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In-vitro Antitumor Activity and Metabolic Finger Printing of the Actinomycetes Isolated from Various Ecological Niches in Pakistan

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A B S T R A C T

Actinomycetes are Gram positive bacteria with high G+C content in their DNA and are capable of producing variety of secondary metabolites. Many of these metabolites possess different biological activities and have the potential to be developed as therapeutic agents. The aim of the present study was to screen actinomycetes of various ecological niches in Pakistan for cytotoxic and antitumor compounds. Several water and soil samples were collected from different areas in Pakistan and more than 500 actinomycete isolates were recovered by selective isolation techniques. Among them 120 isolates were selected on different morphological basis for identification and cytotoxicity. These isolates were identified on the basis of their Microscopy (Scanning electron microscopy), morphological, biochemical and physiological behavior's along with genetic characterization. The isolates such as MKA 17, SSA 13 and KML 2 showed highest mortality rate with having 92%, 84% and 84% dead larvae (*Artemia salina*) after 24 hours respectively. Theses potent isolates were tested against proliferative cell lines by Methyl Thiazolyl Tetrazolium (MTT) bioassay method. Isolate KML 2 and SSA 13 showed highest antitumor activity with having IC_{50} values of 12.17µg/ml and 16.4 µg/ml against HeLa cell line respectively. Chemical screening profile by TLC and robust UPLC-MS showed distinctive chemical diversity in the extracts of selected isolates. Indeed, preparative screening of isolated actinomycetes strains delivered bioactive compounds including actinomycin D and resistomycin from SSA 13 and MKA 17 respectively. The genetic characterization by 16S rRNA gene sequencing exhibited maximum genetic similarity with different species of the well-known genus of actinomycetes named as *Streptomyces*.

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Authors' Contributions
UA and IS conceived and designed the study and also analyzed the data. UA executed the experimental work and wrote the article. ZAQ helped in cell culture experiments.

Key words
Actinomycetes; Streptomyces, MTT assay, Cytotoxicity, Antitumor activity

INTRODUCTION

Among the different human diseases, cancer is still the major health threat worldwide. The most effective treatment for cancer is still the chemotherapy (Cocco *et al.*, 2003). Nature is the most promising source of novel therapeutic anti-cancer compounds and almost 74.8% of the small molecules used for the treatment of cancer are derived from natural sources (Newman and Cragg, 2012). Among nature, microbial source is the most efficient in the battle against cancer and other diseases, as it continues to add unique compounds in the panel of chemotherapeutic drugs. A large number of anticancer compounds have been isolated from the famous group of microbes, the actinomycetes, which is a continuing source of biologically diverse compounds including different antibiotics, antitumor agents and enzymes (Xu *et al.*, 2005). Actinomycetes have proved themselves for having competitive biosynthetic machinery by which they produce many chemically distinct anticancer compounds like Anthracycline, Bleomycin, Actinomycin and Mitomycin (Cragg and Newman, 2005).

As part of the endeavor for search of effective chemotherapeutic treatment, unexplored habitats are recommended for finding the new and rare species of actinomycetes strains, having the ability to produce novel anticancer compounds. Very few members of this group are reported from extreme environments such as cold forests, hypersaline, volcanic zones, extreme islands, hyperarids and glaciers. (Balagurunathan and Radhakrishnan, 2007; Hamedi *et al.*, 2013; Takahashi and Omura, 2003). There is no doubt that up to 1980s major focus for finding valuable antibiotics was limited to terrestrial actinomycetes. After that researchers move on to other habitats like ocean floor, because of thinking that terrestrial environment is now exhausted for isolating unique actinomycetes strains, which can give new valuable metabolites (Fenical and Jenson, 2006; Zaehner and Fiedler, 1995).

Different members of actinomycetes were found to be numerously present in the marine ecosystem and attest themselves as an active member of marine microbial communities (Jensen *et al.*, 2005). Vast differences in the

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environmental parameters of terrestrial and marine environment pose a massive impact on the behavior of actinomycetes isolated from respective samples (Ramesh and Mathivanan, 2009). This is also confirmed by the discovery of novel taxa of actinomycetes with having novel metabolic activities being isolated from different marine samples which includes deep sea water, sponges, water from intertidal zones and ocean sediments (Magarvey *et al*., 2004; Sun *et al*., 2010; Xiao *et al*., 2011). In principal marine actinomycetes strains which live under extreme conditions which include low temperature, high pressure, variable salinity, variable oxygen concentrations and lack of light have proven themselves for having unique metabolic machinery for the production of novel compounds with various biological activities (Bull *et al*., 2000). If we look only in the last 10 years of screening of marine environment, we come up with several novel compounds isolated from actinomycetes with having potent activity against different human cancers. These compounds includes Chinikomycins from *Streptomyces* sp. (Li *et al*., 2005), Mechercharmycins from *Thermoactinomyces* sp. (Kanoh *et al*., 2005), Salinosporamide A from *Salinispora tropica* (Jensen *et al*., 2007), Arenimycin from *Salinispora arenicola* (Asolkar *et al*., 2010), Proximicins from *Verrucosispora* sp. (Fiedler *et al*., 2008), Caboxamycin from *Streptomyces* sp. (Hohmann *et al*., 2009).

So in considering the above hypothesis that unique ecological sources, which are still unexplored will contain unique actinomycetes strains having the ability of producing potent anticancer compounds. We start isolation of actinomycetes from six different sources viz., marine, forest, terrestrial, hypersaline, extraordinarily hot niches and dry stressed areas. By following this type of strategy we not only been able to get some useful information about ecological distribution of actinomycetes across the different habitats of Pakistan, but it would also open the ways for us to find new natural compounds with a higher hit rate against different cancers. We Screen several actinomycete strains for their capacity to produce compounds active against tumor as well as normal cell lines.

MATERIALS AND METHODS

Sample collection and selective isolation of actinomycets

Soil and water samples were collected from marine (Karachi sea), forest (Naran Kaghan Forest), terrestrial (Cotton, Sugar cane and botanical fields from Rahim yar khan and Lahore), hypersaline environment (Khewra Salt mines), extraordinarily hot niches (Thal desert) and dry stressed areas (Quetta mountains) of Pakistan in sterile sampling bag. The samples were then treated using different physical and chemical methods for the enrichment of actinomycetes (Hayakawa *et al.*, 2004). Actinomycetes strains were isolated using selective isolation media Glycerol-Casein-KNO_3 agar (glycerol 10 g, KNO_3 2 g, casein 0.3 g, NaCl 2 g, K_2HPO_4 2 g, $MgSO_4.7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4.7H_2O$ 0.01 g, agar 18 g in one liter) containing nystatin (50µg/ml) as an antifungal agent (Küster and Williams, 1964). About one gram of soil was dissolved (10^{-2} to 10^{-4}) in autoclaved distilled water and serial dilutions were spread on Glycerol-Casein-KNO_3 agar plates. The spread plates were incubated for 7-21 days at 28 °C. The presumptive actinomycetes colonies were selected and purified by repeated sub-culturing on GYM agar (10 g malt extract, 5 g yeast extract, 5 g glucose, 15 g agar in one liter of tap water (Shirling and Gottlieb, 1966). Later the selected isolates were preserved in liquid nitrogen for future use.

Identification of isolated actinomycetes

The isolated actinomycetes were identified based on their morphological, biochemical, physiological and genetic characterization. The morphology, biochemical and physiological characteristics were determined according to the guidelines given in International *Streptomyces* Project (ISP), which includes colony characteristics such as size, consistency, shape, elevation, margins, color of aerial/substrate mycelium, along with production of soluble pigments into the medium, formation of melanin, utilization of different sugars as sole source of carbon, utilization of organic acids, hemolysis, utilization of oxalates (Shirling and Gottlieb, 1966; Williams *et al*., 1983).

Scanning electron microscopy (SEM)

In order to see the deep morphological pattern (substrate & aerial mycelia) of selected actinomycetes strain scanning electron microscopy (SEM) was performed following the method described by Cavaletti *et al.* (2006). The strains were grown on GYM agar, after that sharp piece of agar containing sufficient growth of strains was cut with sterile cutter. The cell mass or mycelium and spores were fixed with glutaraldehyde (2 %) and formaldehyde (5%), later by 0.1 M sodium cacodylate buffer (0.1 M cacodylate, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, 0.09 M sucrose; pH 6.9) and left at 4 °C for overnight. After overnight fixation, strains were washed with cacodylate buffer. Agar pieces containing growth were then dehydrated with in a graded acetone series (10, 30, 50, 70, 90, and 100% acetone). After acetone dehydration agar pieces were subjected to critical-point drying with liquid CO_2. Before loading the agar pieces in the scanning electron microscope (SEM) they were covered with 10 nm thick gold film by sputter coating

(Hummer-V Sputter Coater). After coating deep morphological patterns of the isolated actinomycetes strains were analyzed by using scanning electron microscope (HITACHI S-2300 SEM).

16S rRNA gene sequencing

Total genomic DNA was isolated after growing the stains in GYM broth. Genomic DNA was extracted by the phenol/chloroform method as described by Hopwood *et al.* (1985). The 1.5 kb 16S rDNA fragment was amplified using universal primers
(27F 5'AGAGTTTGATCCTGGCTCAG3' and
1522R 5'AAGGAGGTGATCCARCCGCA3')
by the polymerase chain reaction (PCR) (PeQ lab, Primus 96 advanced). Each PCR reaction vial contains approximately 300 ng genomic DNA, 2 µl of each primer having working concentration of 10 pmol and 25 µl of 2x PCR master Mix (Merck-GeNei^{TM}). The PCR conditions were adjusted as, Lid Heat 99 °C, Initial denaturation: 94°C for 5 min; Denaturation: 94 °C for 20 sec; Annealing 50°C for 20 sec; Extension; 72 °C for 2 min for 30 cycles and final extension at 72 °C for 5 min. After amplification, the reaction product was analyzed on 1% agarose gel and purified using minielute^{TM} PCR purification kit (Qiagen, USA). The whole gene product was sequenced using dye terminator chemistry on an automated sequencer (ABI-PRISM® BigDye® Terminator version 3.1 Cycle Sequencing Kit, Applied Biosystems, USA), and the sequence data was compared to the sequences already present in the database using blastn analysis at NCBI (www. nih.nlm.gov/blast.cgi). The sequences were then deposited to NCBI GenBank.

Cultivation of strains

The selected actinomycetes strains were grown in 250 ml GYM broth in 1 liter Erlenmeyer flasks (pH was adjusted to 7.8 before sterilization). The flasks were incubated at 28°C on a Rotary shaker at 100 rpm for 5-7 days. After incubation the cells were disrupted by sonication (*Iso-temp* sonicater water bath). The resulting broth containing all the intra and extracellular metabolites were mixed with ethyl acetate 1:1. The mixture was again sonicated and the organic layer of ethyl acetate was collected by using separating funnel. The organic layer was evaporated on rotary vacuum evaporator (Heidolph® 4000 efficient) and a small amount of crude extract was obtained. These crude extracts were then analyzed for cytotoxic and antitumor activities against different cell lines.

Brine shrimp microwell cytotoxicity assay

The microwell cytotoxicity assay as described by Sajid *et al.* (2009) was used to determine the nonspecific cytotoxicity of crude extracts against brine shrimp (*Artemia salina*) larvae. Dead larvae were counted (value N) before adding 20 µg of the crude extract in 5 µl of DMSO and the plate was kept at room temperature in the dark. The test was performed in triplicate; each test row was accompanied by a blind sample containing pure DMSO as negative control. Actinomycin D (10µg/ml) was used as a positive control with 100% mortality rate. In order to calculate the percentage mortality of the larvae after 24 hours, following formula was used.

$$M = [(A - B - N)/ (G - N)] \times 100$$

Where, M is percent of the dead larvae after 24 h, A is number of the dead larvae after 24 h, B is average number of the dead larvae in the blind samples after 24 h, N is number of the dead larvae before starting of the test, G is total number of larvae.

In vitro screening for antitumor activity

The antitumor assay was performed on HeLa, MDBK and Vero cell lines (ATCC) using MTT assay (Mosmann, 1983). The cells were grown in 96 well plate in Dulbecco's modified Eagle's medium (Invitrogen, NY, USA) supplemented with 10% fetal bovine serum and 1% antibiotics (streptomycin and penicillin-G) by incubating at 37 °C for 24 h in 5% CO_2 (Sanyo CO_2 incubator MCO-15AC) under humid conditions. After the formation of confluent monolayer of the actively dividing cells, trypsinization was done and cell suspension (10^5 cells/ml) was then seeded in the wells containing culture media and different concentrations of the extracts obtained from the shaking culture of isolated actinomycetes strains. The plate was then incubated at 37 °C for 48 h in 5% CO_2 environment. After incubation cellular viability for each concentration of the extracts was measured as described by Mosmann, 20 µl of MTT (5 mg/ml) were added in each well and plates were incubated again at 37 °C for 3 h in 5% CO_2 environment. After incubation the media was carefully removed and 100 µl of DMSO was added in order to solubilize the formazan crystals produced by metabolically active cells. After that optical density (O.D) of the wells were measured with microplate reader (Epoch BIOTEK®) at 570 nm with 655 nm as reference. Cell controls were maintained throughout the experiment and the assay was performed in triplicates. Calculations of IC_{50} were done through dose dependent curve. The growth inhibition rate for each dilution was calculated by the following formula:

$$\text{Inhibition rate} = \frac{\text{O.D (control well) - O.D (treated well)}}{\text{O.D (control well)}} \times 100$$

Determination of antimicrobial activity of the selected strains

Antimicrobial activity of the extracts obtained from the culture broth of isolated actinomycetes strains was determined by disc diffusion method as described by Sajid *et al.* (2009) against a set of test organisms including *Bacillus subtilus, Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), Methicillin resistant *Staphylococcus aureus (MRSA), Acinetobacter, Pseudomonas aeruginosa* and *Klebsiella pneumonia* (ATCC 706003).

Thin layer chromatography (TLC) analysis

Initial chemical screening of the extracts obtained from isolated actinomycetes was done by using the simple technique of TLC. Small droplets of crude extracts were subjected to the TLC plate (Merck-Silica with Aluminum base TLC plate's 20×20cm, 20 µm thickness with binder Polymeric fluorescent indicator) at a specific point in a superimposing fashion. In this way almost 2-5 µg of the extract was adsorbed on the surface of TLC plate. After loading of sample the TLC plate was developed by using CH_2Cl_2/5% MeOH solvent system. When the solvent front attains reasonable height the plate was removed and air dried. Fluorescence activated compounds were detected by visualizing the plate under short (254 nm) and long (366 nm) u.v. For detecting the presence of specific compounds TLC plate was sprayed with two different detection reagents including anisaldehyde/H_2SO_4 and Ehrlich reagents. The colored spots produced due the reaction between the staining reagent and the active metabolites were scanned and documented.

Ultra performance liquid chromatography™ -mass spectrometry (UPLC-MS) analysis

For UPLC-MS analysis samples were prepared carefully by dissolving the crude extracts in 500 µl methanol. Furthermore this methanolic crude mixture was 5 times diluted in 50% acetonitrile. The diluted samples were then filtered through 2 µm pore size disposable syringe filters (Sartorius Minisart® SRP-15 syringe filters) and then centrifuged at 14000 *g* in order to remove any particle. Samples were then transferred to special LC-MS glass vials which were further placed in the sample tray of UPLC-MS chromatography system (Waters® Acquity UPLC-MS System).

Analytical conditions for the UPLC-MS analysis

The analytical conditions for the UPLC-MS analysis were as follows: Waters ACQUITY UPLC H-class BEH-C18, (2.1 × 100 mm; particle size, 1.7 µm) column; Column temperature: Ambient; Gradient Elution: A is Acetonitrile, B is Acetonitrile + 0.5% acetic acid, C is MilliQ water + 0.5% acetic acid, D is MilliQ water; Gradient Profile: 0-1.5min 5% B and 95% C, 1.5-7 min is 95% B and 5% C, 7-10 min 95% B and 5% C, Before and after run column was re-equilibrate by eluting with 100% A and B; Flow rate: 0.5 ml/min; m/z monitoring range: 200-2000 m/z ratio; Wavelength monitoring range: 220-800 nm; Total run time: 10 min; Data: Continuum; Seal wash period: 5 min; Waters SQ detector mass spectrometer; Ionization mode: ESI positive; Scan Duration: 0.5sec; Capillary voltage, 3.3 kV; Cone voltage ramp: on; Source temperature: 150 °C; Desolvation temperature: 400 °C; Desolvation gas flow: 800 L/h; Cone voltage; 0V; Desolvation gas: liquid nitrogen; Mass lynx software V 4.1 (waters).

RESULTS

In our search for indigenous actinomycetes showing potent cytotoxic and antitumor activities, several water and soil samples were collected from untapped unique ecological habitats of Pakistan. Initially about 500 actinomycetes strains were isolated from the samples collected from various ecological niches in Pakistan, among them 120 isolates were selected based on different parameters *viz.,* origin, morphology, physiology, culture behavior and level of activity (crowded plate technique) (Fig. 1). In prescreening studies which includes antimicrobial activity and nonspecific cytotoxicity, 10 strains were selected with highest cytotoxicity. These promising strains with strong cytotoxicity were further investigated to explore their potential against cancerous and normal cell lines. As depicted in Table I, strains MKA 17, SSA 13 and KML 2 showed good cytotoxic activity with 92%, 84% and 84% larval mortality respectively, against *Artimia salina*. The other isolates including, LSA 14, BLH 1, KMB 1, KME 1, KMF 2, KMJ 8, SSA 17 exhibited larval mortality in the range of 70 to 80% . As shown in the results (Table I) the strain KMJ 8 exhibited antibacterial activity against all the test strains with having maximum activity against *Escherichia coli* and *Acinetobacter.* Strains MKA 17, SSA 13, SSA 17 and KMB 1 all were moderately active against maximum of three test strains. Based on the assumption that the strains with high nonspecific cytotoxicity may lead to some useful antitumor activity, we move forward for screening the strains for *in-vitro* antitumor activity through MTT assay method. The Table II shows that several strains possess impressive activities which are in accordance to guide lines set by American National Cancer Institute. In case of HeLa cell line, the strains KML 2, SSA 13 and MKA17 were found to be very active with IC_{50} values of 12.17 µg/ml, 16.40 µg/ml

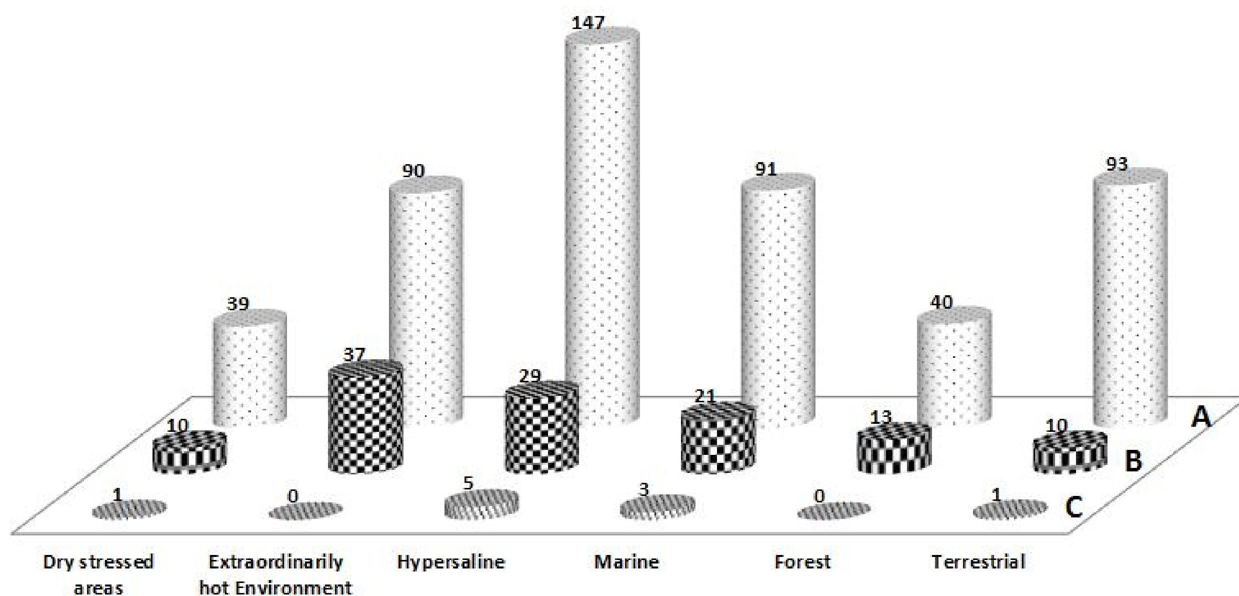

Fig. 1. Number of actinomycetes isolates associated with different habitats of Pakistan (A is Total No. of isolated actinomycetes strains from respective environment, B is number of unique actinomycetes strains from respective environment, C is No. of actinomycetes strains come under top 10 on the basis of highest percentage cytotoxicity from respective environment).

Table I.- **Antimicrobial activity and cytotoxicity of the selected *Streptomyces* strains.**

Streptomyces Strains	Antimicrobial activity test organisms zone of inhibition (mm)							Cytotoxicity test organism (% age mortality) *Artemia salina*
	B. subtilus	E.coli	S. aureus	MRSA[a]	Acinetobacter	X4[b]	k. pneumonia	
LSA 14	-	10	-	-	10	-	-	73
MKA 17	5	-	8	-	-	-	19	92
SSA 13	10	12	-	-	18	-	-	84
SSA 17	6	10	14	-	-	-	-	72
KMB 1	10	-	-	-	10	-	10	73
KME 1	20	-	-	-	13	-	-	75
KMF 2	-	14	-	-	-	-	17	71
KML 2	-	-	-	-	-	-	-	84
KMJ 8	10	18	8	11	15	11	13	70
BLH 1	-		9	-	12	-	-	71

[a]Means Methicillin resistant *Staphylococcus aureus*; [b]means *Pseudomonas aeruginosa*

and 36.00 μg/ml respectively (Table II). Others strains including KMB 1, KMF 2 and KMJ 8 were found to be less active with having IC_{50} values greater than 100 μg/ml for all of them. Strains KME 1, BLH 1, LSA 14 and SSA 17 showed moderate activity against HeLa cells. Activity against normal cells including Vero and MDBK cells were also checked and found that MKA 17, KML 2 and SSA13 all three showed impressive IC_{50} values (>100 μg/ml, 56.0 μg/ml and 64.8 μg/ml respectively) against Vero cells and IC_{50} values are greater than 100 μg/ml for the strains KMF 2, KME 1 and LSA 14 against MDBK cells (Table II).

The morphological, biochemical and physiological characterization of the selected strains showed various distinctive actinomycetes like characteristics (Table III and IV). One out of 10 strains named SSA 17 produce melanin, while six out of ten strains named MKA 17, SSA 13, KML 2, KMB 1, KME 1 and SSA 17 showed

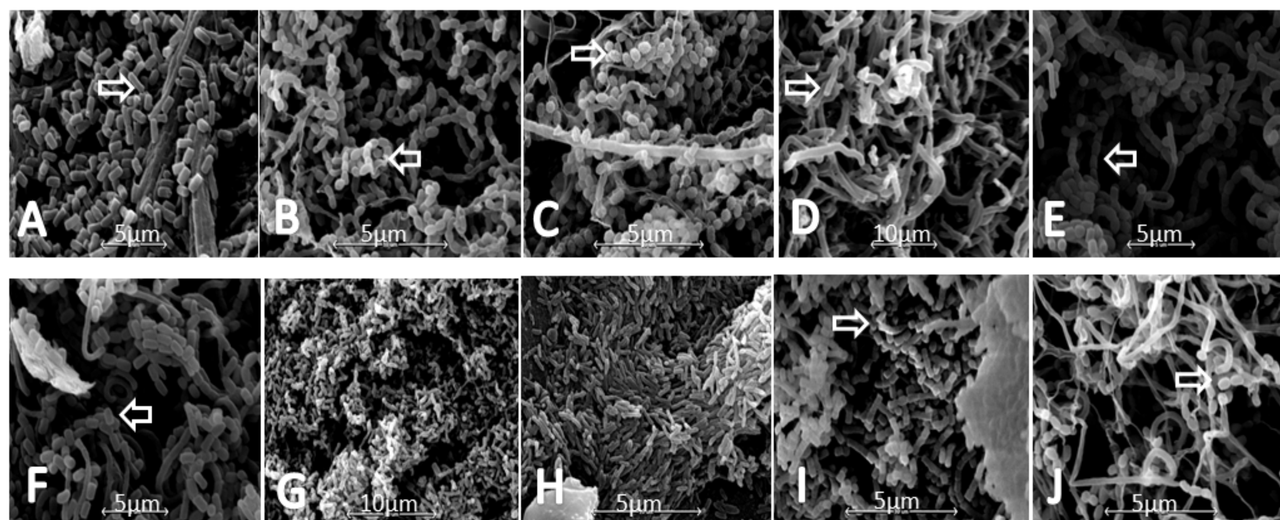

Fig. 2. Scanning electron microscope images of selected *Streptomyces* strains. A (LSA 14); B (MKA 17); C (SSA 17); D (SSA 13); E (KMB 1); F (KME 1); G (KMF 2); H (KML 2); I (KMJ 8); J (BLH 1) (White arrows from A to J indicate the spores attached to substratum mycelia).

Table II.- IC_{50} values of isolated *Streptomyces* strains against different Cell lines.

Isolate	IC_{50} (µg/ml)		
	HeLa cells	**MD-BK cells**	**Vero cells**
LSA 14	54	>100	>100
MKA 17	36	86	>100
SSA 13	16.4	>100	64.8
SSA 17	58.9	<6.25	>100
KMB 1	>100	55.2	<6.25
KME 1	42	>100	74.2
KMF 2	>100	>100	>100
KML 2	12.17	47.88	56.12
KMJ 8	>100	57	11.4
BLH 1	53	58	70.7

strong urease production, only three strains SSA 17, KMJ 8, BLH 1 were able to produce strong hemolysis pattern, remaining seven were negative for hemolysis except KMF 2 which produce incomplete hemolysis. All the strains were able to grow on glucose and galactose. Two strains KML 2 and KMJ 8 were not able to utilize fructose as a carbon source, while only four strains LSA 14, MKA 17, KMF 2 and BLH 1 exhibited growth on sucrose. The strains LSA 14, MKA 17, KMF 2 and BLH 1 were able to utilize raffinose as a carbon source; however, in case of L-arabinose seven strains were found unable to grow on it. The isolated actinomycetes strains were found to be active against a panel of different indicator microorganisms.

The scanning electron microscope images (Fig. 2) shows, that the spore chains of isolate KML 2 are straight, smooth, thick, branched with very little coiling. Diameters of the spores are same with having attached to definite mycelia. The spore chains of strains KMB 1, KME 1 and BLH 1 are highly coiled with moderate branching patterns. Most of the strains possess smooth spores except SSA 17 which have hairy spores with thick appearance. Culture characteristics of the strains were compared with those already reported in *Bergey's Manual of Systematic Bacteriology* (Locci, 1989). Results obtained after comparison clearly demonstrated that these strains belong to a well-known family of actinomycetes known as Streptomycetaceae. These results were also confirmed by the comparison of 16S rRNA gene sequences of the selected strains with those already present in the GenBank through BLAST analysis (Table V). The accession numbers for selected *Streptomyces* strains obtained from GenBank after submission of 16S rRNA gene sequences and their closest similarity member were reported in Table V.

In chemical screening several UV absorbing spots were detected on TLC plates, when examined under short (254 nm) and long (366 nm) UV. Distinguished patterns of different color bands were observed after spraying the TLC plates with Ehrlich and Anisaldehyde/H_2SO_4 reagents. Each spot after reacting with spraying reagents produces different colors including red, yellow, blue and brown. Maximum chemical diversity was observed in the extracts of LSA 14, SSA 17, KML 2 and KMB 1 (Fig. 3). Samples for the spectroscopic analysis were prepared very carefully keeping in mind that the recommended

Table III.- Biochemical and physiological characteristics of the selected *Streptomyces* strains.

Biochemical properties		Isolates									
		LSA 14	MKA 17	SSA 13	SSA 17	KMB 1	KME 1	KMF 2	KML 2	KMJ 8	BLH 1
Utilization of sugars	D-Glucose	+	+	+	+	+	+	+	+	+	+
	D-Fructose	+	+	+	+	+	+	+	-	-	+
	L-Arabinose	-	+	-	+	-	+	-	-	-	-
	D-Galactose	+	+	+	+	+	+	+	+	+	+
	Raffinose	+	+	-	+	-	+	+	-	-	-
	D-Mannitol	+	-	+	+	+	-	+	+	+	+
	Sucrose	+	+	-	-	-	-	+	-	-	+
	Mannose	+	+	+	+	+	+	-	+	+	+
Utilization of organic acids	Potassium gluconate	-	-	-	+	MP	-	-	MP	-	+
	Trisodium citrate	-	+	-	+	+	-	-	+	+	+
	Sodium malate	MP	MP	+	-	+	+	+	-	+	-
	Sodium lactate	-	MP	+	MP	-	-	+	-	-	MP
	Sodium malonate	-	MP	MP	-	MP	MP	MP	-	MP	-
	Oxalate	-	+	+	+	+	+	+	-	-	+
Production of Melanin		-	-	-	+	-	-	-	-	-	-
Urease		MP	SP	SP	SP	SP	SP	MP	SP	MP	MP
Hemolysis		-	-	-	SH	-	-	IH	-	SH	SH

+mean positive; -means negative; MP means Moderately Positive; SP means strongly positive; SH means Strong Hemolysis; IH means Incomplete Hemolysis.

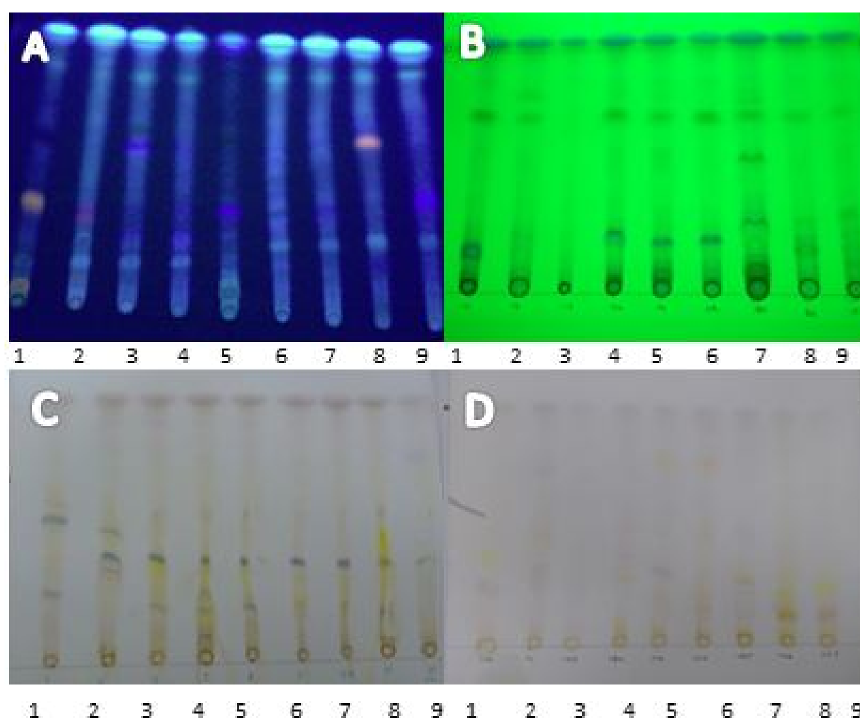

Fig. 3. Chemical screening using Thin Layer Chromatography (TLC). TLC plates a, under u.v. at 366nm, b, under u.v. at 254nm, c, after treatment with anisaldehyde/H_2SO_4 solution, d, after treatment with Ehrlich's reagent. Numbers 1-9: crude extracts of *Streptomyces* strains 1= LSA 14; 2, MKA 17; 3, SSA 17; 4, SSA 13; 5, KMB 1; 6, KME 1; 7, KMF 2; 8, KML 2; 9, KMJ 8.

Fig. 4. *In-vitro* activity of *Streptomyces* Extracts against HeLa, MDBK and Vero cell lines determined by MTT assay.

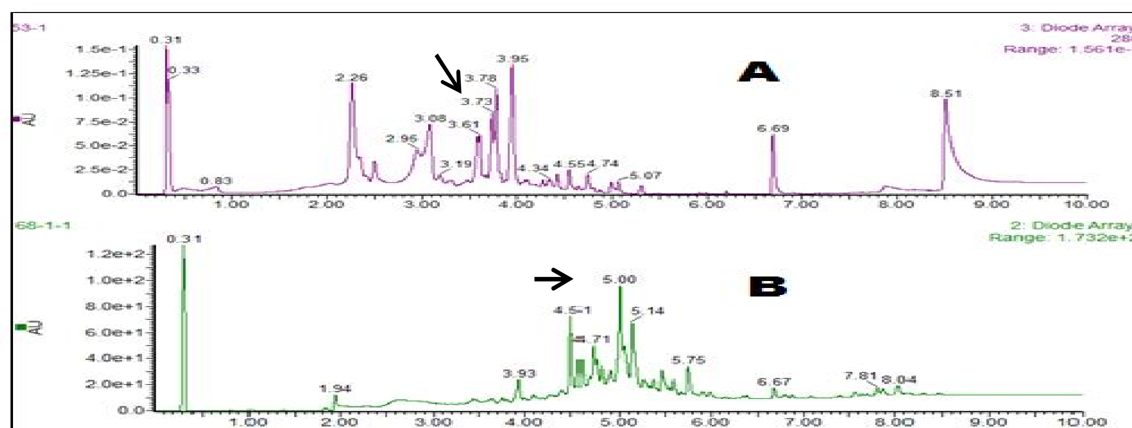

Fig. 5. Diode array chromatogram of extracts from MKA 17(A) and SSA 13(B).

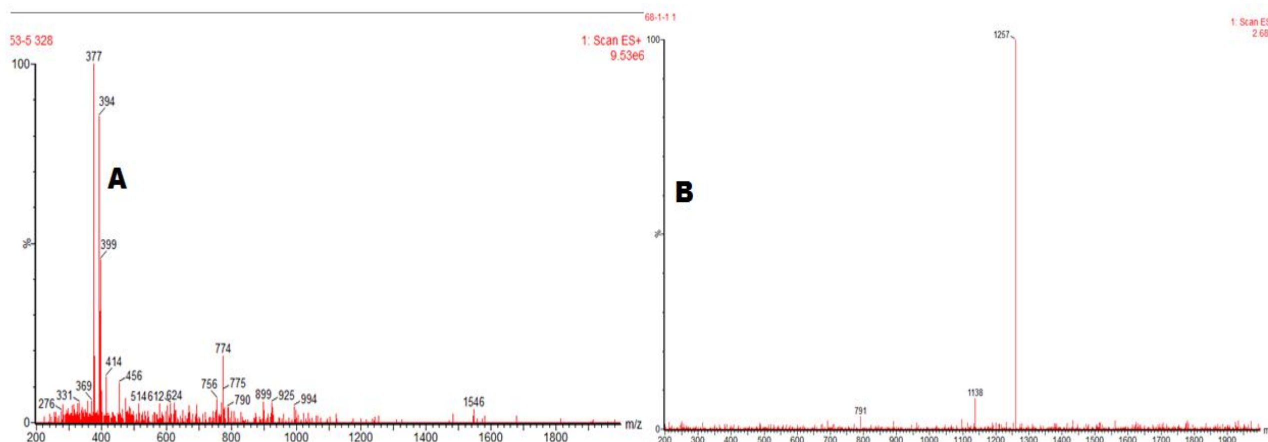

Fig. 6. Mass spectrum of the peaks having retention time of 3.61min and 5.0 min from the extracts of MKA 17(A) and SSA 13(B) in ES positive mode respectively.

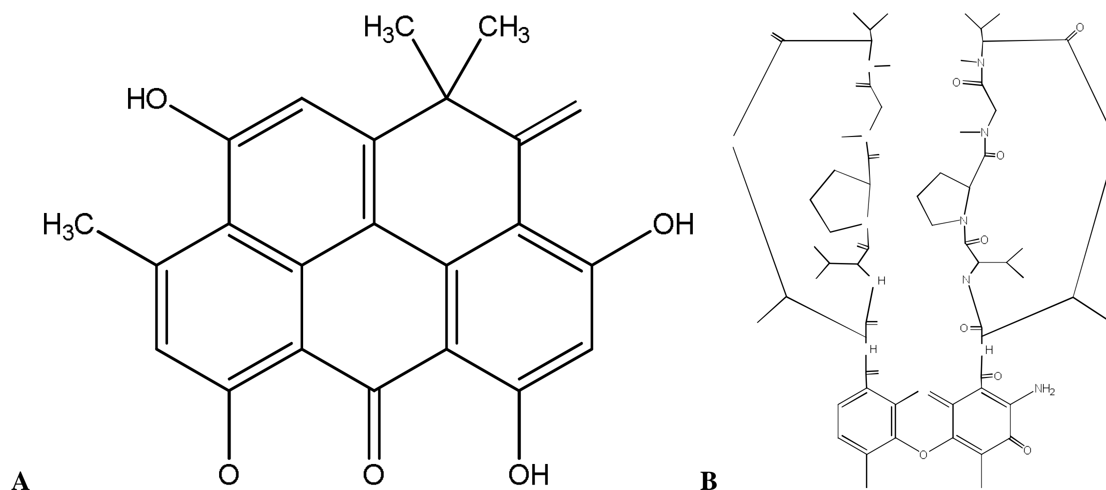

Fig. 7. Molecular structures of Resistomycin (A) and Actinomycin D (B) isolated from MKA 17(A) and SSA 13(B) respectively.

concentration for good analysis is 1 to 5000 ng/ml. UPLC-MS chromatograms of extracts from different *Streptomyces* strains revealed peaks of several bioactive compounds concurrently with good concentration eluting from the column at different retention times (R_t). The UPLC-MS results of MKA 17 and SSA 13 extracts are shown in Figure 5. Several prominent peaks for MKA 17 at the retention time of 2.26, 2.95, 3.08, 3.19, 3.61, 3.73, 3.78, 3.95, 4.34, 4.55, 4.74, 5.07, 6.69, 8.51 minutes were observed while monitoring through diode array detector. Each of the latter peaks corresponds to the compounds with the m/z ratio of 230, 1801, 818, 234, 376, 1345, 267, 1086, 333, 210, 238, 434, 1201 and 492 in ES positive mode respectively. Similarly chromatogram of the strain SSA 13 also showed peaks at the retention time of 1.94, 3.93, 4.51, 4.71, 4.59, 5.00, 5.14, 5.59, 5.75, 7.81, 8.04 with each peak containing compounds with m/z ratio of 237, 285, 386, 478, 1044, 1256, 544, 1070, 1023 and 1037 in ES positive mode respectively. The UPLC-MS-DAD and NMR spectroscopic examination of extracts obtained after pilot scale fermentation of selected strains reveals potent antitumor compounds which includes resistomycin at 3.61 Rt from MKA 17 extract and actinomycin D at 5.00 Rt from SSA 13 (Figs. 6, 7). Same kind of metabolic diversity was observed in the UPLC-MS analysis of remaining 8 extracts of isolated *Streptomyces* strains.

DISCUSSION

Since very long actinomycetes are famous for their unprecedented ability to provide multitude of bioactive compounds. These bioactive compounds serve their role by combating at different fronts related to human health problems. They have the potential to be active as herbicidal, insecticidal, immunosupressants, antihelmintic, antiparasitic, antimicrobial, anticancer, antiviral and antifungal agents. Actinomycetes from normal habitats are well studied. So in order to find out unique strains with unique abilities, we need to examine untapped ecological niches. In this connection the present work describes the isolation and screening of the strains of actinomycetes especially the members of genus *Streptomyces* for antibacterial and antitumor activity from six different habitats of Pakistan. Among these six different habitats incredible diversity with respect to isolation of bioactive actinomycetes was seen in the marine and salt mine samples. Marine environment snatches the focus of researchers for isolating actinomycetes strains that can produce novel, biologically active metabolites. Another reason for this inclination towards this saline environment is the consistent redundancy in the screening of terrestrial actinomycetes for bioactive compounds. Since the last few decades marine actinomycetes strains serves a stimulating role by providing a novel compounds with attractive bioactivities. So in connection to growing need of new and potent therapeutic agents, we should keep on focusing marine environment for isolating the different family members of actinomycetes group (Hozzein *et al*., 2013; Subarmani and Aalbersberg, 2012).

Streptomyces have already proved themselves for the production of unique compounds with diverse biological activities. More than half of the antibiotics used at present for curing human illnesses are produced by different *Streptomyces* species (Liu *et al*., 2013). The potential of *Streptomyces* against super bugs can easily be judged by the fact that almost 10,000 new compounds of immense biological interest have been isolated from their crude extracts (Watve *et al*., 2001). We were quite successful for having actinomycetes strains with high cytotoxic activity after preliminary screening of 120 out of 500 isolated actinomycetes strains. Cytotoxicity testing by using larvae of *Artemia salina* is used since very long for cytotoxic evaluation purpose (Mclaughlin, 1991). Among 120 isolates the extracts of the strains MKA 17, KML 2 and SSA 13 showed promising cytotoxic activity with values of 92%, 84% and 84% respectively. On the basis of the results of cytotoxicity against *Artemia salina* ten different strains with maximum larval mortality were selected for further investigation. In order to find out the antitumor activity of these selected strains, one cancerous (HeLa) and two normal cell lines (Vero and MDBK) were cultured in the presence of increasing concentration of isolated extracts. To check the effectiveness of extracts against proliferating power of cell lines, IC_{50} values were determined by dose response curve method. IC_{50} values clearly indicate that antitumor activity of the extracts are variable and dependent on factors including concentration of extracts, cell line used *etc.* As shown in Fig. 4 strains KML 2 and SSA 13 are most effective against HeLa cells with the IC_{50} values less than 30 µg/ml. According to guide lines set by the American National Cancer Institute a potent preliminary extracts should possess IC_{50} value less than 30 µg/ml. In order to eliminate the chance that these isolated extracts were toxic to normal cells, we have also checked their effect against Vero and MDBK cell lines. The strains KML 2 and SSA 13 which were most active against HeLa were found to be less toxic to normal cell lines with having IC_{50} greater than 45 µg/ml in both cases. Several mechanisms for this anti-proliferative activity can be speculated by the compounds present in these crude extracts. Most common mechanisms reported by the compounds obtained from actinomycetes are apoptosis, mitochondria permeabilization, blockage of signal transduction pathways by inhibiting key enzymes and tumor induced angiogenesis (Olano *et al*., 2009).

While on the way to prescreen any microorganisms, some time we underestimate its potential and not consider it in the list of potential microorganisms by only looking at the results of one bioassay which we have applied. So we should always be conscious about the fact that may be if our isolated strain did not showed good antibacterial activity, then it may have good antitumor potential (Taddei *et al*., 2006). Best way to solve that kind of problem is to focus on horizontal screening methods in which we use combination of tests with low selectivity. In this regard we also check the antibacterial activity of isolated actinomycetes strains which already showed good cytotoxic activities in brine shrimps assay. Among different strains KMJ 8 showed antibacterial activity against all the test organisms. This strain did not showed good antitumor activity against HeLa cells.

The morphological physiological and biochemical characteristics of all the active strains strongly suggest their relationship with the well-known genus of actinomycetes, the *Streptomyces*. This strong relationship was also seen in the images obtained from scanning electron microscope. These images clearly describe the morphological features of spores having connection with substrate mycelia which is the unique morphological characteristic of all the actinomycetes strains. Later the genetic approach is used to confirm the taxonomic status of these isolated strains. 16S rRNA gene sequencing tool

was used to identify these strains. The results obtained from the BLAST analysis of 16S rRNA gene of all the strains proved that, all the isolates were belonging to different species of the genus *Streptomyces*. The sequences of 16S rRNA gene of these *Streptomyces* strains were submitted in GenBank with accession numbers mentioned in Table V.

In order to investigate the metabolic fingerprinting pattern of each of the selected *Streptomyces* strain, we utilized the chemical screening approach. Chemical screening was done by TLC and UPLC-MS analysis. TLC analyses give us opportunity to investigate the unique pattern of secondary metabolites present in the methanolic extracts of each strain. This information is sufficient enough to make a decision for selecting or deselecting of the strains for further studies. Several colorful bands were observed when the TLC plate, which was developed with CH_2Cl_2/5%MeOH and sprayed with anisaldehyde/H_2SO_4 and Ehrlich's reagents. The Banding pattern was good in case of anisaldehyde/H_2SO_4 as various spots with different colors appeared after spraying the TLC plate. These spots were marked and scanned for record of metabolic diversity among the selected *Streptomyces* strains. Extensive picture of chemical screening was obtained by UPLC-MS analysis of the microbial extracts. UPLC-MS technique is very power full technique which help us in identifying compounds with satisfactory knowledge for efficient dereplication. It has several advantages over conventional HPLC-MS which includes quality of results, efficiency, speed, resolution, sensitivity, pressure limit, versatility of solvents used and column chemistry. An UPLC-MS technique is pretty much suitable for the study of peptides and proteins of natural origin because the results obtained from it give excellent resolution with high peak capacity. It is very easy to interpret this high resolution data which contains 20 fold more spectral information. This technique is useful for both low and high molecular weight compounds with no optimization needed in ion source collision induced dissociation because it is intelligent enough for producing monomer ions. In short this technique give us sufficient information about chemical nature of our *Streptomyces* extracts, as it gives increased peak concentrations with low chromatographic dispersion and artefacts (Novakova *et al.*, 2006).

As shown in Figure 5 diode array and total ion chromatogram of SSA 13 and MKA 17, several compounds with different molecular masses were eluted at different retention time from the column. The molecular masses resulting from the UPLC-MS analysis of the extracts were searched in several databases for effective dereplication. Several hits were observed for the compounds present at different retention times. In case of MKA 17, compound with the molecular weight of 376 eluting at 3.61 min came up with only one hit with reference to resistomycin. Compound with this molecular weight already reported from *Streptomyces* is resistomycin which is a potent antitumor compound against HeLa cell (Vijayabharathi *et al.*, 2011). The Structure confirmation was also done by subsequent characterization through Mass and NMR spectroscopic technique. Resistomycin compound is composed of malonyl and acetyl unit, both of which come under the category of acetogenins. Resistomycin is also reported for to be active against different cancers cell lines, including breast tumor cell line MCF-7 etc. (Kock *et al.*, 2005). Similarly in case of SSA 13, compound with the molecular weight of 1256 having retention time of 5.00 min came up with the hit pointing towards the Actinomycin D, which is well known compound reported from several members of actinomycetes. Peptide test also confirmed the presence of peptide by giving blue spot on TLC plate on reacting with o-dianisidin. For confirmation structure elucidation was done by performing UPLC-MS-DAD and NMR analysis. Actinomycin D is a well-known polypeptide with all of its variants termed as A, B, C, D, I, X, Z having same phenaxazinone chromophore and are reported for to be used as anticancer drugs. Since very long this compound is known for its effectiveness against HeLa cells (Di Marco *et al.*, 1965). Actinomycin D is also famous for its used against soft tissue sarcoma and Wilm's tumor (Lackner *et al.*, 2000). In this way we come up with the confirmed reason of high cytotoxic behavior of both of these strains.

Strains of *Streptomyces* isolated and characterized in this study have already known for their other activities as well. *S. coelicoflavus* MKA 17 isolated from marine water is also reported for its antioxidant activity by the production of Surugapyrone (Sugiyama *et al.*, 2010). Strain named as *S. resistomycificus* SSA 13 isolated from Karachi sea soil is already known as a stable producer of resistomycin, but in our case we have isolated actinomycin D from its extract after bioassay guided fractionation. As such this is the first report of isolation of actinomycin from any *S. resistomycificus* strain. Several other strains of *Streptomyces* are reported for the production of resistomycin and actinomycin at a time (Sajid *et al.*, 2011). Both of these strains are isolated from a marine habitat which clearly shows its great potential for having tremendous microbial diversity.

Another interesting fact is the presence of five strains belonging to the same habitat (Khewra salt mine) among the top ten group with respect to highest cytotoxicity. May be the limiting factors present in the mines are the probable reason for harboring of these kind of unique actinomycetes strains. In literature several

Table IV.- Morphological characteristics of the selected *Streptomyces* strains.

Isolate	Morphological parameters										
	Aerial mycelium	**Substrate mycelium**	**Diffusible pigments**	**Spores**	**Size (mm)**	**Consistency**	**Odour**	**Elevation**	**Margins**	**Surface**	**Growth pattern**
LSA 14	Detachable/ yellow	Abundant/ White	Nil	Abundant/ rough	5	hard / embedded	None	Convex	Entire	Rough	Moderate growth
MKA 17	Detachable/ brown	Abundant/ Red	Nil	Abundant/ smooth	3	hard / embedded	Earthy	Raised	Entire	Rough	Well growth
SSA 13	Detachable/ white	Abundant/ greenish	Green pigment	Abundant/ smooth	9	hard / embedded	Fruity	Raised	Entire	Smooth	Well growth
SSA 17	Detachable/ grayish	Abundant/ black	Nil	Abundant/ smooth	4	hard / embedded	None	Raised	Entire	Rough	Moderate growth
KMB 1	Detachable/ pink	Abundant/ grey	Nil	Abundant/ smooth	3	hard / embedded	Earthy	Umbonate	Entire	Rough	Moderate growth
KME 1	Detachable/ grey	Abundant/ brown	Nil	Abundant/ smooth	3	hard / embedded	Earthy	Raised	Entire	Rough	Moderate growth
KMF 2	Detachable/ bluish	Abundant/ white	Nil	Abundant/ smooth	5	hard / embedded	None	Raised	Entire	Rough	Moderate growth
KML 2	Detachable/ pale yellow	Abundant/ Red	Nil	Abundant/ smooth	3	hard / embedded	Earthy	Raised	Entire	Rough	Well growth
KMJ 8	Detachable/ creamy	Abundant/ brown	Nil	Abundant/ smooth	3	hard / embedded	None	Convex	Entire	Rough	Moderate growth
BLH 1	Detachable/ grey	Abundant/ brown	Nil	Abundant/ smooth	5	hard / embedded	Earthy	Umbonate	Entire	Smooth	Well growth

Table V.- Results of 16S rRNA gene sequencing of the isolated *Streptomyces* strains.

Isolate	Source	No. of nucleotides sequenced (bp)	% homology with		GenBank Accession Number
LSA 14	Rahim Yar khan sugar cane fields	1452	*Streptomyces parvus*	99	GenBank:KJ020692
MKA 17	Marine water from Karachi	1429	*Streptomyces coelicoflavus*	99	GenBank:KJ020691
SSA 13	Karachi sea shore soil sample	1455	*Streptomyces resistomycificus*	99	GenBank:KJ020688
SSA 17	Karachi sea shore soil sample	1449	*Streptomyces griseorubens*	99	GenBank:KJ020693
KMB 1	Khewra mines soil sample	1445	*Streptomyces rochei*	99	GenBank:KJ020689
KME 1	Khewra mines soil sample	715	*Streptomyces griseoincarnatus*	100	GenBank:KJ020696
KMF 2	Khewra mines soil sample	1438	*Streptomyces minutiscleroticus*	99	GenBank:KJ020685
KML 2	Khewra mines soil sample	1437	*Streptomyces griseus*	99	GenBank:KJ009562
KMJ 8	Khewra mines soil sample	1440	*Streptomyces microflavus*	99	GenBank:KJ020690
BLH 1	Quetta, Baluchistan soil sample	1422	*Streptomyces mutabilis*	99	GenBank:KJ020694

limiting factors which includes, low level of organic nutrients, slight or no light, extraordinary mineral concentrations and presence of competition for inorganic energy sources make these kinds of ecological biotopes suitable only for specialized microorganism. Presence of double competitions, one for food among different microorganisms and other for combating with stress in hypersaline environments are also marked as probable reasons for immense diversity of actinomycetes (Anwar *et al.*, 2014; Peck, 1986).

Several studies reports the isolation of different halophilic actinomycetes strains from salt rich environments, which can produce novel potent compounds having unique bioactivities (Aftab *et al.*, 2015). Existing isolation studies from mines of different countries demonstrate the abundance and novel biosynthetic potential of different isolated actinomycetes strains (Urzì *et al.*, 2008). Large number of novel actinomycetes taxa has been reported from unique habitats of mines and caves which includes *Knoellia* and *fodinibacter* etc. (Saiz-Jimenez, 1999; Wang *et al.*, 2009). Some studies also report the isolation of actinomycetes from different mines, which are difficult to isolate from other environments such as *Nocardia*, *Amycolatopsis*, *Isoptericola*, *Jiangella, Pseudonocardia* (Groth *et al.*, 2007).

These results also highlight the importance of khewra salt mines habitat in our region for future sampling. It is a unique ecological niche which can help us in novel drug discovery, as already several studies reports the isolation of novel genus, species and compounds from chemically-talented Actinomycetes strains, isolated from mines and caves. These kinds of biotopes should be examined through both culture and culture independent techniques for the complete exploration of potential present in inhabitant actinomycetes strains (Carlsohn *et al.*, 2007).

After detailed investigation of the results obtained from chemical and biological screening we reach at a point that in general *Streptomyces* are good source for finding novel antitumor compounds. On one hand extracts of these potent strains showed very promising antitumor activity against proliferating cell lines and on the other hand Mass and NMR spectroscopic analysis revealed many impressive compounds with different molecular weights. Further, detailed investigation of these isolated *Streptomyces* strains is under consideration. In conclusion these results provide an insight into an untapped ecological niches present in Pakistan which harbor unique pool of actinomycetes strains. Screening of indigenous actinomycetes which are yet to be explored in these climatic regions might be a promising source of novel potent antitumor compounds. In addition we need to make the systematic strategies in order to truly explore the potential of unique habitats such as khewra salt mine and Arabian Sea marine environment in our country.

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Conflict of interest

The author(s) declare that they have no conflict of interests.

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