

Molecular Identification, Bioactivity Screening and Metabolic Fingerprinting of the Actinomycetes of Chenab River Sediments

Abstract:

Aims: The mortality, morbidity and health cost have been increased due to multiple antimicrobial drug resistance in pathogens across the world. So it is important to discover new active compounds. This study was designed to screen the Chenab river actinomycetes for antimicrobial activity against various MDR bacterial pathogens and preliminary cytotoxicity.

Study Design: Collection of water and sediments samples, sample enrichment and selective isolation of the actinomycetes, laboratory scale cultivation, solvent extraction, determination of antimicrobial activity and cytotoxicity, chemical profiling and metabolic fingerprinting by TLC and HPLC-UV.

Place and Duration: All the work was done during July 2013 to July 2015 in the Department of Microbiology and Molecular Genetics, University of the Punjab Lahore, Pakistan.

Methodology: The water and sediments samples of Chenab river were collected from the Sialkot region (Punjab, Pakistan) and were processed for the selective isolation of actinomycetes. The isolated strains were identified by morphological, biochemical, physiological characterization and by 16S rRNA gene sequencing. The selected strains were screened for antimicrobial activity and cytotoxicity using agar diffusion assays and by microwell cytotoxicity assay against *Artemia salina* larvae. For metabolic fingerprinting the methanolic crude extracts obtained from the selected strains were analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC-UV).

Results: A total of 30 actinomycetes strains were isolated and were identified as different species of genus *Streptomyces*. Among all the selected strains, the isolates M72, M1, M71, W38, W108 and M93 were found to exhibit potent antimicrobial activity against the test strains including *Klebsiella* sp., methicillin resistant *Staphylococcus aureus* (MRSA), *Acinetobacter* sp., *E. coli*, and *B.cereus*. Similarly, the isolates M54, W19 and M81 demonstrated significant cytotoxicity (up to 80% larval mortality) against *Artemia salina*.

Conclusion: The study suggested that actinomycetes flora of Chenab river is rich source of useful natural compounds and should be continuously isolated characterized and investigated for novel antibiotics and other chemotherapeutic agents.

Keywords: Chenab River actinomycetes, antimicrobial activity, cytotoxicity, chemical screening by TLC and HPLC-UV/RI

Introduction

Antibiotics are the most recommended medications worldwide, however their viability is decreasing, which is creating genuine clinical concerns particularly because of the rise of resistant microorganisms [1]. Novel methodologies for the advancement of new antimicrobials have been sought, but just a couple of new antibiotics have been introduced by the pharmaceutical industry these days [2]. Regardless of the tremendous expectations on synthetic drugs with successful antimicrobial properties, products from natural sources still deserve attention and guarantee the presence of new and unidentified active compounds [3]. Continuous isolation and screening of bacteria from different ecological niches which has not been explored yet could be a promising source of novel bacteria [4], which can produce novel drug [5]. Examining the potential of microorganisms from different niches is a sensible idea since different environmental conditions can impact microbial metabolism system which can result in the production of unique metabolites. Microorganisms producing variety of antimicrobial products have been isolated from different sources, for example, from fresh water bodies, endophytes from various plants and from oceans etc. [6, 7].

Actinomycetes are the most prominent and versatile producer of secondary metabolites among the all living groups. The *Streptomyces* species produce about 70-80% of the all active microbial natural compounds discovered to the date [8]. The distribution and diversity of actinomycetes in freshwater bodies have not been investigated widely yet, however some studies have been conducted on water reservoirs containing high level of organic matter and the littoral zones of rivers. Principally these studies concentrated on the taste, discoloration and smell of water. Recent studies of freshwater actinomycetes are coordinated towards the new possibilities of producing unique proteins and secondary metabolites [9]. The other reason behind is because of their filamentous nature actinomycetes attach firmly with substrate which makes them difficult to remove from the source material. The isolation techniques for actinomycetes are more similar to fungi rather than other bacteria due to their filamentous and spore forming nature. Some scientists question the presence of actinomycetes in fresh water and believe they are not truly aquatic in nature and may be artifact of run-off or spore survival. But most of the scientists reinforced the presence of actinomycetes in fresh water. The aquatic actinomycetes have been overlooked because they grow slowly and do not dominate the culture plate. There is general assumption regarding aquatic actinomycetes that they are not ecologically important and it is due to lack of data available. Aquatic actinomycetes should not be ignored in the journey of exploring novel bioactive microbial compounds and understand the interaction of aquatic microbial community [10].

Like any other country, the micro flora of Pakistan has not been fully explored yet. There are diverse ecological niches in Pakistan e.g. deserts, forest soil, salt ranges, agricultural soil, mountains, marine water of Arabian sea and fresh water bodies. In this study we focused the aquatic actinomycetes of Chenab river water and sediments and tried to explore their antimicrobial and cytotoxic potential. As far as we know, the biodiversity of aquatic actinomycetes in Chenab river has not been reported before. The Chenab river originates from upper Himalayas in Himachal Pradesh, India and enters Sialkot district of

Pakistan. It is major tributary of Indus river and 1086-kilometer long. This place is hot and humid in summer and temperature may rise to over 40 °C while in winter temperature drops to -2 °C. The samples were collected from Chenab river to isolate the aquatic actinomycetes and explore their antimicrobial and cytotoxic potential. An impressive actinomycetes diversity and a promising antimicrobial response and metabolic profile of the isolated strains have been observed against various pathogenic bacteria.

Materials and methods:

Sample collection:

The water and mud/sediments were collected from Chenab river at various sites in District Sialkot, Punjab, Pakistan. Water samples were taken from approximately 0.5m depth in autoclaved screw capped test tubes. Sediment samples were collected from the depth of the water in sterile polythene bags using a sediment corer to ensure maximum microbial diversity and were labeled accordingly. The Samples were transferred to the laboratory and were processed further within one week.

Sample enrichment for actinomycetes

The samples were treated by both physical and chemical treatment methods. All the collected sediment samples were heat treated at 55 °C for 3 days according to the methods described by Sajid, et al. [11]. This treatment reduced the vegetative cell of general microbial flora considerably without affecting the colony counts of actinomycetes. In chemical treatment, samples were mixed with CaCO₃ (10:1 w/w) and were incubated at room temperature (28°C) for 7-9 days in a water saturated atmosphere [12].

Selective Isolation and Identification of the Presumptive Actinomycetes

After physical and chemical treatment, serial dilutions of each sample were prepared and spreading was done by plating 50µl of 10⁻³ dilutions on the selective media (glycerol casein KNO₃ agar) using glass spreader. The plates were labelled properly and incubated at 28°C for two weeks. After incubation the plates were observed for actinomycetes colonies, which were differentiate from general bacteria on the basis of non-slimy (or powdery if sporulation has occurred) and of embedded nature of colonies. These colonies were transferred to the enrich media i.e. glucose yeast extract malt extract agar (GYM agar) and the plates were incubated at 28°C for 5-7 days to obtain pure culture [13]. The isolates were identified on the basis of morphological, biochemical and physiological characteristics and by 16S rRNA gene sequencing. Biochemical and, physiological characterization such as melanin production, utilization of nine different sugars as carbon source was performed according to the “conventional” methods [13]. Formation as well as utilization of organic acids and oxalate, hydrolysis of esculin, arbutin, urea and allantoin was also determined. The 16S rRNA gene sequencing was done commercially at the 1st Base facility (1st Base, Singapore) to determine the final taxonomic status. The gene sequence data obtained was first analyzed using the advanced BLAST search at NCBI: <http://www.ncbi.nlm.nih.gov/BLAST/>. The nucleotide sequence data was deposited to GenBank, and the accession numbers for each of the strains were obtained.

Cultivation and preparation of methanolic crude extracts

The selected strains were first grown as subcultures on agar plates for 3 days using GYM agar. The resulting sub-cultures were then investigated microscopically to confirm the purity of the culture. The subcultures were used to inoculate 250 ml of GYM broth in 1 L Erlenmeyer flasks (the pH was adjusted to 7.3 before sterilization), the flasks were sterilized and followed by incubation as shaking culture at orbital shaker (Sanyo orbital shaking incubator, Japan) for 7 days at 28°C. The resulting culture broth was sonicated in a sonicating bath for cell breakage (UltrasonaMedi-II, J.P Selecta S.A) and mixed with equal amount of ethyl acetate, the mixture was sonicated again for 10 min. The organic layer (solvent having secondary metabolites) was separated in a separating funnel and the solvent was evaporated on rotary vacuum evaporator (Laborta 4000 efficient, Heildolph Germany). The crude extracts were dissolved in 1-5 ml methanol and were stored at 4°C.

Determination of antimicrobial activity against MDR bacterial pathogens and cytotoxicity against *Artemia salina*

For the detection of bioactive secondary metabolites produced by these *Streptomyces* isolates, agar diffusion method was used. The plates for the test organisms were prepared by pouring 14ml of Luria Bertani agar (LB-agar) [14] as base layer; after solidifying, this was overlaid with 4ml of inoculated seed layer. Agar wells (5 mm diameter) were made by using sterile cork borer and 60µl of the methanolic crude extract was loaded to these wells, the plates were left at room temperature for 2 hours for the dilution to diffuse and were incubated at 37°C for 18- 24 hours. After incubation zones of inhibition were measured in mm. Test organism used for antimicrobial activity were *Bacillus cereus*, *Acinetobacter* sp., MRSA, *Klebsiella* sp. and *E. coli*. Brine shrimp microwell cytotoxicity assay was performed using eggs of *Artemia salina* as described by McLaughlin [15].

Metabolic fingerprinting of the active actinomycetes strains

Thin Layer Chromatography (TLC) analysis of the methanolic extracts

A line was drawn at one inch from the base of TLC plate and sample numbers were labelled on it with lead pencil. With the help of a capillary tube each sample extract was spotted on the TLC plate drop wise, with allowing the former spot to dry before super imposing it. The plate was air dried after loading the samples, and developed with 10% MeOH/CH₂Cl₂ solvent system. The TLC plate was placed in the tank and it was made sure that spots were not dipped in the solvent. The solvent was allowed to rise about one third of the plate, and then plate was taken out and allowed to dry. The air dried plates were visualized using UV light at 254 nm and 366 nm. The component showing UV absorbance and fluorescence were marked and scanned. The TLC plate was stained by spraying with anisaldehyde/H₂SO₄ and Eherilisch's reagents separately. The plates were dried under a hot air drier and were observed for color bands. The clear bands of different colors were visible in each sample.

High Performance Liquid Chromatography (HPLC-UV) analysis of the methanolic extracts

The crude extracts were analyzed by the HPLC-UV system (Sykam HPLC) using the software clarity. The column used was C18 from Hypersil with 30 cm length and 4.5mm ID. Mobile phase used was methanol and water (95:5) and the flow rate was adjusted to 1 ml/min. The crude extract was dissolved in HPLC grade methanol and 20 µl was injected through a microsyringe. The sample was run for 15 minutes and UV absorbance was determined at 254 nm. The peaks of sample components were analyzed and were compared at different retention times with standard UV absorption data of secondary metabolites. The peaks at different retention times (t_R) were later compared with the standard UV absorbance data of secondary metabolites in order to get the idea about the nature of compounds produced by the selected actinomycetes strains.

Results and Discussion

In this study a total of 30 actinomycetes strains were isolated, among them 9 strains were recovered from water samples including W2, W211, W101, W38, W102, W101, W124, W19 and W127 and 21 actinomycetes strains from sediments collected from various sites of Chenab river near Sialkot, Punjab, Pakistan which includes M5, M72, M5ii, M715, M1, M71, M714, M51, M712, M713, M54, M714, M713, M32, M7, M92, M93, M99, Pi and M81. Many studies have reported that *Streptomyces* species have found to be most abundant among actinobacterial community in isolates from lakes and wetlands [16, 17]. On the other hand, Terkina, et al. [18] studied the prevalence of *Streptomyces* sp. and *Micromonospora* in lake water and lake sediments. Rifaat [19] reported similar results when they observed diversity of actinomycetes in Nile river. The collected samples were treated physically and chemically, in physical treatment best results were obtained by extended heat treatment at 55 °C for 2-3 weeks. This technique worked very well in selective isolation of actinomycetes from mud samples. Nüesch (1964) reported that drying of samples relatively increases the micro flora of samples. Radhakrishnan, et al. [20] reported that physical treatment of mud samples at 40-50°C considerably reduces the microbial flora without disturbing population of actinomycetes. In chemical treatment CaCO_3 enrichment method gave best results. Air dried samples were mixed up with CaCO_3 (10:1 w/w) and incubated at 28°C for 7-9 days. Studies have reported that this method is efficient and increases the colonies of actinomycetes to many folds and also reduces the fungal contamination. Selected strains were purified by repeated sub culturing. Kumar, Kannabiran [21] reported that the medium having glycerol or starch as sole carbon source and arginine or nitrate as nitrogen source can serve as best selective isolation media for actinomycetes.

In macroscopic features color of aerial mycelium, substrate mycelium, and consistency of mycelium morphology of colonies, size and subtle pigments production were observed. The colonies of all the selected isolates were embedded in media, rough, dry, colored having regular or irregular margins and colony size from pinpoint to large up to 3mm. Unlike other bacteria which produce endospore, actinomycetes produce exospores which are visible in growth culture with naked eye and spore color is a

distinctive property of actinomycetes. However, consistency and color of spore was almost different in all isolated strains. The isolates W38, W102, W108, W124, M93 and Pi showed grey colored spores. In case of M5ii, W211, M1, W101, M715, M712, M710, M711, M54, M713, M7, W19, W127, M99 and M81 the color of spore was white. In other strains color varies from pink, light brown, purple to yellow. None of the selected strains produced exopigments (Table 1; Fig1).

Table 1 Taxonomic characteristics of the selected actinomycetes strains determined after 5-7 days incubation at 28°C on GYM agar

Strains	Morphological characteristics						
	Size of Colony	Shape	Margin of Colony	Texture	Substrate Mycelium	Aerial Mycelium	Color of diffused pigment
M5	2	Round	Regular	Dry & Hard	Light Brown	Brown	No pigment
M72	3	Round	Irregular	Dry & Hard	White	Light Brown	No pigment
M5ii	2	Round	Irregular	Dry & Hard	White	White	No pigment
W2	2	Round	Irregular	Dry & Hard	Yellow	Light Brown	No pigment
W211	2	Round	Irregular	Dry & Velvety	Brown	White	No pigment
M1	2	Round	Irregular	Dry & Hard	Yellow	White	No pigment
M715	2.5	Irregular	Irregular	Dry & Velvety	Off white	White	No pigment
W101	3	Round	Regular	Dry & Velvety	White	Yellow	No pigment
W38	2	Round	Irregular	Dry & Velvety	White	Grey	No pigment
M71	2.5	Irregular	Regular	Dry & Hard	White	Pink	No pigment
M714	2.2	Round	Irregular	Dry & Hard	White	Off white	No pigment
M51	3	Round	Regular	Dry & Hard	Brown	Yellow	No pigment
M712	2.3	Irregular	Irregular	Dry & Hard	Light Brown	White	No pigment
M713	2.6	Irregular	Irregular	Dry & Hard	Yellow	White	No pigment
W102	2.2	Round	Irregular	Dry & Hard	White	Grey	No pigment
W108	2.4	Irregular	Irregular	Dry & Hard	White	Grey	No pigment
M54	2	Round	Regular	Dry & Hard	Brown grey	White	No pigment
M710	3	Round	Irregular	Dry & Hard	Off white	Brown	No pigment
M711	3	Round	Regular	Dry & Hard	Off white	White	No pigment
W124	2	Irregular	Irregular	Dry & Hard	Brown	Grey	No pigment
M32	2	Round	Irregular	Dry & Velvety	Light Brown	Yellow	No pigment
M91	3	Round	Irregular	Dry & Velvety	White	Purple	No pigment
M7	2	Irregular	Irregular	Dry & Hard	Dark Brown	White	No pigment
M92	3	Round	Regular	Dry & Hard	White	Light Purple	No pigment
M93	3	Round	Regular	Dry & Hard	Brown	Grey	No pigment
W19	3	Irregular	Irregular	Dry & Hard	Brown	White	No pigment
W127	2	Round	Irregular	Dry & Hard	Dark brown	White	No pigment
M99	2	Round	Regular	Dry & Hard	Yellow	White	No pigment
M81	2	Round	Regular	Dry & Hard	Brown	White	No pigment
Pi	2	Round	Regular	Dry & Hard	White	Grey	No pigment

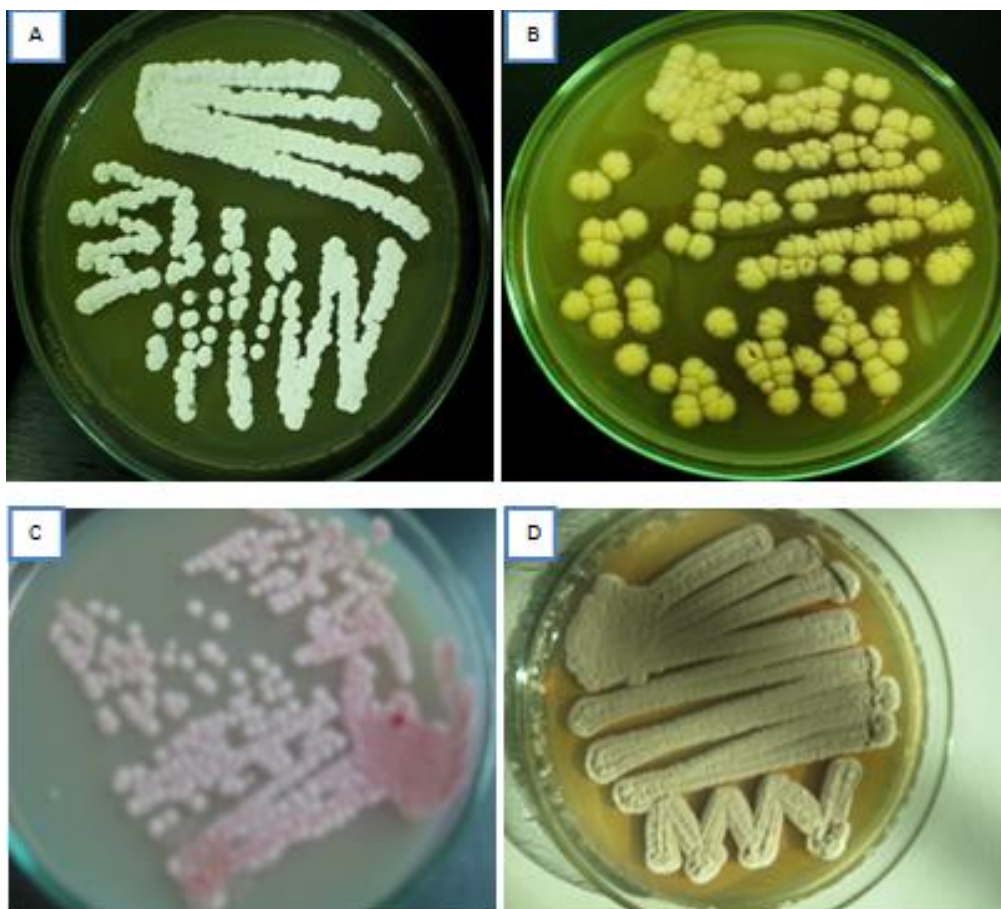


Fig 1 Pure cultures of actinomycetes strains on GYM agar (A: M713, B: M1, C: M71, D: M710) after incubation at 28°C for 7 days

For the biochemical identification of selected actinomycetes melanin formation and sugar utilization profile was determined. Melanin formation is most reliable test in characterization of genus *Streptomyces* and was included in International *Streptomyces* project (ISP). The isolates M5, M72, M5ii, M712, W101, M54, M710 and M91 showed strong positive results by turning media into black and these strains were characterized as belonging to genus *Streptomyces*. Some of the isolates showed moderate melanin production like W2, W211, M51, W102, M711, W124, M7, M92 and W19. Some strains M7, Pi, W19, M92 and W124 did not produce melanin (Table 2). All isolates efficiently utilized glucose as carbon source and grew on glucose supplemented media rapidly. Almost all isolates well utilized fructose except M710 and M711. Mannitol was also a good carbon source for isolates as most of them were able to utilize it except M71, M51, W102, W108, M710, M711, M92, W19 and M8 (Table 2). Glucose was found to be best carbon source as all isolate strains were able to utilize it, followed by fructose (94%), sucrose (73%), mannitol (70%), arabinose (57%) and galactose (27%). The results show that galactose was not efficiently utilized by selected actinomycetes (Fig 2). This study leads to the conclusion that fresh water actinomycetes are non-specific to the carbon source and can use wide range of carbon sources. Almost

204 similar pattern of carbon source assimilation was reported by Oskay et al. (2005) and Varghese et al. (
205 2012).

206 **Table 2** Biochemical characteristics and sugar utilization profile of selected actinomycetes strains

Actinomycetes Strains	Melanin Formation	Sugar Utilization Profile					
		Glucose	Fructose	Galactose	Arabinose	Sucrose	Mannitol
M5	++	+	+	-	+	+	+
M72	++	+	+	-	+	+	+
M5ii	++	+	+	+	+	+	+
W2	+	+	+	-	-	+	+
W211	+	+	+	-	-	+	+
M1	+	+	+	-	-	-	+
M715	++	+	+	+	+	+	+
W101	++	+	+	+	+	+	+
W38	++	+	+	-	+	+	+
M71	++	+	+	-	+	+	-
M714	-	+	+	+	+	+	+
M51	+	+	+	-	+	-	-
M712	++	+	+	-	-	+	+
M713	-	+	+	-	+	+	+
W102	+	+	+	-	+	+	-
W108	++	+	+	-	+	+	-
M54	-	+	+	+	+	+	+
M710	++	+	-	-	-	-	-
M711	+	+	-	-	-	+	-
W124	+	+	+	-	-	+	+
M32	++	+	+	-	-	-	+
M91	-	+	+	-	+	-	+
M7	+	+	+	-	-	+	+
M92	+	+	+	-	-	+	-
M93	++	+	+	-	-	+	+
W19	+	+	+	-	+	-	-
W127	-	+	+	+	+	+	+
M99	++	+	+	+	-	+	+
M81	++	+	+	+	-	-	-
Pi	-	+	+	-	+	-	+

207 (Key: ++ = Good growth, + = Average growth, - = No or poor growth)

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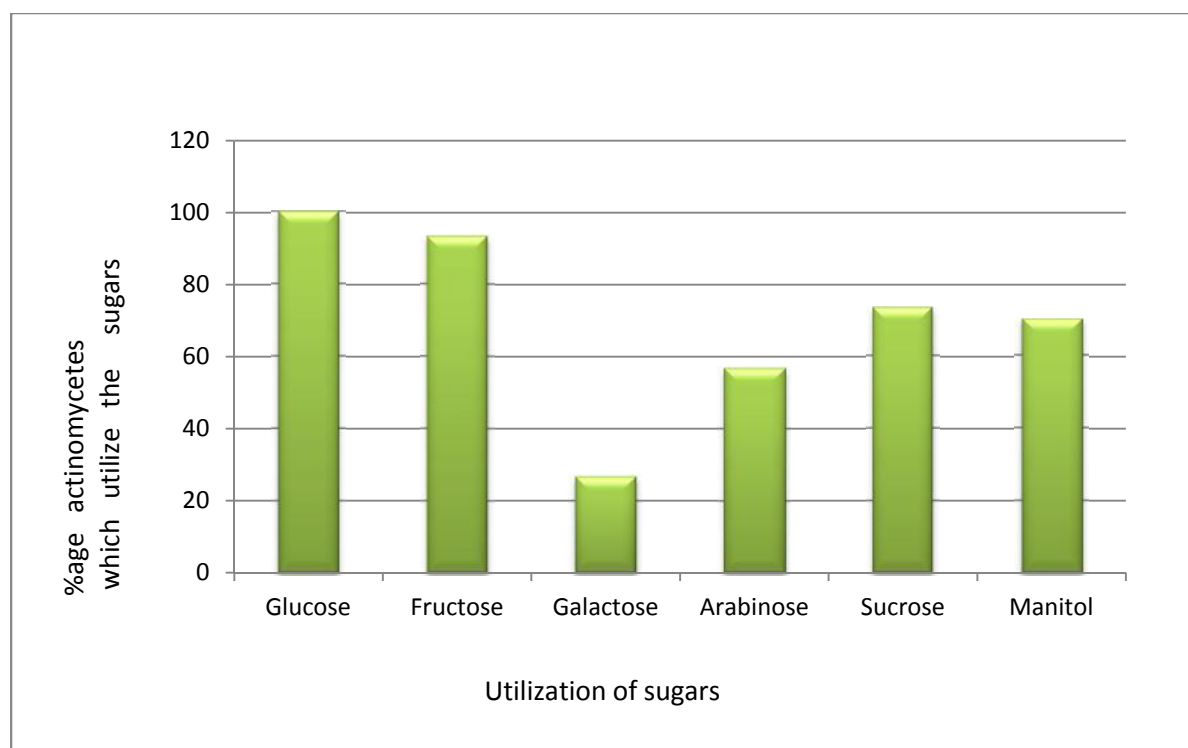


Fig 2 Percentage sugar utilization by selected actinomycetes strains

In agar diffusion method, five test strains *B. cereus*, *Acinetobacter* sp., MRSA, *Klebsiella* sp. and *E. coli* were used in agar diffusion method. Incubation was given at 37°C for 24 hours. The clear zones around wells were observed. The diameter of zones of inhibition was measured in mm. Among all the selected actinomycetes strains, the isolates M72, M1, M71, W38, W108 and M93 were found to be more biologically active as they exhibited activity against all the five test organisms. In case of *B. cereus*, the isolates M54, M714, W124, and M99 exhibited interesting antimicrobial activity showing larger zones of inhibition up to 18mm, 17mm, 17mm and 16mm respectively. The actinomycetes isolates M5, W211, M71, M51, M713, W108, M710, M711, M93, W19, W127 and M81 showed inhibition zones up to 15mm, 13mm, 15mm, 14mm, 15mm, 13mm, 14mm, 13mm, 13mm, 15mm, 12mm and 11mm respectively against *B. cereus*. In case of *Acinetobacter* sp., the isolates M5, M72, M713 and W127 exhibited larger zones of inhibition up to 18mm, 16mm, 17mm and 17mm respectively. The isolates M5ii (14mm), W2 (16mm), M1 (14mm), M715 (12mm), W38 (13mm), M71 (15mm), M714 (12mm), W102 (13mm), W108 (15mm), M54 (12mm), M710 (12mm), M711 (15mm), W124 (9mm), M91 (14mm), M7 (12mm), M93 (15mm), W19 (13mm), M99 (12mm) and Pi (14mm) showed significant activity against *Acinetobacter* sp. The isolates W211, M712, M92 and M81 did not show activity against *Acinetobacter* sp. (Table 3; Fig 3). In case of MRSA, maximum activity was exhibited by the isolates M72 (17mm), W2 (16mm), M81 (17mm), M7 (15mm), W102 (15mm), M714 (15mm), W38 (15mm) and M715 (15mm). The isolates M5ii, M51, M712, M54, W101, M714, M93 and W19 showed zones of inhibition of 11mm, 11mm, 8mm, 10mm, 13mm, 8mm, 11mm and 11mm respectively. No activity was shown by the isolates M32, M91 and W127 against MRSA. In case of *Klebsiella* sp., the isolates M71, M714, W101, M1, M32, M91 and M93

exhibited the zones of inhibitions 13mm, 12mm, 8mm, 12mm, 13mm, 12mm and 12mm respectively. The isolates M5, M72, M5ii, M715, W38, M712, W102, M710, M711, W124, M7, M92, M93, W127, M99, M81 and Pi did not show activity against *Klebsiella* sp. In case of *E. coli*, the isolates M5ii, Pi, M92 and W38 showed the zones of inhibition of 18mm, 20mm, 17mm, and 15mm respectively. The isolate M5 (8mm), W2 (13mm), W211 (14mm), M1 (11mm), M715 (9mm), W108 (14mm), W38 (15mm), M71 (13mm), M714 (9mm), M51 (12mm), M712 (11mm), M713 (8mm), M710 (13mm), M32 (12mm) and M91 (14mm) showed significant activity against *E. coli* (Table 3; Fig 3).

The Brine shrimp cytotoxicity assay was performed to determine the cytotoxicity of the active extracts of the selected actinomycetes strains to kill the laboratory cultured *Artemia salina* larvae. The maximum cytotoxicity was observed in case of actinomycete strains M81 (90 %), W19 (85 %), M71 (80 %), M54 (80 %), W124 (78 %), M7 (77 %), Pi (75 %), M92 (73 %), W108 (71%) and M72 (70 %). The cytotoxicity of isolates M5ii, W2, M714, M712, M32, M91 and M93 was 65 %, 40%, 40 %, 46 %, 10 %, 43 %, 32 % and 53 % respectively. Some isolate did not show any cytotoxic effect including M1, W105, M51 and W102 (Table 3).

Table 3 Antimicrobial activity of the selected actinomycetes strains against MDR bacterial pathogens and cytotoxicity against *Artemia salina*

Actinomycetes Strains	Zone of Inhibition (mm)					Cytotoxicity % Mortality
	<i>B.cereus</i>	<i>Acinetobacter</i>	MRSA	<i>Klebsiella</i>	<i>E.coli</i>	
M5	15	18	12	-	8	30
M72	10	16	17	11	10	70
M5ii	7	14	11	-	18	65
W2	5	16	16	8	13	40
W211	13	-	14	5	14	20
M1	14	14	12	12	11	-
M715	9	12	15	-	9	30
W101	10	9	13	8	12	-
W38	13	13	15	11	15	10
M71	15	15	13	13	13	80
M714	17	12	15	12	9	40
M51	14	8	11	9	12	-
M712	8	-	8	-	11	46
M713	15	17	12	14	8	31
W102	11	13	15	-	15	-
W108	13	15	10	13	14	71
M54	18	12	10	12	8	80
M710	14	12	8	10	13	10
M711	13	15	12	-	11	30
W124	17	9	11	-	9	78
M32	11	7	-	13	12	43
M91	9	14	-	12	14	32
M7	5	12	15	-	11	77
M92	7	-	13	-	17	73
M93	13	15	11	12	12	53
W19	15	13	11	10	14	85
W127	12	17	-	-	12	12
M99	16	12	14	-	9	23
M81	11	-	17	-	14	90
Pi	9	14	11	-	20	75

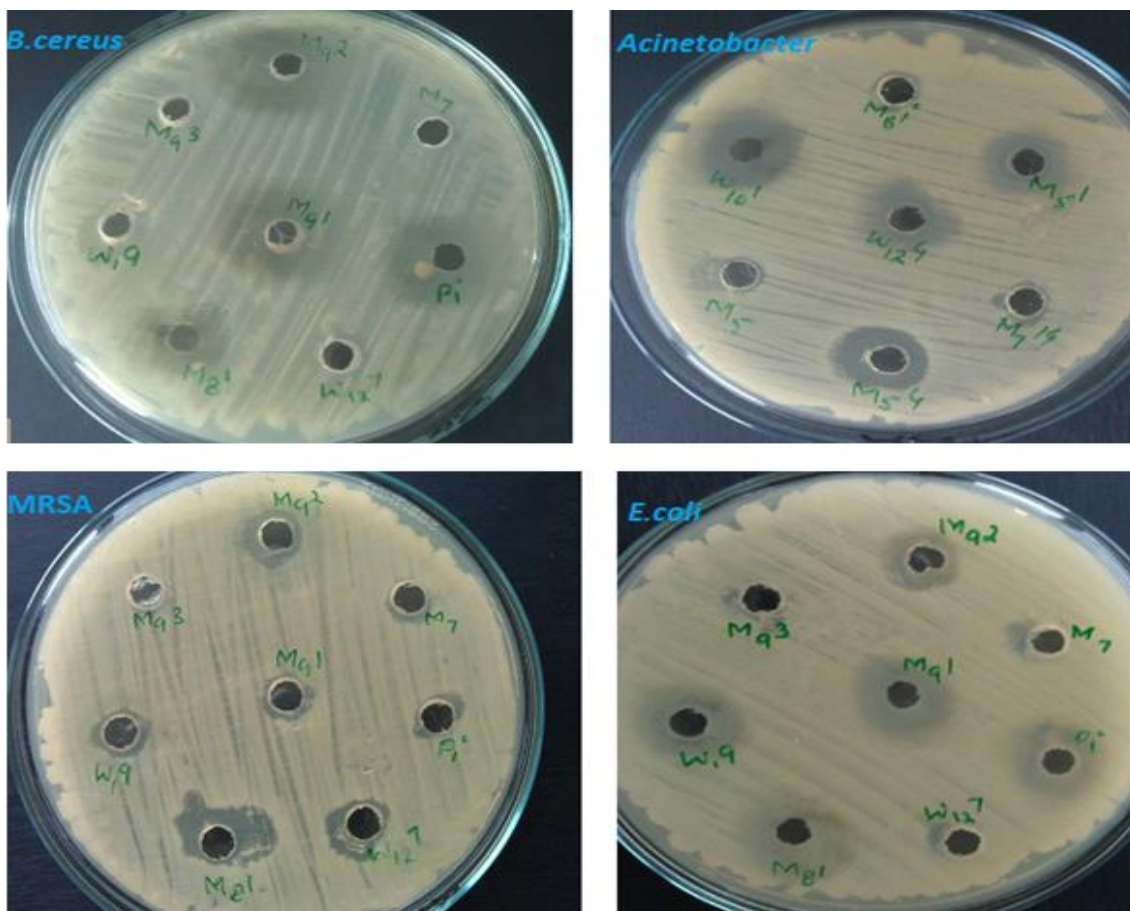


Fig 3 Antimicrobial activity of the selected actinomycetes strains determined by agar diffusion method using methanolic crude extracts

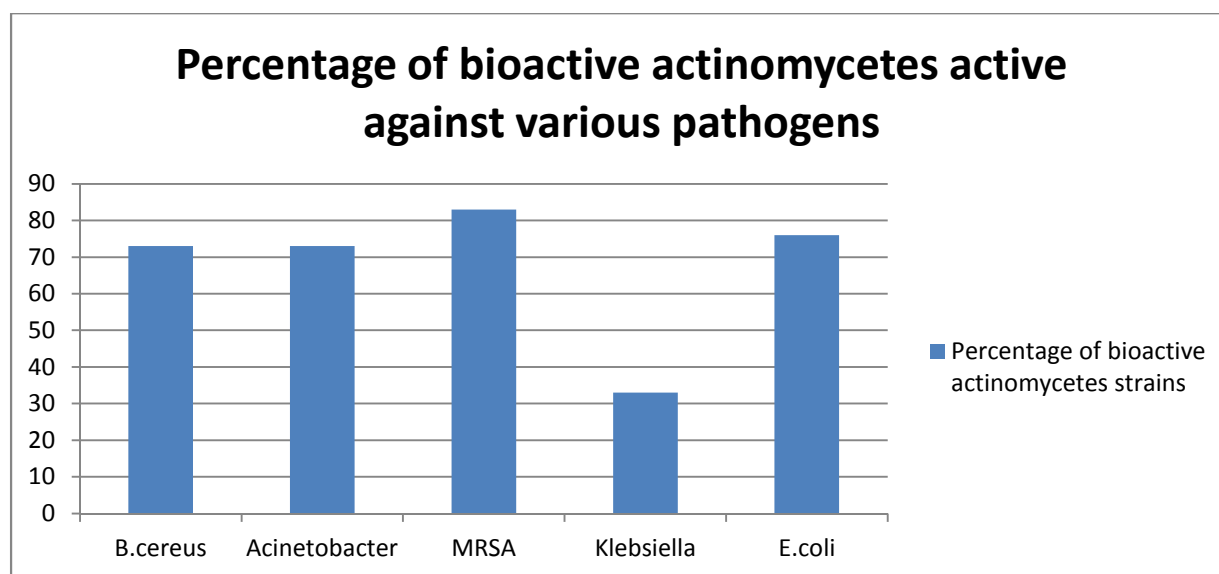


Fig 4 Graph showing the percentage of bioactive actinomycetes strains active against different pathogenic test organisms

The 16S rRNA gene sequencing is a powerful tool, used to study the phylogenetic and taxonomic associations among bacteria from different origins. Studies have been reported that 16S rRNA gene sequencing provides with identification 90% at genus level and 65% at specie level [22]. In a routine antibiotic screening stratetgy, the isolates showing interesting activities are identified up to species level. In our study, 8 isolates showing significant antibacterial and cytotoxicity were identified by 16S rRNA gene sequencing. The morphological, physiological and biochemical characterization strongly suggested that these isolates are the members of genus *Streptomyces* sp. (Table 4).

Table 4 Genetic similarity of the selected isolates with different *Streptomyces* Sp. already reported in Genbank, determined by BLAST analysis of 16S rRNA gene

Actinomycetes strains	BLAST analysis		GenBank Accession number
	Similarity with	% similarity	
W127	<i>Streptomyces puniceus</i>	100%	KM062031
M93	<i>Streptomyces laurentii</i>	100%	KM062032
M71	<i>Streptomyces vitaminophilus</i>	100%	KM062033
M54	<i>Streptomyces hypolithicus</i>	100%	KM062034
M51	<i>Streptomyces chartreusis</i>	100%	KM062035
W211	<i>Streptomyces hypolithicus</i>	100%	KM062036
M32	<i>Streptomyces sparsogenes</i>	100%	KM062037
M714	<i>Streptomyces diastaticus</i>	100%	KM062038

The crude extracts of the strains were analyzed by thin layer chromatography using anisaldehyde/H₂SO₄ and Eherilisch's reagent as staining reagents. The bands of the separated fractions were observed on TLC plate under UV at short and long wavelengths (254 nm, 366 nm). Most of the constituents of the crude extract exhibited UV absorbance however most prominent bands were observed in crude extracts of the isolates W101, M713, M91, W19, M72, M715 and W38 (Fig 5). The pattern of color bands after staining with anisaldehyde/H₂SO₄ and Eherilisch's reagents was observed. Crude extracts of M72, W105, M71, M713, M54, M711 and W211 gave bands of different colors like violet, orange, blue, pink and brown with anisaldehyde/H₂SO₄ reagent. After staining with Eherilisch's reagent, M7, M93, M81 and M32 gave yellow spots which were indicating the presence of N-heterocycles and purple due to some peptides or related compounds (Fig 5). Taddei, et al. [23] studied that if secondary metabolites produce particular colors after staining then it's obvious that compounds belong to a specific chemical group. Solanki, et al. [24] described that chemical screening by thin layer chromatography is a basic tool to find metabolite pattern which is very important to select useful strains. They reported that appearance of grey and brown

color after staining with anisaldehyde/H₂SO₄ may be due to the presence of sugar moieties in the molecules, while the appearance of pink and yellow color of the fractions indicate the absence of sugar moieties in the molecules. In our case fractions exhibiting grey, brown, pink and yellow have been observed and it can be predicted that these fractions may belong to aminoglycosides, anthracyclines, macrolides and glycopeptide structural classes etc.

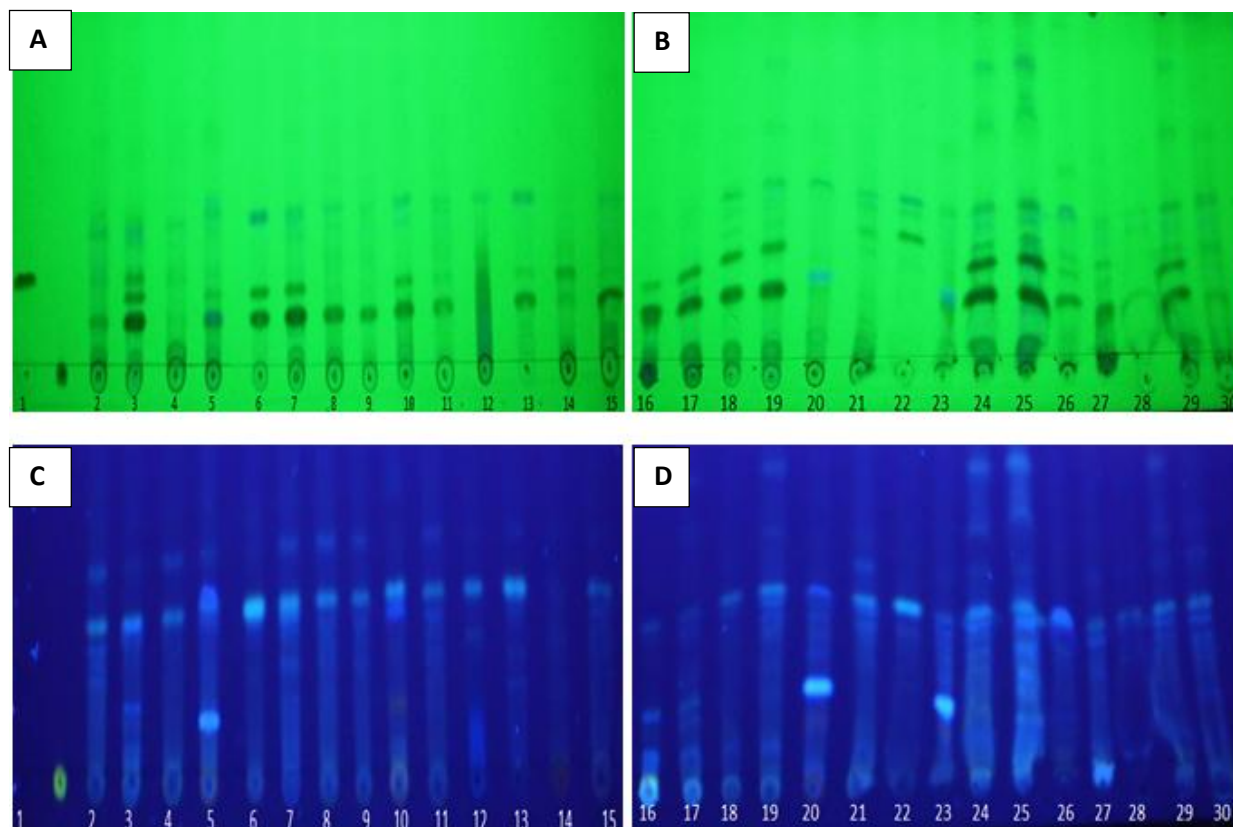


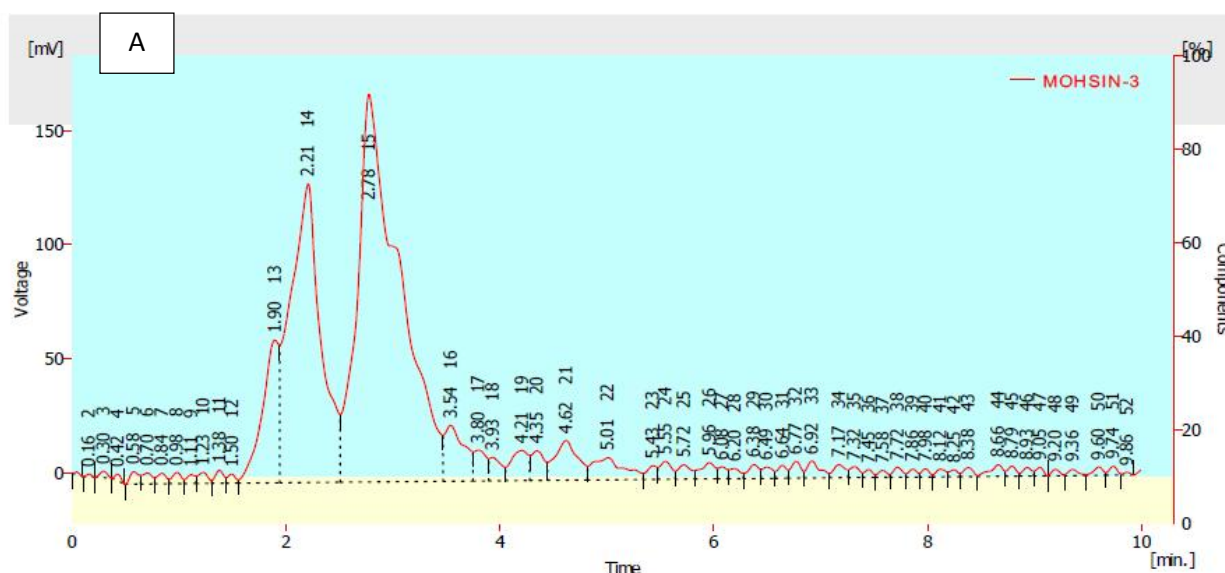
Fig 5 TLC plates under UV at 254nm (A & B) and 366 nm showing UV absorbance and fluorescence (C & D), **Key*** 1 (M5), 2 (M72), 3 (M5ii), 4 (W2), 5 (W211), 6 (M1), 7 (M715), 8 (W101), 9 (W38), 10 (M71), 11 (M714), 12 (M51), 13 (M712), 14 (M713), 15 (W102), 16 (W101), 17 (M54), 18 (M710), 19 (M711), 20 (W124), 21 (M32), 22 (M91), 23 (M7), 24 (M92), 25 (M93), 26 (W19), 27 (W127), 28 (M99), 29 (M81), 30 (Pi)

The crude extracts of most promising bioactive strains were analyzed on the HPLC-UV. The peaks of different active components in each sample were observed at different retention times (t_R) and were analyzed. The crude extract of the isolate W101 showed three major peaks at retention time (t_R) 2.060 min, 2.864 min and 3.576 min with a peak area of 499.014 [mV.s], 3197.365[mV.s] and 548.9 [mV.s] respectively. In case of crude extract of M713, major peaks appeared at retention time (t_R) 2.896 min, 3.200 min and 3.504 min with peak area of 524.29 [mV.s], 529.07 [mV.s] and 382.601[mV.s] respectively. In case of isolate M91, major peaks were observed at retention time (t_R) 1.896 min, 2.208 min, 2.776 min, 3.54 min and 4.620 min with peak area of 665.629 [mV.s], 2601.886 [mV.s], 4616.599 [mV.s],

314.520[mV.s], and 258.736 [mV.s] respectively. In case of isolate W19, the major peaks were observed at retention time (t_R) 2.020 min, 2.688 min, 2.896 min and 3.632 min with peak area of 757.754[mV.s], 245.799 [mV.s], 2243.657[mV.s] and 386.208 [mV.s]. In case of isolate M72, two peaks were appeared at the retention time (t_R) 2.080 min and 2.884 min with peak area of 611.689 [mV.s], and 3710.437 [mV.s]. The appearance of the active components in the extracts at different retention times (t_R) in HPLC chromatograms of different strains shows that these actinomycetes isolates produce more than one active compounds with different concentrations which is visible by different peak areas in each case. Similar results have been reported by Fiedler, et al. [25], they concluded that by using HPLC-UV we can identify new metabolites with respect to their retention time additionally HPLC-UV is a very cost effective method for chemical screening which does not replace other techniques of structure elucidation but is very helpful in resolving unknown peaks regarding their association with specific group of natural compounds.

306 Conclusion

307 Overall the study indicated many actinomycetes isolates from Chenab river (Punjab, Pakistan) showing significant antimicrobial activity and cytotoxicity. The outcome of the study emphasized that actinomycetes from Chenab river should be continuously isolated, characterized and investigated for their antimicrobial potential. Considering the cytotoxicity of isolates, we strongly believe that these isolates should be exploited further which may result in the isolation of some useful antitumor compounds. Hence these strains proved to be the potential source of commercially useful secondary metabolites.



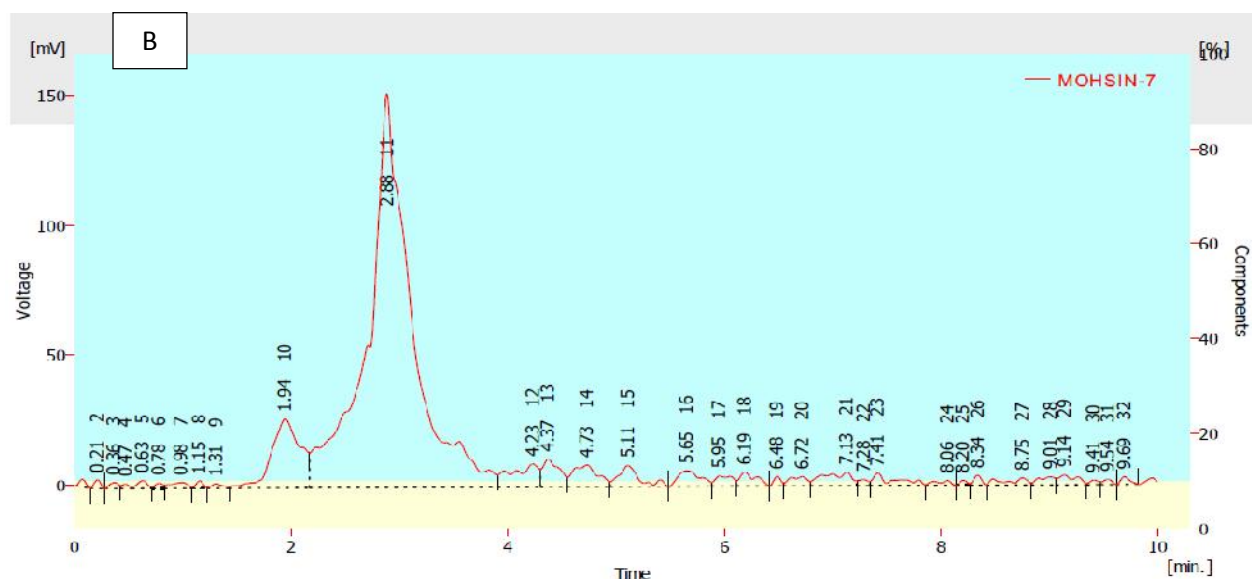


Fig 6 HPLC-UV chromatogram of the selected actinomycetes isolates which exhibited most prominent activity against the pathogenic test organisms (A= M713, B = W101)

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