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Antibacterial and Larvicidal Activity of Ethyl Acetate Extract of Actinomycetes from Soil Samples

Iram Liaqat[1]*, Noor Muhammad[1], Muhammad Mubin[2], Najma Arshad[3], Tehreema Iftikhar[4], Sumera Sajjad[5] and Farzana Rashid[5]

[1]*Microbiology Laboratory, Department of Zoology, Government College University, Lahore-54000, Pakistan*
[2]*Centre of Agricultural Biochemistry and Biotechnology, University of Agriculture, Faisalabad, Pakistan*
[3]*Department of Zoology, University of Lahore, Lahore, Pakistan*
[4]*Department of Botany, Government College University, Lahore, Pakistan*
[5]*Department of Zoology, Lahore College for Women University, Lahore, Pakistan*

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ABSTRACT

This study aimed to explore the antibacterial and larvicidal potential of actinobacterial strains isolated from soil samples of Pakistan. Out of fourteen purified actinobacteria, antibiotic susceptibility profile confirmed five isolates showing resistance against tested antibiotics (ampicillin; lincomycin; rifampicin and erythromycin). Ribotyping confirmed that these isolates belong to *Streptomyces* species and were identified as *S. monticola*, *S. septentrionalis*, *S. polaris*, *S. desertarenae* and *S. lutosisoli*. Primary screening of the five isolates using cross streak method showed excellent zone of inhibition (ZI 10-27 mm) against tested pathogens (*Escherichia coli, Klebsiella pneumoniae, Bacillus licheniformis, Pseudomonas aeruginosa* and *Bacillus subtilis*). Secondary screening using ethyl acetate extracts also showed significant ZI in the range of 06 – 14.0 mm ($P \leq 0.05$) against these pathogens thus confirming their bioactive potential. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of screened Actinomycetes was in the range of 1.3 - 3.5 $mgmL^{-1}$ and 1.9 – 4.0 $mgmL^{-1}$ of bacterial biomass, respectively. Three isolates, *S. monticola*, *S. septentrionalis* and *S. polaris* showed 100 % mortality at 1000 ppm against *Anopheles* 3rd instar larvae. These findings indicated that actinobacterial isolates possess antibacterial and larvicidal potential. Further extraction and purification of bioactive components from these bacterial may be a good source of novel antibiotics and natural-insecticides.

INTRODUCTION

Actinomycetes are free living, Gram-positive and saprophytic bacteria (Rahman *et al*., 2011). They possess secondary metabolites, novel antibiotics and other bioactive molecules against pathogenic bacteria (Chaudhary *et al*., 2013). Actinomycetes are abundantly found throughout the earth including oceans but most of them inhabit terrestrial environment (Ceylan *et al*., 2008).

Phylum Actinobacteria contains 80 genera, among which major ones include *Streptomyces, Micromonospora, Propionibacterium, Salinispora, Nocardia, Mycobacterium, Gordonia, Corynebacterium, Frankia, Gardnerella, Bifidobacterium, Leifsonia* and *Rhodococcus* and other are minor genera (Barka *et al*., 2016). Various secondary metabolites, including novel antibiotics, anticancer agents, antifungal and other pharmaceutically as well as industrial compounds such as enzymes are being produced by organisms belonging to this phylum (Shivlata and Tulasi, 2015). Additionally, many medically useful antitumor drugs for example, anthracyclines (*e.g*., aclarubicin), peptides (*e.g*., actinomycin D), enediynes (*e.g*., neocarzinostatin), aureolic acids (*e.g*., mithramycin), carzinophilin and mitomycins were also isolated from Actinomycetes (Newman and Cragg, 2007; Olano *et al*., 2009).

Importantly, Phylum Actinomycetes is responsible for producing more than 80% of total antibiotics available in the market today. That is why Actinomycetes are

* Corresponding author: iramliaq@hotmail.com
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considered as golden microorganisms of the 21st century because of their capacity to produce different broad-spectrum antibiotics, anticancer and other compounds of therapeutic importance (Aliero *et al*., 2017). Owing to the fact that pathogenic bacteria are increasingly developing resistance against multiple antibiotics and inducing major health problems, scientists are forced to discover novel broad-spectrum antibacterial compounds with significant antibacterial property (Payne *et al*., 2007; Chambers and Deleo, 2009; Michael, 2017).

Unfortunately**,** since the last twenty years, there is not much progress in the discovery of novel antibiotics (Silambarasan *et al*., 2012). Increased number of multi-drug resistant bacteria (Charousová *et al*., 2017) and vector borne diseases in developing country like Pakistan, pose common threat to public health. The discovery of new antibacterial and larvicidal compounds is urgently needed. Ullah *et al*. (2012) checked antibacterial activity of actinobacterial strains isolated from soil samples collected from forest of north western, Pakistan. Fatima *et al*. (2019) performed antibacterial activity of actinobacterial strains isolated from soil samples of Cholistan, Pakistan. Adeela *et al*. (2018) also isolated actinobacterial strains from Cholistan, Pakistan and characterized for methicillin resistant *Staphylococcus aureus* (MRSA). Aslam and Sajid (2016) also checked antibacterial activity of actinobacterial strains but they isolated these strains from water samples collected from Kalar Kahar, Salt range of Pakistan. Aftab and Sajid (2016) isolated actinobacterial strains from various sites of Pakistan like Lahore, Rahim Yar Khan, Sea and Quetta but they performed anti-tumor activity. Anwar *et al*. (2014) checked insecticidal activity of actinobacterial strains isolated from soil samples collected from salt range of Pakistan. That's why in current study, we isolated and screened the actinobacteria from soil samples of Nankana Sab and Kasur, Pakistan. Ethyl acetate extract of the screened actinobacteria were tested for antibacterial and larvicidal potential. To, our knowledge, this is the first study which utilized the indigenous soil actinobacteria to explore antibacterial and larvicidal potential. In future, extraction and purification of bioactive components from these potential Actinomycetes may be a good source of novel antibiotics and natural-insecticides.

MATERIALS AND METHODS

Collection of soil samples

In total, six soil samples were collected from various sites (including fertile agriculture land) of Nankana Saab and Kasur, Punjab, Pakistan. The samples were collected in sterile plastic bags from 15cm depth by removing upper layer of soil, aseptically transported to the Microbiology lab, Department of Zoology, GC University, Lahore and stored at -20°C for future study (Ganesan *et al*., 2017). Following method by Sheik *et al*. (2017), samples were air dried at room temperature for one week, crushed properly in cotton cloth using a piece of wood and sieved through a steel sieve prior to isolation purpose.

Isolation and characterization of pure Actinomycetes

Fourteen actinobacterial strains were isolated and purified following standard microbiological method following Rahman *et al*. (2011). All actinobacterial strains were characterized morphologically and biochemically. Following the morphological characterization, Gram positive actinobacterial strains were selected for biochemical studies. Biochemical test performed included starch hydrolysis, urea hydrolysis, carbohydrates fermentation, citrate utilization, indole, MRVP, catalase and oxidase tests as described by Reddy *et al*. (2011).

Antibiotic susceptibility testing

The antibiotic susceptibility test was performed to check the susceptibility pattern of isolated actinobacteria against commercially available antibiotics and considering that resistant bacteria might possess potential bioactive compounds, conferring these antibacterial and larvicidal potential. The resistance of screened actinobacteria against antibiotics was checked using Kerby-Bauer disc diffusion method (Hudzicki, 2009). Briefly, bacterial cultures adjusted to 0.5 McFarland turbidity standard and spread on the Mueller-Hinton agar (MHA) plates. Four antibiotic discs *i.e*., ampicillin (Am-50 μgmL^{-1}), rifampicin (Rif-50 μgmL^{-1}), erythromycin (Ery-20 μgmL^{-1}) and lincomycin (Linc-50 μgmL^{-1}) were aseptically placed on inoculated plates and incubated for 5 days at 28 ± 2 °C. Appearance of zone (on the basis of written specifications of commercial discs) around the disc showed sensitivity to the antibiotics and vice versa (Hamid, 2011). The zone of inhibition (ZI) was measured in mm.

Ribotyping

Genomic DNA from the five antibiotic resistant actinobacterial strains was extracted using Gene JET Genomic DNA Purification Kit (Thermo Fisher Scientific) following manufacturer's guidelines. Universal primers. Forward primer (5'-AGAGTTGATCCTGGCTCAG-'3) and reverse primer (5'- AAGGAGGTGATCCAGCCGCA-'3) were used for amplification of 16S rRNA gene sequencing. This reaction was carried out using Q thermocycler in a 25 µL volume consisting of genomic DNA (50 ng), Taq DNA polymerase (1 U/µL) and MilliQ grade water. PCR was performed under standard conditions. Amplified PCR product

was checked using 0.9 % agarose gel electrophoresis. The product was purified with Purelink TMquick Gel Extension Kit (Ref K210012, Invitrogen) and sent for sequencing to Axil scientific, Singapore. The sequences were compared with the reference strains from genomic database banks for similarity index and the isolates were taxonomically identified up to species level. The sequences were submitted to GenBank and accession numbers were obtained.

Test organisms

Pathogenic test organisms used in antibacterial study were *B. subtilis* (MN900684), *B. licheniformis* (MN900686), *E. coli* (MN900682), *K. pneumonia* (MN900695) *and P. aeruginosa* (MN900691).

Screening of actinobacterial isolates

The antibacterial activity of actinobacterial isolates was done in two stages *i.e.,* primary screening and secondary screening (Chaudhary *et al*., 2013).

Primary screening

In primary screening, the five actinobacterial strains were cross streaked against pathogenic bacteria following standard cross streak method (Oskay, 2009). In short, the MHA media plates were inoculated by single streak of actinobacterial strains in the center and incubated for 12-14 days at 28 °C (Kumar *et al*., 2014). Following that, pathogenic bacteria were streaked horizontally to former streak. Plates were incubated at 37 °C for further 24 h (Kumar *et al*., 2012). ZI were recorded in mm. Experiment was run in triplicates.

Ethyl acetate extraction and secondary screening

Following antibiotic susceptibility and primary screening of five actinobacterial stains, next step was the secondary screening. Ethyl acetate extracts were prepared following methods by Vinodhkumar *et al*. (2015) and Balakrishnan *et al*. (2017). Secondary screening was performed following standard agar well diffusion method (Chaudhary *et al*., 2013). Antibacterial activity of crude ethyl acetate extract was determined by dissolving extract in DMSO at 3 $mgmL^{-1}$ concentration. Sterile cork borer was used to made wells on MHA plates. The culture of pathogenic bacteria (already adjusted to 0.5 McFarland turbidity standard) was spread on the plates in uniform manner. Following that, 100 µL of each ethyl acetate extract (3 $mgmL^{-1}$) was poured in the wells, except for control. Dimethylsulfoxide (DMSO) and rifampicin (Rif-50 μgmL^{-1}) were used as positive and negative controls, respectively. Plates were incubated for 24 h at 37 °C and ZI were observed in mm.

Measurement of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC of ethyl acetate extracts of five Actinobacterial strains was determined following Ramachandran *et al*. (2018) with slight modification. In short, 24 h old cultures of test bacteria were prepared and optical density (OD_{523}) was adjusted to 0.08 ± 0.2. Three mL of freshly prepared nutrient broth was added in sterile test tubes and 30 µL of bacterial culture was inoculated. Various concentrations (4.5 $mgmL^{-1}$, 4.0 $mgmL^{-1}$, 3.5 $mgmL^{-1}$, 3.0 $mgmL^{-1}$, 2.5 $mgmL^{-1}$, 2.0 $mgmL^{-1}$ and 1.5 $mgmL^{-1}$) of ethyl acetate extracts of five selected isolates were added. Test tube having nutrient broth only was kept as control. Test tubes were incubated at 37 °C for 24 h and OD_{523} was measured. In order to determine MBC, 100 µL of MICs was spread on nutrient agar plate and incubated at 37 °C for 24h.

Collection of anopheles mosquito's larvae and anti-larvicidal assay

The *Anopheles* larvae were collected from water reservoir around the agricultural form Kangan Pur Punjab, Pakistan (geographical coordinates: latitude 30.77°N and longitude 74.07°E) as described by Vijayakumar *et al*. (2010) and Anwar *et al*. (2014). The place was selected because of standing water and large number of available mosquito's larvae. Larvicidal activity of ethyl acetate extract against *Anopheles* larva was assessed using the standard method described by WHO (1996). In short, 100 mg of ethyl acetate extract of Actinomycetes was dissolved in 100 mL of tap water to obtain 1000 parts per million (ppm) concentrations in a beaker. This mixture was serially diluted to obtain 500, 250, 125 ppm concentrations. The ppm values were calculated as described by WHO using Guidelines for laboratory and field testing of mosquito larvicides. According to this manual, 0.1% solution of any extract is equal to 1000 ppm. A control using 1 mL of DMSO was run in parallel. Six early third instar *Anopheles* larvae were added to every beaker, separately. Larvae were fed with dog biscuits and pinch of brewer's yeast in (1:3) ratio. The experiment was performed in triplicates. The beakers were provided with 12:12 light, dark cycle (humidity 30-60 %) at room temperature. The death of the larvae was observed after 24 h.

Statistical analysis

Data were presented as mean and standard error of mean (SEM). Using SPSS Version, 20.0), one-way analysis of variance (ANOVA) followed by a post hoc Tukey test was applied to establish the level of significance ($P \leq 0.05$).

RESULTS

Fourteen Actinomycetes strains (AB1, AB3, AB4, AB5, AB7, GB1, GB3, GB6, GB7, GB8, SCA C1, SCA2, SCA C3 and SCA C7) were isolated and purified on starch casein agar plates. All strains were Gram positive. Morphologically all were irregular and most colonies having gray substrate mycelium and dark gray aerial mycelium. Results for morphological characterization are summarized in Supplementary Table I. Biochemical results evidenced that most of the strains were positive for methyl red/voges-proskauer (MR/VP) test. Biochemical characterization revealed that all the strains hydrolyzed starch, urea and fermented carbohydrates (Supplementary Table II). Biochemical study of Actinomycetes isolates was comparable to Dhananjeyan *et al*. (2010) who isolated Actinobacteria from soil and identified these strains as Gram positive, rod shape having filamentous ones with ability to ferment carbohydrates, starch and urea. Similar to our findings, their isolates also showed positive results for catalase, MRVP and belonged to *Streptomyces*.

Antibiotic susceptibility test

Out of fourteen actinobacterial isolates, only five isolates (AB7, AB1, GB6, GB8 and GB7) were highly resistant against the tested four antibiotics; ampicillin, lincomycin, rifampicin and erythromycin without any ZI, while nine isolates (SCA C3, SCA C7, SCA C1, GB3, SCA2, AB4, AB5 and AB3) showed least resistance with upto 6 mm ZI (Fig. 1). Previously, Kamble and Kulkarmi (2012) reported the antibiotic resistance of soil Actinomycetes against commercial antibiotics (ampicillin, penicillin, chloramphenicol and tetracycline). Also, Hamid (2011) reported similar antibiotic resistant pattern of *Streptomyces* sp. and found that *Streptomyces* sp. was resistant against amphotericin B, penicillin and sulphamethoxazole. Authors concluded that significant differences in antibiotic susceptibility patterns and resource utilization within and among *Streptomyces* species may be linked with local adaptations.

Molecular identification and phylogenetic study

Ribotyping of antibiotic resistant Actinomycetes subjected to BLAST confirmed their homology to *Streptomyces* sp. Isolates AB1, AB7, GB6, GB7, and GB8 showed 100 % homology to *S. monticola* (MN865480), *S. septentrionalis* (MN865593), *S. polaris* (MN865672), *S. desertarenae* (MN865690) and *S. lutosisoli* (MN865691), respectively. Phylogenetic tree was shown in Figure 2. Similar findings were reported by Ganesan *et al*. (2017) who isolated actinobacterial strains from soil and confirmed their homology to *Streptomyces* sp. on the basis of ribotyping. Our finding corroborated with Abdelfattah *et al*. (2016), who reported soil *Streptomyces* sp. exhibiting inhibitory potential against pathogenic bacteria.

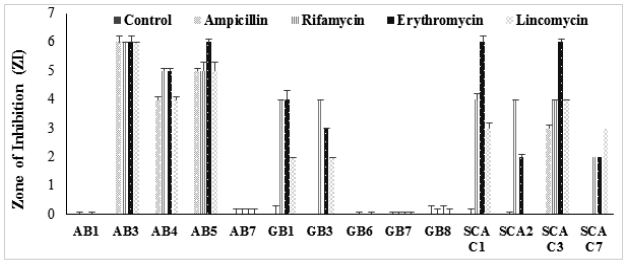

Fig. 1. Antibiotics susceptibility test. Actinobacterial stains (AB1, AB7, GB6, GB7 and GB 8) showed resistance against selected antibiotics (rifampicin, lincomycin, erythromycin, amplicillin). ZI was measured in mm. Experiment was run in triplicates.

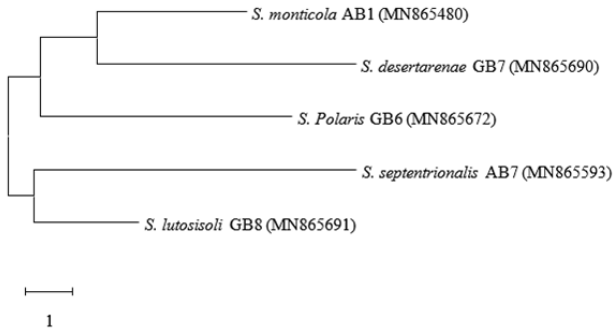

Fig. 2. Phylogenetic tree based on 16S rRNA ribotyping showed genetic variability among the 05 Actinomycetes strains. The tree was constructed using MEGA X software by neighbor joining tree method.

Antibacterial activity using primary and secondary screening

In primary screening, five strains *i.e*., *S. monticola* AB1, *S. septentrionalis* AB7, *S. polaris* GB6, *S. desertarenae* GB7 and *S. lutosisoli* GB8 showed ZI upto 24.0 mm against *B. subtilis,* 27.0 mm against *B. licheniformis*, 19.0 mm against *E. coli* and 7.0 mm against *P. aeruginosa,* respectively. All the actinobacterial strains showed statistically significant antibacterial activity ($P \leq 0.05$) against all the test pathogens in primary screening (Table I). Significant ZI observed by actinobacteria against test pathogens observed in current study are in agreement with the findings by Pushpa and Doss (2016) who described that Actinomycetes sp. isolated from soil sample showed remarkable antibacterial activity against human pathogens (*Klebsiella* sp., *P. aeruginosa* and *B. subtilis*) and fish pathogens (*Aeromonas hydrophila*, *B. subtilis*, *P.*

aeruginosa, *Vibrio harveyi* and *V. alginaticus*).

In secondary screening, five ethyl acetate extracts of five Actinomycetes strains showed significant antibacterial activity ($P \leq 0.05$) against test pathogens. Previously, Maleki *et al*. (2013) also reported ethyl acetate as better solvent for extraction compared to diethyl ether, dichloromethane, ethyl acetate, n-Hexane, chloroform, methanol, and water extract. Table II indicated that *S. monticola* AB1, *S. septentrionalis* AB7 and *S. desertarenae* GB7 showed significantly high ($P \leq 0.05$) ZI (12.0 mm) against *P. aeruginosa* 14.0 mm by *S. polaris* GB6 against *P. aeruginosa* and 10.0 mm by *S. lutosisoli* GB8 against both *B. licheniformis* as well as *P. aeruginosa*. Kumar and Rao (2012) reported that ethyl acetate extracts of actinobacteria possess strong antibacterial potential. It is established that *Streptomyces* isolates produce active and enhanced antibiotics in different nutritional media with various carbon sources (glucose, starch) and growth promoters ($CaCO_3$). Also, in some cases the extraction of secondary metabolites from liquid culture affects the antimicrobial activity of active substance (Dezfully and Ramanayaka, 2015), however this was not observed in current study. These results are comparable with Basavaraj *et al*. (2010) and Valli *et al*. (2012) who reported that actinobacterial sp. showed excellent ZI against *K. pneumonia, E. coli, B. subtilis* and *Pseudomonas* sp. Likewise, Maleki *et al*. (2013) reported that *Streptomyces* isolates were effective in growth inhibition of *E. coli, K. pneumonia, Shigella flexneri, Listeria monocytogenes, B. cereus, Yersinia enterocolitica* and *Staphylococcus aureus*.

MIC and MBC concentration

The MIC values were in the range of 1.3-3.4 $mgmL^{-1}$ and MBC were in the range of 1.9-4.0 $mgmL^{-1}$ (Table III). The results indicated that MIC and MBC of five actinobacterial strains were also significant ($P \leq 0.05$) against all the test bacteria. These results are in accordance to Chaudhary *et al*. (2013), who published that crude ethyl acetate extracts of actinobacterial strains showed MIC between 1.5 to 2.5 $mgmL^{-1}$ and MBC between 2.0 to 3.5 $mgmL^{-1}$ against *K. pneumonia, E. coli, B. licheniformis* and *P. aeruginosa*. All this data, collectively suggested that isolated Actinomycetes possessed significant antibacterial potential and led us to investigate the larvicidal activity.

Table I. Antibacterial activity of Actinomycetes isolates by cross streak method.

Sr. No.	Strains	Zone of inhibition in mm (Mean±S.E)				
		B. licheniformis	***B. subtilis***	***E. coli***	***P. aeruginosa***	***K. pneumonia***
1	*S. monticola* AB1	19.33 ± 0.3^{cd}	20.33 ± 0.3^{b}	18.3 ± 0.3^{c}	5.33 ± 0.3^{d}	2.3 ± 0.3^{a}
2	*S. septentrionalis* AB7	27.33 ± 0.0^{d}	24.33 ± 0.3^{c}	7.33 ± 0.1^{b}	6.33 ± 0.1^{ab}	5.0 ± 0.3^{a}
3	*S. polaris* GB6	5.66 ± 0.3^{a}	18.66 ± 0.3^{b}	18.33 ± 0.3^{b}	5.33 ± 0.3^{a}	4.33 ± 0.3^{a}
4	*S. desertarenae* GB7	22.33 ± 0.3^{d}	19.0 ± 0.0^{c}	19.33 ± 0.3^{c}	7.33 ± 0.3^{b}	4.33 ± 0.3^{a}
5	*S. lutosisoli* GB8	18.66 ± 0.3^{c}	17.66 ± 0.3^{c}	18.66 ± 0.3^{c}	6.33 ± 0.3^{b}	3.0 ± 0.0^{a}

The data was expressed as mean ± SEM of all isolates. One-way analysis of variance (ANOVA) with post hoc Tukey was used to determine the antibacterial effect of 14 actinobacterial isolates against 05 test bacteria *i.e. B. licheniformis, B. subtilis, E. coli, K. pneumonia* and *P. aeruginosa*. $P \leq 0.05$ were considered significant in all tests.

Table II. Antibacterial activity of ethyl acetate extract of five Actinomycetes isolates by secondary screening method.

Sr. No.	Actinomycetes strains	Zone of inhibition in mm (Mean ± S.E)			
		B. licheniformis	***B. subtilis***	***E. coli***	***P. aeruginosa***
1	*S. monticola* AB1	8.16 ± 0.1^{cd}	7.0 ± 0.2^{de}	8.0 ± 0.2^{de}	12.03 ± 0.0^{d}
2	*S. septentrionalis* AB7	8.66 ± 0.3^{de}	8.0 ± 0.5^{e}	7.0 ± 0.2^{cd}	12.06 ± 0.0^{d}
3	*S. polaris* GB6	6.83 ± 0.4^{c}	4.8 ± 0.4^{c}	5.0 ± 0.2^{b}	14.0 ± 0.1^{e}
4	*S. desertarenae* GB7	11.0 ± 0.5^{f}	6.02 ± 0.1^{cd}	9.0 ± 0.2^{e}	12.0 ± 0.1^{d}
5	*S. lutosisoli* GB8	10.0 ± 0.2^{ef}	7.96 ± 0.3^{e}	6.0 ± 0.2^{bc}	10.0 ± 0.2^{c}
6	P.C	3.1 ± 0.1^{b}	2.06 ± 0.0^{b}	1.03 ± 0.0^{a}	6.03 ± 0.0^{b}
7	N.C	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

The data was expressed as mean ± SEM of ethyl acetate extract active actinobacterial isolates. One-way analysis of variance (ANOVA) with post hoc Tukey was used to determine the independent antibacterial effect of 05 active actinobacterial isolates against 04 test bacteria *i.e. B. licheniformis, B. subtilis, E. coli* and *P. aeruginosa*. $P \leq 0.05$ was considered significant in all tests.

Table III. MIC and MBC values of ethyl acetate extracts of five Actinomycetes isolates against four test pathogens.

Bacterial extract	Test strains	MIC (mgmL^{-1}) (Mean ± S.E)	MBC (mgmL^{-1}) (Mean ± S.E)
S. monticola AB1	*E. coli*	2.4 ± 0.0^{a}	3.0 ± 0.5^{a}
	P. aeruginosa	2.9 ± 0.0^{c}	3.5 ± 0.0^{b}
	B. subtilis	2.2 ± 0.3^{c}	2.7 ± 0.5^{d}
	B. licheniformis	2.2 ± 0. 5^{b}	2.6 ± 0.0^{b}
S. septentrionalis AB7	*E. coli*	2.3 ± 0.3^{a}	3.0 ± 0.5^{a}
	P. aeruginosa	2.4 ± 0.0^{a}	3.0 ± 0.5^{a}
	B. subtilis	1.6 ± 0.5^{b}	2.3 ± 0.0^{ab}
	B. licheniformis	2.0 ± 0.0^{b}	2.5 ± 0.0^{b}
S. polaris GB6	*E. coli*	3.4 ± 0.1^{c}	4.0 ± 0.0^{c}
	P. aeruginosa	3.2 ± 0.0^{d}	3.8 ± 0.5^{c}
	B. subtilis	2.0 ± 0.5^{c}	2.4 ± 0.3^{cd}
	B. licheniformis	2.6 ± 0.0^{c}	3.2 ± 0.5^{c}
S. desertarenae GB7	*E. coli*	3.4 ± 0.1^{c}	4.0 ± 0.0^{c}
	P. aeruginosa	3.2 ± 0.0^{d}	3.8 ± 0.5^{c}
	B. subtilis	2.0 ± 0.5^{c}	2.4 ± 0.3^{cd}
	B. licheniformis	2.6 ± 0.0^{c}	3.2 ± 0.0^{c}
S. lutosisoli GB8	*E. coli*	2.9 ± 0.3^{b}	3.5 ±0. 5^{b}
	P. aeruginosa	2.7 ± 0.0^{b}	3.2 ± 0.0^{a}
	B. subtilis	1.3 ± 0.5^{a}	1.9 ± 0.5^{a}
	B. licheniformis	2.4 ± 0.3^{c}	3.0 ± 0.0^{c}

The data was expressed as mean ± SEM of MIC and MBC of active actinobacterial isolates. One-way analysis of variance (ANOVA) with post hoc Tukey was used to determine the independent MIC and MBC effect of 05 active actinobacterial isolates against 04 test bacteria *i.e. B. licheniformis, B. subtilis, E. coli* and *P. aeruginosa*. P ≤ 0.05 was considered significant in all tests.

Larvicidal assay

Ethyl acetate extracts of five actinobacterial isolates showed highly significant (P ≤ 0.05) larvicidal activity against *Anopheles* larvae. Three actinobacterial isolates (*S. monticola* AB1, *S. septentrionalis* AB7 and *S. polaris* GB6) extracts showed 100 % mortality at 1000 ppm after 24 h of incubation. Isolate, *S. desertarenae* GB7 extract showed 83.1% and *S. lutosisoli* GB8 showed 68.7% mortality at 1000 ppm (Table IV). Similar observations were made by Jiang and Mulla (2009), who isolated *Streptomyces* (*Saccharopolyspora spinosa*) from soil and reported its potential to kill *Anopheles* larvae. Likewise, Dhanasekaran *et al*. (2009) also isolated actinobacterial strains from soil having larvicidal activity. Authors observed effective killing of *Anopheles* larvae by one isolate (*S. bikiniensis*) identified by DNA-DNA homology. All these finding support our study that these all five soil Actinomycetes strains possess l insecticidal activity. To our knowledge, this is the first study to report the larvicidal potential of indigenous *S. monticola, S. septentrionalis, S. polaris, S. desertarenae* and *S. lutosisoli*.

Table IV. Larvicidal activity of ethyl acetate extracts of five Actinomycetes isolates against *Anopheles* 3rd instar larvae.

Actinobacterial strains	Ethyl acetate extract concentration (ppm)	Mortality (%) of 6 larvae after 24 h
S. monticola AB1	1000	100.0 ± 0.0^{L}
	500	85.6 ± 0.1^{k}
	250	66.8 ± 0.1^{h}
	125	37.6 ± 0.1^{e}
S. septentrionalis AB7	1000	100.0 ± 0.0^{L}
	500	83.3 ± 0.0^{j}
	250	52.0 ± 0.0^{g}
	125	33.3 ± 0.0^{d}
S. polaris GB6	1000	100.0 ± 0.0^{L}
	500	83.3 ± 0.0^{j}
	250	50.0 ± 0.0^{f}
	125	18.7 ± 0.1^{c}
S. desertarenae GB7	1000	83.1 ± 0.5^{j}
	500	52.0 ± 0.0^{g}
	250	33.3 ± 0.0^{d}
	125	16.7 ± 0.1^{b}
S. lutosisoli GB8	1000	68.7 ± 0.0^{i}
	500	37.1 ± 0.5^{e}
	250	16.2 ± 0.6^{b}
	125	0.0 ± 0.0^{a}

Four different concentrations *i.e.*, 1000, 500, 250 and 125 ppm of Actinomycetes ethyl acetate extract tested against 6 *Anopheles* larvae. The data was expressed as mean ± SEM of antilarvicidal activity of active actinobacterial isolates extracts. One-way analysis of variance (ANOVA) with post hoc Tukey test was used to determine the independent antilarvicidal effect of 05 active actinobacterial isolates against *Anopheles* 3rd instar larvae. P ≤ 0.05 was considered significant in all tests.

CONCLUSION

It can be concluded from the present study that actinobacterial flora of Kasur and Nankana Sab, Pakistan is a large source of *Streptomyces* sp. having antibacterial and larvicidal potential. Future studies regarding identification and structure interpretation of the novel bioactive components of screened Actinomycetes are recommended to overcome the great dilemma of increasing antimicrobial/ larvicidal resistance by surrounding opportunistic

microbes/insects all over the globe.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20200526130518

Statement of conflict of interest

The authors have declared no conflict of interest.

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