *et al*. (2011). Zhang *et al*. (2019) isolated talaromycin A and B from endophytic fungus, *Talaromyces aurantiacus*.

The UPLC-MS analysis of partially purified compounds from SAK-12 and subsequent database search also suggested the presence of a variety of bioactive

compounds (Table IV, Fig. 5B) including Songorine, Isoderrone, Kumujancine, Serratine, Clausine F, Boldine, Horsfiline and Pachydictyol A. The pharmacological potential of these compounds has been reported by various researchers (Paylovska *et al*., 2015; Brein *et al*., 2006; Piereira *et al*., 2017). Songorine has found to exhibit anti-cancer and anti-inflammatory effects (Khan *et al*., 2018). Maximo *et al*. (2002) reported the antifungal activity of isoderrone against *Cladosporium cucumerinum*.

CONCLUSION

The study revealed that Kallar Kahar Lake contains an untapped diversity of rare actinobacteria with extraordinary potential to produce a variety of bioactive metabolites. The most frequently isolated strains belonged to the genus *Streptomyces,* however other rare genera such as *Pseudonocardia*, *Lentzae*, and *Nocardiopsis* were also identified. Overall, this study constitutes a comprehensive and in-depth secondary metabolite profiling of partially purified compounds produced by the isolated actinobacteria from a saline lake of Pakistan. Thus, the exploration of untapped aquatic ecosystems with extreme environment warrants considerable attention as a reservoir of novel species of actinomycetes which may unlock the avenue for the discovery of novel pharmaceutically significant products.

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Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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