

# Antibiotics Resistance Profiling and In-Vitro Inhibition of Clinical *Klebsiella* Strains by Actinomycetes Isolated From Different Ecological Niches in Pakistan

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## Abstract

**Background:** Multidrug resistance among different pathogens is increasing immensely day by day. To control these problems, we need new potent antimicrobial agents in repository of antibiotics.

**Objectives:** This study aimed at investigation of antibiotics resistance pattern of pathogenic *Klebsiella* strains isolated from clinical samples in Lahore region Pakistan and study inhibition of resistant strains by natural extracts obtained from actinomycetes isolated from different ecological niches in Pakistan.

**Materials and Methods:** The isolated *Klebsiella* strains were identified by morphological, biochemical and physiological characterization along with 16S rRNA gene sequencing. Antibiotics susceptibility was determined by standard Kirby Bauer disc diffusion assay. The biological and chemical screening was performed for detection of active secondary metabolites produced by actinomycetes against resistant *Klebsiella* strains. Biological screenings include antimicrobial activity by agar diffusion assay and brine shrimp microwell cytotoxicity assay. In chemical screening, the crude extracts of actinomycetes strains were analysed by TLC and HPLC-UV techniques.

**Results:** The isolated *Klebsiella* strains showed resistance against most of the antibiotics as follows; ceftriaxone > cephalixin > cefpirome > ceftazidime > levofloxacin > ciprofloxacin = ceftriaxime = fusidum > cefoperazone > ampicillin sulbactam. The actinomycetes strain A19, A20, A2, A10, A6 and A8 exhibited remarkable activity against resistant *Klebsiella* strains. The strains A19 and 20 showed excellent inhibitory effects on all isolated multidrug resistant *Klebsiella* strains.

**Conclusions:** The clinical *Klebsiella* strains isolated from Lahore region, Pakistan exhibited resistance to most commonly used antibiotics, which can be a serious threat to public health. The study reported some potential actinomycetes strains, which exhibit promising activity against multidrug resistant *Klebsiella* strains.

**Keywords:** Antimicrobial Agents, Actinomycetes, Cytotoxicity, HPLC-UV, *Klebsiella*

## 1. Background

*Klebsiella* resistance has been increased immensely during the last few decades, so the need for new antimicrobial agents is greater than ever due to emergence of multidrug resistance in communal pathogens (1). Resistance of bacteria against antibiotics has been a major problem in the treatment of disease. Infectious diseases are still the second principal cause of death worldwide (2). Hospital-acquired urinary tract (UTI) infections are caused by medically important bacteria named *Klebsiella pneumoniae*, which accounts for a greater proportion of pneumonia, septicaemia and soft tissue infections. Nosocomial outbreaks occur in hospitals because of rapid spread of different *Klebsiella* species (3). Increased resistance against specific antibiotics is the result of self-medication, overuse of drugs and mutations in specific genes (4). In Pakistan, ESBL producing strains of *K. pneumoniae* have been reported from different hospitals and centers;

however, due to less knowledge of resistance mechanism in *K. pneumoniae* ESBL, attention has not been paid properly in Pakistan. On the other hand, carbapenem resistant strains are among the emerging resistant strains reported in few hospitals of Pakistan as well. As such yet there is no report of Carbapenem resistance in *K. pneumoniae* from Pakistan, but rapid emerging resistance of these antibiotics among different nosocomial pathogens is an alarming sign for infectious control authorities to expect carbapenem resistant *K. pneumoniae* in the near future (5).

To control this emerging antibiotic resistance scenario, we need adding new potent bioactive compounds. To accomplish this, several microbial groups help us by providing bioactive compounds with miraculous activity against superbugs. Actinomycetes is one of the famous and remarkable groups. It contains several genera, which

all play their role in controlling antibiotic resistant superbugs. One of its genera known as *Streptomyces* produces most important bioactive products. About 22,500 bioactive compounds are reported and only 16,500 compounds have revealed antibiotic producing ability. Among them about 10,100 (45%) are discovered from actinomycetes, in which 7,630 from *Streptomyces* and 2,470 from other rare actinomycetes (6).

In the last few decades, for production of novel secondary metabolites, unique species of actinomycetes were isolated that are difficult to culture and can grow under harsh conditions such as alkaline and acidic environment (7). These actinomycetes species give us compounds with unique chemical moieties having remarkable activity against multidrug resistant bacteria. These compounds are identified using several chromatographic techniques among which high performance liquid chromatography (HPLC) is consistently used for identification of active microbial secondary products (8).

In this study *Klebsiella* strains were isolated from clinical samples collected from a tertiary care hospital. The isolates were identified by morphological, physiological biochemical and genetic characterization. Antibiotic susceptibility of selected *Klebsiella* isolates was determined using standard Kirby Bauer disc diffusion assay. The genomic DNA of the isolated strains was extracted and 16S *rRNA* gene was amplified and purified by gel purification technique. To screen bioactive compounds, chemical screening of extracts obtained from actinomycetes was performed by thin layer chromatography and high performance liquid chromatography. Cytotoxicity of the crude extracts was determined against brine shrimps (*Artemia salina*) using a microwell cytotoxicity assay. Biofilm formation studies and effect of the crude extracts of actinomycetes on *Klebsiella pneumoniae* tested on 96-well microtiter plates.

## 2. Objectives

The current study aimed at investigating antibiotics resistance in *Klebsiella* in our region and determining inhibitory potential of different actinomycetes strains against isolated drug-resistant *Klebsiella* strains.

## 3. Materials and Methods

### 3.1. Selective Isolation of *Klebsiella* Strains

From the tertiary care hospital (Punjab institute of cardiology, Lahore), different clinical samples were collected and processed. Samples including urine, pus and wound swabs were cultured using routine bacteriological media including MacConkey's agar (peptone 17.0 g, proteose peptone 3.0 g, lactose 10.0 g, NaCl 5.0 g, crystal violet 1.0 g, neutral red 30.0 g, bile salts 1.5 g, agar 13.5 g and distilled water up to 1,000 mL) and CLED agar (enzymatic digest of gelatin 4 g, enzymatic digest of casein 4 g, beef extract 3

g, lactose 10 g, L-cysteine 0.128 g, bromothymol blue 0.02 g and agar 15 g in 1 L). About 30 isolates were selected on CLED agar. Selective isolation of lactose fermenter (LF) from non-lactose fermenter (NLF) Gram-negative microorganisms was performed on MacConkey's medium. About 25 of 30 isolates were selected; the selected isolates belong to the genus *Klebsiella*, which was selected for further screening experiments.

### 3.2. Morphological, Biochemical and Physiological Characterization

A small inoculum of each strain was picked and plated by streaking on MacConkey's agar for Gram-negative strains and incubated at 37°C for 24 hours. The purified colonies obtained were studied for different morphological characteristics including colony size, consistency, shape, elevation, margins, colour and pigments diffusing into the medium. The colonies were observed visually and under stereoscopic microscope for in-depth analysis. For cell shape, arrangement of cells, size of the cells, branching pattern and chains of cells, 24 hours incubated cultures at 37°C observed under microscope after performing Gram's staining. Biochemical characterization includes the cytochrome oxidase and IMViC test criteria; a series of tests were performed for characterization of selected enteric family members (9).

### 3.3. Antibiotics Susceptibility Test

Antibiotics susceptibility test was performed by swabbing the test organism on Muller Hinton agar (casein hydrolysate 17.5 g, beef extract 2.0 g, starch 1.5 g, agar 17.0 g in 1 L) and the respective antibiotics were placed on the agar surface with proper distance. After, these plates were incubated at 37°C for 24 hours. After incubation, zone of inhibition was measured and isolated strains were reported as sensitive or resistant according to the clinical laboratory standards institute's guidelines (CLSI, 2013).

### 3.4. Genetic Characterization, 16S *rRNA* Sequencing

After genomic DNA extraction, PCR amplification of 16S *rRNA* was performed using forward and reverse primers (27F 5'-AGAGTTGATCCTGGCTCAG-3' and 1522R 5'-AAGGAGGTGATCCARCCGCA-3'). The amplified products were purified using FavorPrep MicroElute Gel Extraction Kit®. The selected cleaned gene products were sequenced by dye terminator chemistry using an automated sequencer. After sequencing, results of nucleotide BLAST were obtained to check the percentage homology with other reported microorganisms. The selected strains showed maximum homology with *Klebsiella pneumoniae*. The sequences were submitted to Genbank (through BANKit) to get accession number of submitted sequences of 16S *rRNA* genes.

### 3.5. Preparation of Small Scale Cell Extracts

After inoculating actinomycetes strains (obtained

from MMG department bioactive culture collection) in 100 mL GYM-broth, the flasks were incubated on rotary shaker for seven days at 28°C. After incubation, 100 mL of ethyl acetate was poured in every flask and mixture was sonicated for 30 minutes. After sonication, the cells were poured in separating funnel and vigorously shaken for 15 minutes and the separating funnels kept undisturbed to separate the organic and inorganic layers. Upper layer of ethyl acetate was removed carefully and evaporated on rotary evaporator to get the crude extract and about 4 mL methanol was added to obtain the methanolic extracts. These extracts were further used for biological and chemical screening through HPLC-UV analysis (10).

### 3.6. Biological Screening

#### 3.6.1. Screening for Antimicrobial Activity

Muller Hinton agar plates were prepared and 24-hour broth culture of test strains of *Klebsiella* swabbed on these plates. Streaked plates were placed on the bench top for 10 minutes at room temperature. After then sterile pasture pipette was used for making wells in the agar plates. About 60 µL of the supernatant from actinomycetes shaking cultures was loaded in each well and incubated for 24 hours at 37°C. Plates were observed for clear zones around the wells, which are indicative for sensitivity of test strains for the respective actinomycetes extract.

#### 3.6.2. Brine Shrimp Microwell Cytotoxicity Assay

Cytotoxicity of the crude extracts of actinomycetes was determined by the brine shrimps microwell cytotoxicity assay. Dried eggs of *Artemia salina* were added to 400 mL of artificial seawater. Tank was left aerated for 24 - 48 hours at room temperature. After that tank was kept undisturbed, to settle down the dead larvae. The tank was covered with aluminium foil and kept in the light to collect all of the live phototrophic larvae. About 30 larvae were transferred to each microtiter well previously filled with 200 µL of artificial sea water. About 20 µL of the crude extract dissolved in DMSO was added. After 24 hours, with the help of microscope, number of dead larvae (A) was counted. The remaining alive larvae killed by adding 100 µL methanol to determine the total number of larvae.

$$M = \frac{A-B-N}{G-N} \times 100$$

Where M = percent of the dead larvae after 24 hours; A = number of the dead larvae after 24 hours; B = average number of the dead larvae in the blind samples after 24 hours; N = number of the dead larvae before starting the test; G = total number of larvae.

### 3.7. Chemical Screening

#### 3.7.1. Thin Layer Chromatography (TLC)

The crude extracts were spotted on the TLC plate in a superimposed manner. The plates were let to dry and developed with CH<sub>2</sub>Cl<sub>2</sub>/10% MeOH solvent system. The developed air dried plates were visualized under UV light at 254 nm and 366 nm. The components showing UV absorbance and fluorescence were marked and scanned. The TLC plate was then stained by spraying with anisaldehyde/H<sub>2</sub>SO<sub>4</sub> and Ehrlich's reagents separately.

#### 3.7.2. High Performance Liquid Chromatography (HPLC-UV)

The crude extracts were analysed on the HPLC system (Sykum HPLC system) using the software clarity. The column used was reverse phase C18 from phenomenex with 30 cm length. Mobile phase used was methanol and water (95:5) and the flow rate was adjusted to 1 mL/minute. The crude extract was dissolved in HPLC grade methanol and 50 µL was injected through the microsyringe injector. The sample was run for 15 minutes and UV absorbance was determined at 254 nm. The peaks of each sample were analyzed by comparing different retention times (R<sub>t</sub>) with standard UV absorption data of secondary metabolites.

### 3.8. Detection of Biofilm Formation and Its Disruption by the Natural Extracts

#### 3.8.1. Congo Red Agar Method (CRA)

The medium was prepared (brain heart infusion broth 3.7 g, Congo red 0.08 g, glucose 10 g, agar 1.5 g and water 100 mL) and poured. Plates were inoculated with test organism and incubated at 37°C for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicate biofilm production; weak producers usually remain pink, though occasional darkening at the centre of colonies observed in some cases.

#### 3.8.2. Effect of Crude Extracts of Actinomycetes on Biofilm Formation

The effect of the crude extracts of actinomycetes against biofilm forming capability of *Klebsiella pneumoniae* was tested on 96-well microtiter plates. The crude extracts at concentration of 50 µL were added in LB broth containing bacterial suspension of 1% inoculum from overnight culture of *Klebsiella pneumoniae*. Each test row was accompanied by blank sample. The plates were incubated for 3 days at 37°C on rotary shaker. After incubation, the cells and media were discarded, and weakly adherent cells were removed by washing with deionized water and allowed to air dry before being stained. The biofilms were

stained by 200 µL of 0.4% crystal violet solution for 10 minutes. After that, the crystal violet was discarded and the wells were washed twice with water. The wells were then allowed to dry. The crystal violet was solubilized with 1 mL of methanol. At the end the optical density was determined at 570 nm (11).

#### 4. Results

Total of 30 *Klebsiella* strains were isolated from clinical samples; only 25 strains were selected based on preliminary biochemical screening tests. Furthermore, only 10 different strains were selected for detailed analysis of antibiotics susceptibility experiments, biological screening, comparative efficacy and biofilm forming ability. After sequencing, nucleotide BLAST was performed to check the percentage homology with other already reported organisms. The selected strains showed maximum homology with 16s rRNA gene sequence of *Klebsiella pneumoniae* already present in the NCBI database. After confirmation, the sequences were submitted to Genbank (BANKit). Accession number for the strains K1, K5, K6 and K13 are shown in Table 1.

The results of antibiotics susceptibility testing indicated that *Klebsiella* isolates showed resistance against most of used antibiotics. They showed significant resistance to the following antibiotics: amoxicillin, ceftriaxone, cephalixin, cefpirome and cefoxitin. The order of resistance is as follows; amoxicillin = ceftriaxone > cephalixin > cefpirome > cefoxitin = cefepime > levofloxacin > ciprofloxacin = ceftazidime = fusidum > cefoperazone > ampicillin sulbactam (Table 2).

The results of biological screening (well diffusion assay) indicated that actinomycetes strains were found to be effective against isolated multidrug resistant test organisms of *Klebsiella* genus. Extracts that exhibited the maximum activity on *Klebsiella* were of following actinomycetes strains; A19, A10, A20, A1, A2, A6 and A5. The activity shown by the actinomycetes crude extract has proven to be effective as they inhibit the growth of test *klebsiella* strains significantly (Figure 1). The strains A19, A20, A2 and A10 have shown remarkable zone of inhibition against *Klebsiella* strains and zones were about 20, 19, 18 and 22 mm, respectively. Strains A19 and 20 showed inhibitory effects on almost all of the isolated *Klebsiella* strains.

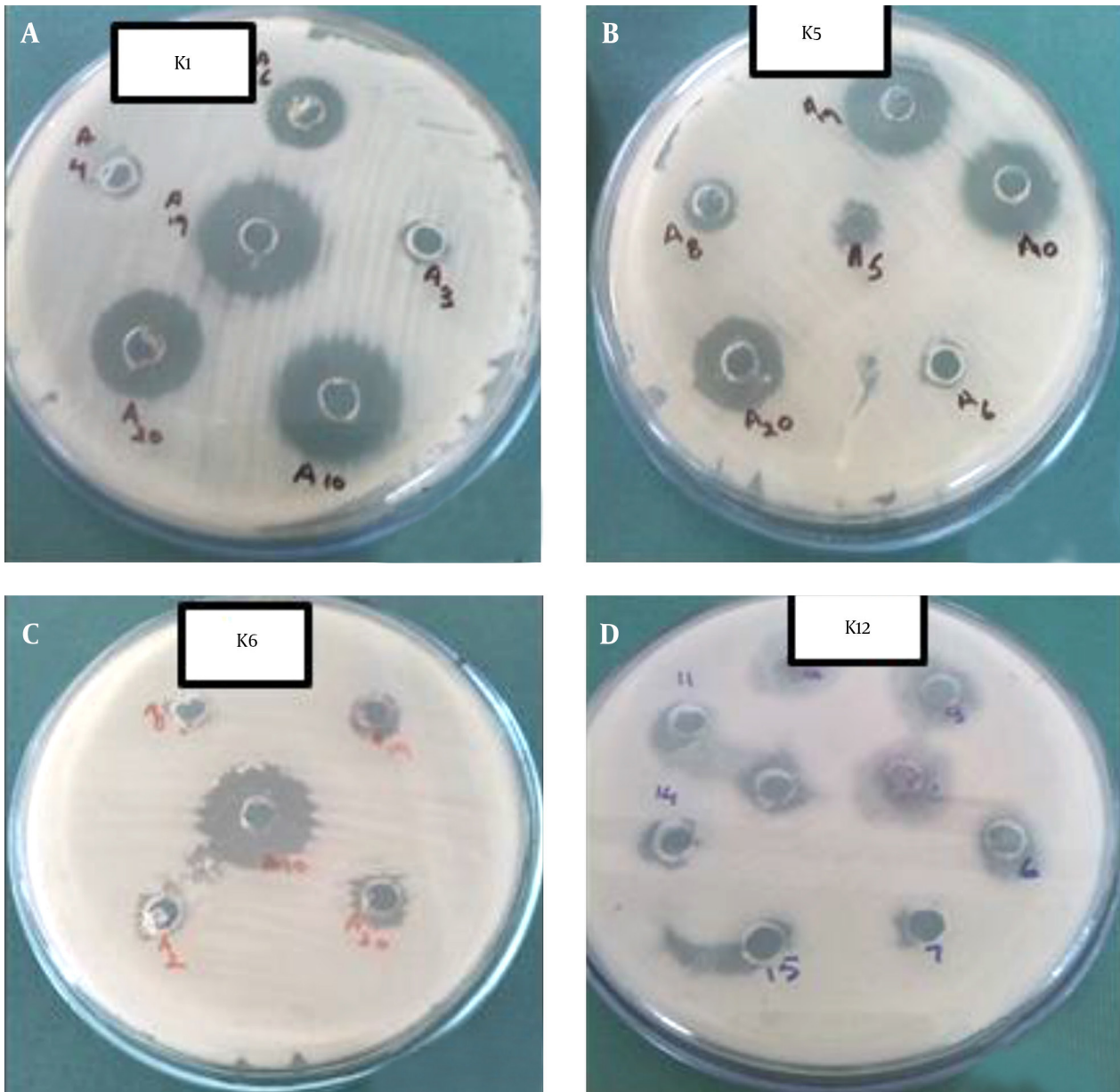
**Table 1.** GenBank Accession Numbers and the Percentage Homology With *Klebsiella pneumoniae*

| S. No. | Isolates | Nucleotide Length, bp | Age Homology, % | Organism                     | Genebank Accession No. |
|--------|----------|-----------------------|-----------------|------------------------------|------------------------|
| 1.     | K1       | 1530                  | 99.0            | <i>Klebsiella pneumoniae</i> | KR822682               |
| 2.     | K5       | 1411                  | 99.0            | <i>Klebsiella pneumoniae</i> | KR822680               |
| 3.     | K6       | 403                   | 98.0            | <i>Klebsiella pneumoniae</i> | KR822681               |
| 4.     | K13      | 498                   | 98.0            | <i>Klebsiella pneumoniae</i> | KR822679               |

**Table 2.** Antibiotics Resistance Pattern of *Klebsiella* Against Different Antibiotics

| S. No. | Antibiotics Applied  | Percentage, % |           |
|--------|----------------------|---------------|-----------|
|        |                      | Resistant     | Sensitive |
| 1.     | Cefepime             | 76.9          | 23.0      |
| 2.     | Cefpirome            | 84.6          | 15.3      |
| 3.     | Ciprofloxacin        | 61.5          | 38.5      |
| 4.     | Cefoxitin            | 76.9          | 23.0      |
| 5.     | Ceftriaxone          | 100           | 0         |
| 6.     | Fusidum              | 61.5          | 38.4      |
| 7.     | Levofloxacin         | 69.2          | 30.7      |
| 8.     | Amoxicillin          | 100           | 0         |
| 9.     | Ceftazidime          | 61.5          | 38.5      |
| 10.    | Ampicillin sulbactam | 46.2          | 53.8      |
| 11.    | Cefoperazone         | 53.8          | 46.2      |
| 12.    | Cephalexin           | 92.3          | 7.6       |
| 13.    | Imipenem             | 30.7          | 62.9      |

**Figure 1.** Antimicrobial Activity With Crude Extract



A, Antimicrobial activity shown by A19, A20, A6, A10, K1 is more susceptible to the crude extracts compared to other *Klebsiella*; B, antimicrobial activity against K5; C, antimicrobial activity against K6; D, antimicrobial activity against K7.

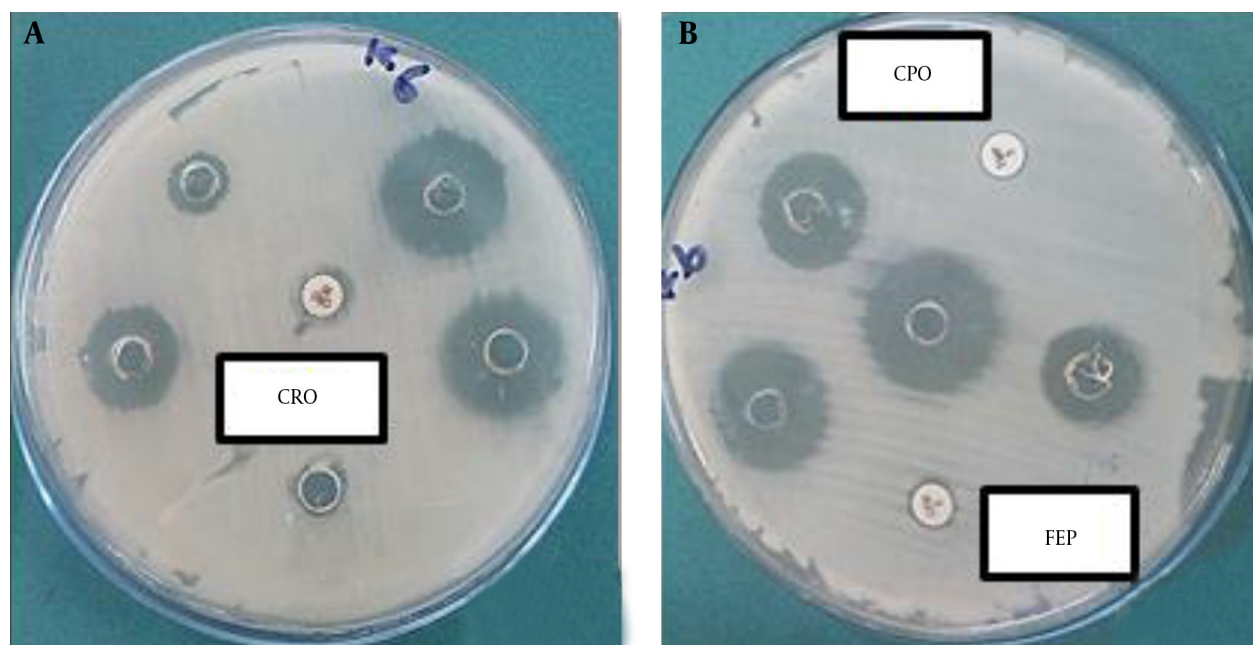
In brine shrimp cytotoxicity assay, the cytotoxic effect of different active strains was evaluated and shown in Table 3. Among all active extracts of actinomycetes, A16 showed the maximum cytotoxicity as about 54%. A19 strain had the lowest cytotoxicity as about 15%. The strains A1, A2, A20 and A10 had mortality percentages of 28%, 26%, 30% and 41%, respectively and considered safe to be used as antimicrobial agents. The strains that showed highest cytotoxicity generally produced compounds which were useless and did not have properties of potential antibiotics.

In comparative efficacy, some antibiotics active against *Klebsiella* were compared with actinomycetes crude extracts. Used antibiotics were ceftriaxone, cefepime and ceftiofime and the crude extract active against *Klebsiella* i.e. A19, A20, A10, A6, A1, were used. Used antibiotics were ineffective or had no inhibitory effect on *Klebsiella* and crude extracts were pretty much effective and showed considerable zones of inhibitions. The maximum zone of inhibition was shown by strains A19 and A20, which was about 15 - 20 mm (Figure 2).

**Table 3.** Biological Screening (Antimicrobial Activity and Cytotoxicity Assay)

| Experimental Strains | Zone of Inhibition, mm |    |    |    |    |    |    |    |     |     |     |     | Mortality, % |
|----------------------|------------------------|----|----|----|----|----|----|----|-----|-----|-----|-----|--------------|
|                      | K1                     | K2 | K3 | K4 | K5 | K6 | K7 | K8 | K11 | K12 | K13 | K14 |              |
| A1                   | 9                      | 0  | 0  | 11 | 12 | 0  | 11 | 6  | 15  | 14  | 12  | 11  | 28           |
| A2                   | 10                     | 12 | 10 | 12 | 11 | 18 | 0  | 5  | 0   | 5   | 11  | 12  | 26           |
| A3                   | 12                     | 0  | 13 | 11 | 12 | 5  | 12 | 6  | 9   | 8   | 10  | 9   | 20           |
| A4                   | 15                     | 0  | 8  | 12 | 0  | 9  | 17 | 8  | 6   | 9   | 9   | 11  | 19           |
| A5                   | 8                      | 17 | 7  | 0  | 7  | 8  | 9  | 9  | 6   | 10  | 7   | 20  | 30           |
| A6                   | 11                     | 4  | 13 | 5  | 9  | 0  | 5  | 10 | 0   | 11  | 8   | 11  | 50           |
| A7                   | 15                     | 0  | 0  | 11 | 10 | 0  | 0  | 11 | 9   | 0   | 6   | 12  | 32           |
| A8                   | 20                     | 5  | 0  | 3  | 14 | 19 | 12 | 0  | 0   | 0   | 5   | 20  | 23           |
| A9                   | 11                     | 12 | 6  | 5  | 19 | 14 | 17 | 0  | 12  | 11  | 11  | 16  | 24           |
| A10                  | 13                     | 11 | 10 | 7  | 16 | 10 | 22 | 11 | 10  | 10  | 0   | 0   | 41           |
| A11                  | 0                      | 10 | 20 | 12 | 15 | 11 | 4  | 12 | 11  | 10  | 0   | 3   | 19           |
| A12                  | 5                      | 14 | 12 | 10 | 0  | 12 | 9  | 10 | 9   | 11  | 4   | 7   | 25           |
| A13                  | 0                      | 9  | 0  | 9  | 11 | 0  | 0  | 3  | 12  | 5   | 11  | 9   | 30           |
| A14                  | 0                      | 5  | 0  | 0  | 8  | 11 | 5  | 0  | 11  | 8   | 10  | 11  | 18           |
| A15                  | 11                     | 0  | 8  | 5  | 6  | 14 | 11 | 4  | 15  | 9   | 9   | 15  | 40           |
| A16                  | 12                     | 11 | 9  | 14 | 5  | 16 | 0  | 5  | 0   | 0   | 0   | 9   | 54           |
| A19                  | 20                     | 18 | 11 | 9  | 13 | 19 | 8  | 13 | 18  | 11  | 16  | 20  | 15           |
| A20                  | 20                     | 11 | 12 | 20 | 12 | 21 | 20 | 18 | 19  | 16  | 18  | 19  | 30           |

**Figure 2.** Comparative Efficacy



A, Ceftriaxone (CRO) and crude extracts A19, A20, A1 and A2 were used; B, cefepime (FEP), cefpirome (CPO) and crude extracts A10, A19, A20 and A1 were used.

In thin layer chromatography, different biologically active components were analyzed in the crude extracts. The extracts from active actinomycetes isolates gave several bands which were of different colours. When the TLC plate was sprayed with anisaldehyde reagent, several red and pink colour bands were appeared. Ehrlich's reagent spot out the presence of indoles, after staining such compounds appeared yellow. Anisaldehyde spray results indicated the presence of phenols, steroids and terpene in the crude extracts. Strains A19, A20 and A6 generated several red, pink coloured bands after spraying with anisaldehyde reagents. Strains A8, A9, A1 and A2 showed prominent yellow coloured

spots, which indicates presence of indolic compounds.

In HPLC-UV analysis, crude extract indicates variety of peaks at different retention times for potential biologically active compounds (12). The crude extract of A8 exhibited visible peaks at different retention times of 3.004, 3.304, 3.476, 3.672 and 4.002, while the peak height percentage were 3.1%, 6.9%, 9.0%, 3.4% and 4.5% accordingly (Figure 3). The strain A19 showed two prominent peaks at retention times 4.032 and 4.572, while peak height percentage were 14.6% and 36.1%, respectively (Figure 4). Likewise, different strains exhibited peaks at different retention times (Rt).

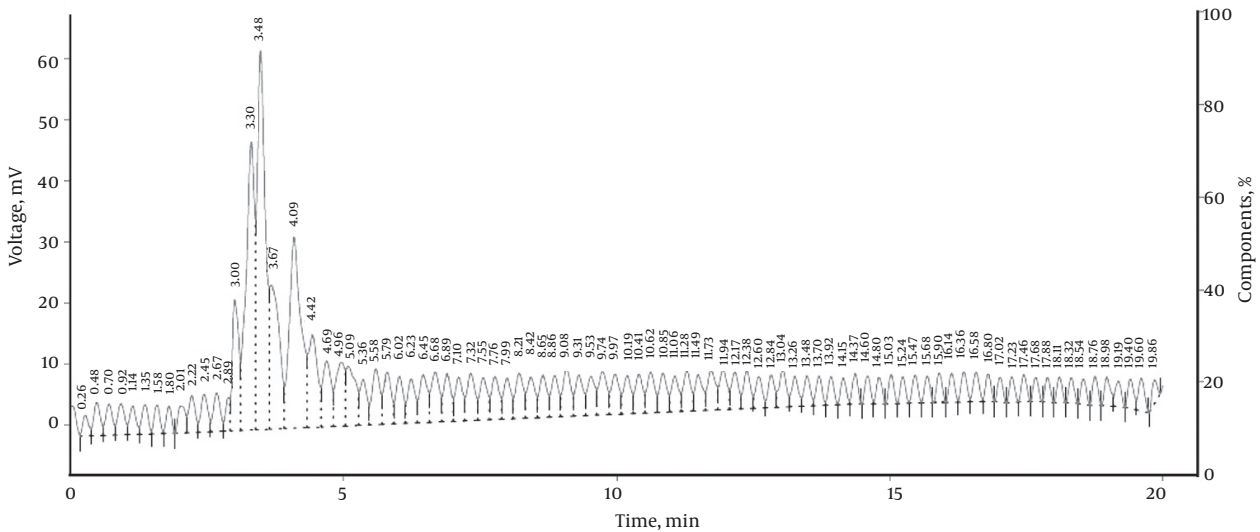


Figure 3. HPLC Analysis of Strain A8 Results Indicates Peaks at Different Retention Times

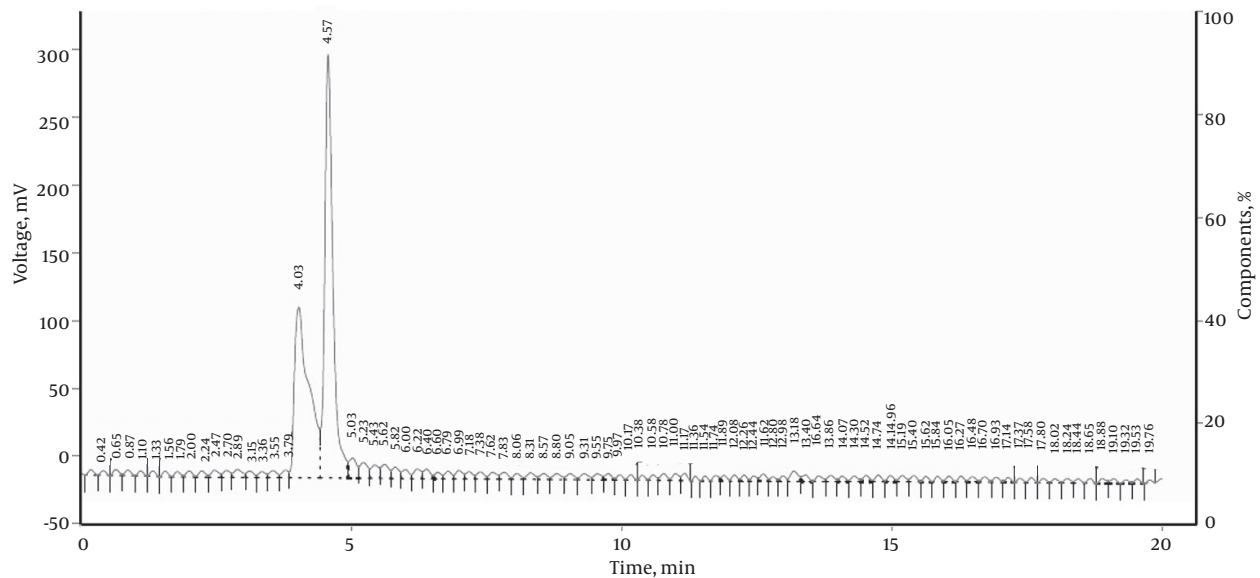


Figure 4. HPLC Analysis of Strain A19 Results Indicates Peaks at Different Retention Times

In biofilm inhibition assay, the crude extracts of strains A19 and A20 showed potent results as there was marked reduction in O.D, which is comparable with the wells without any stress of crude extracts. *Klebsiella* strains K1 and K2 produced strong biofilms when grown without any stress of crude extracts. The optical density was between 1.741 to 2.018 nm, but when crude extract was added and biofilm allowed to be formed in the presence of crude extract, there was a marked reduction in optical density, which clearly depicts that these extracts contain bioactive compounds that start hindering in biofilm formation. The optical density was reduced to 0.57 nm when crude extracts of strain A20 were used.

### 5. Discussion

Nowadays one of the biggest challenges for the treatment of infectious diseases is to handle multidrug resistant pathogens. Drug resistance can be managed by different ways including proper use of antibiotics, intermittent replacement of current antibiotics, antibiotics usage awareness and discovery of novel classes of antibiotics. Several famous superbugs that need serious attention include vancomycin, methicillin resistant *Staphylococcus aureus* and ESBL strains of different *Klebsiella* sp., *E. coli* and *Pseudomonas aeruginosa* (13).

It is reported that different species of genus *Klebsiella* are resistant to several antibiotics commonly used in clinical settings. Mortality rate due to cephalosporin resistant *K. pneumoniae* is very high in hospitalized patients. Commonly reported drug resistant genes are present on plasmids. Several studies reported that the culprit broad spectrum cephalosporins resistant genes present in *Klebsiella* spp. are also plasmid borne (14).

Several factors are declared as reasons for nosocomial *Klebsiella* infections including lengthy stay in hospitals, use of different catheters etc. Hospital acquired pneumonia is the common example of infections caused by genus *Klebsiella*. Mortality rate in this drug-resistant pneumonia is as high as 50%. Furthermore, different studies confirmed isolation of 50% drug resistant *Klebsiella* spp. from patients with nosocomial infections (15).

Several studies confirmed that different classes of antibiotics including macrolides and cephalosporins have no effect in controlling *Klebsiella pneumoniae* infections. Currently, other classes including quinolones, carbapenems and aminoglycosides provide greater coverage to treat infections caused by *Klebsiella* genus. But after emerging global drug resistance among different *Klebsiella* genus, these classes are not sufficient. If proper measures are not taken urgently, we have no active antibiotic to treat infections caused by different *Klebsiella* species.

The current study was attempted to determine antibiotic resistance pattern of different *Klebsiella* sp. isolated from clinical samples and their in vitro inhibition by secondary metabolites of actinomycetes. Major concern of

the present study was to highlight potential of microbial extracts containing lead structures against isolated multidrug resistant nosocomial *Klebsiella* spp.

The morphological, physiological and biochemical characteristics of the selected strains showed that the isolated strains were closely resembled to *Klebsiella pneumoniae*. These selected strains were identified using 16S rRNA gene sequencing, which confirmed their relationship with *Klebsiella pneumoniae* species. GenBank accession numbers along with the percentage homology to different *Klebsiella pneumoniae* species clear taxonomic status of these isolated bacteria. Antibiotic susceptibility test showed 100% resistance against ceftriaxone and amoxicillin antibiotics. Antibiotic resistance pattern of *Klebsiella* against different antibiotics were also calculated. Figure 5 shows the graph for percentage resistance of *Klebsiella* strains against different antibiotics.

The actinomycetes strains found to be effective in biological screening against *Klebsiella pneumoniae* were A19, A20, A3, A16, A10 and A6. Results indicated that about 70% strains showed antimicrobial activity against the test organisms and about 30% were inactive and did not show any antimicrobial activity. The maximum cytotoxicity activity was related to strains A6 and A16, while the minimum cytotoxicity for strain A19.

Selvameenal et al. reported the potential of actinomycetes against extended spectrum  $\beta$ -lactamase producing strain of *Klebsiella* genus. He depicted that the strain *Streptomyces hygroscopicus*, which belongs to the well-known genus of actinomycetes, produces yellow colour compound during solid state fermentation. Although the isolated yellow colour compound did not show significant activity against extended spectrum  $\beta$ -lactamase producing strain of *Klebsiella* (inhibition zone of only 10 mm), but on the same time it was pretty much effective against extended spectrum  $\beta$ -lactamase producing strain of *E. coli* with maximum inhibition zone of 19 mm (16).

While some other studies reported that extracts from different genera of actinomycetes were active against even vancomycin-resistant *Klebsiella* species by MIC value of 2.5 mg/mL (14). Two genera of *Streptomyces* and *Actinopolyspora* were found to be most effective against different multidrug resistant *Klebsiella* species.

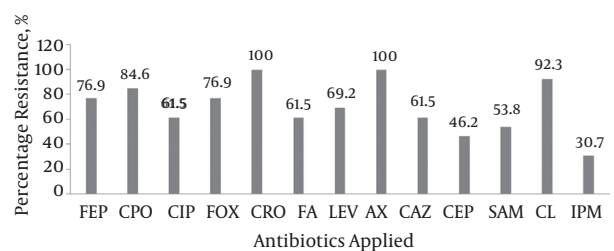


Figure 5. Percentage Resistance of *Klebsiella* Against Different Used Antibiotics



In comparative efficacy, some antibiotics against *Klebsiella* were compared with the actinomycetes crude extract. Used antibiotics were ineffective or had no inhibitory effect on *Klebsiella* strains and crude extracts i.e. A19, A20, A10, A6 and A1, were shown to be highly effective. Therefore, it is proved that the crude extracts have greater efficacy compared to used antibiotics.

Previous studies also supported these results, as in our case *Klebsiella* strains responsible for hospital-acquired infections were 100% resistant to both ceftriaxone and  $\beta$ -lactams (amoxicillin) (17).

In chemical screening, using TLC, different coloured spots were appeared after staining with anisaldehyde and Ehrlich's reagents. Spots of different colour represent different active groups present in the crude extracts, which rendered the crude extract as bioactive. Staining with Ehrlich's reagent indicates the presence of amines and indole. While staining with anisaldehyde indicates existence of phenols, steroids and terpene in the crude extracts (18).

Chromatograms in HPLC-UV indicate the presence of potent bioactive compounds in the crude extracts. The strains A19 and A20 produce two peaks; either the two peaks have bioactive compounds that exhibited antimicrobial activity or both peaks may play their role as a combined effect for observed significant antimicrobial activity. Strain A6 has four peaks; one of the all may have the antimicrobial properties or either all have the antimicrobial activity, which can be confirmed only by subsequent extraction and purification of all the four compounds using different column chromatographic techniques. As such this HPLC-UV-vis chromatographic method helps us to establish chemical fingerprints of crude extracts obtained from our bioactive collection of different actinomycetes strains. This technique also comprehensively depicts the presence or absence of metabolic diversity among our different actinomycetes strains. Different compounds have different  $\lambda_{max}$  and elute at different times from the column, by the help of which we are able to deeply characterize chromatograms of both A8 and A19 as shown in the figures.

The crude extracts of strains A19 and A20 are proved to be effective in biofilm inhibition assay. Since very long *Klebsiella pneumoniae* is known for the production of strong biofilm in vitro. It has been also observed that *Klebsiella pneumoniae* isolated from different clinical samples (sputum, blood, wound swabs and urine) have the ability of biofilm formation. As such biofilm forming ability is one of the invasive characteristics of any pathogenic bacteria, because it made difficult for antibiotics to reach and act upon these pathogens that live in bacterial consortium. Significant biofilm inhibition was observed after comparing the results of control and the test wells, which were without the stress of crude extracts. In short these results indicated that the crude extracts of actinomycetes strains have significant antimicrobial activity against drug resistant clinical isolates of *Klebsiella pneu-*

*moniae* strains, by showing immense inhibition in the biofilm forming ability of test strains. Similar results have been also reported by Saleem et al.; they showed that extracts from different actinomycetes strains can inhibit biofilm formation ability of different bacteria isolated from oral biofilms (19). High rate of inhibition was observed in our results regarding biofilm forming ability of *K. pneumoniae*, which gives us hope as it is recently reported that antibiotic resistance ability increased dramatically; whenever *K. pneumoniae* was able to grow as a part of microbial consortium or biofilm (20). Although to date researchers have not been able to find out the basis of association between biofilm-forming ability of *K. pneumoniae* strains and rapid increase in antibiotic resistance (21).

In short, these results depict the potential of actinomycetes strains as a reservoir of new antimicrobial compounds. These bacteria serve marvellous role in drug discovery against common hospital acquired drug resistant infections. In this way, we can cover our journey towards better human health in an efficient manner. In our case we observed potent bioactivity of ethyl acetate extracts of different actinomycetes against multidrug-resistant *Klebsiella pneumoniae* at very low concentrations of extracts. Comparing our results with other studies, we are quite confident that it would open up the vista for further isolation and purification of responsible compounds present in actinomycetes extracts.

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## Footnotes

**Authors' Contribution:** Study concept and design: Naila Noureen, Ambreen Amjad and Imran Sajid; acquisition of data: Naila Noureen, Ambreen Amjad; analysis and interpretation of data: Naila Noureen, Ambreen Amjad; drafting of the manuscript: Naila Noureen and Usman Aftab; critical revision of the manuscript for important intellectual content: Imran Sajid, Usman Aftab; statistical analysis: Naila Noureen; administrative, technical and material support: Usman Aftab and Imran Sajid; study supervision: Imran Sajid.

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