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Antibacterial activity of *Punnica granatum* methanolic pell extract on multi drug Resistance *Klebsiella pneumoniae*

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Abstract

Background. The increase problems that the world suffers from because they affect health in general, there is an increase in the resistance of most types of bacteria to antibiotics among them is the *Klebsiella pneumonia* bacteria, which poses a great danger to health because it has many virulence factors, especially its formation biofilms from this came this study to use alternatives to antibiotics in treating this bacterium. **Aim.** To compare the effects of antibiotics and nano-extracts of pomegranate peel extract on highly resistant *Klebsiella pneumoniae*. **Methods.** 50 sample were collected from hospitals in Baghdad, such as Ghazi Al-Hariri and Al-Karama Hospital, from patients suffering from burns and wounds, including 22 isolates of *Klebsiella pneumoniae* bacteria, which were examined using biological tests. Bacteriological tests were conducted on them by culturing them on selective media and microscopic examination was conducted using Gram stain then biochemical tests were conducted and they were diagnosed with confirmation using PCR technology to confirm the presence of the diagnostic gene 16 SRNA, and all isolates contained the diagnostic gene. An antibiotic susceptibility test was conducted for 15 antibiotics using the disc diffusion method. **Results**. The level of antibiotic resistance reached 95.45% for both tetracycline and ampicillin, while the low level of resistance reached 36.36% for the antibiotic's chloramphenicol and meropenem then, a test was conducted on the efficacy of the plant extract against a variety of bacteria by etching in agar plates. It showed effectiveness for bacteria the zones of inhibition ranged from (12 to 36) mm. **Conclusion.** This research found that different concentrations of pomegranate peel extract are bacteriostatic for bacteria resistant to multiple antibiotics.

Keywords: *Klebsiella pneumonia*, MDR, 16S rRNA, *punica granatum*

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Introduction

Klebsiella pneumoniae is a Gram-negative bacterial pathogen. It mostly affects those who have impaired immune systems and is frequently linked to nosocomial infections One of the most prevalent organisms causing infections (1) occurs naturally in a variety of environments, including water, animals, soil, and medical equipment (2). It is classed as a member of the Entero-bacteriaceae family, which includes the known genera Salmonella and Escherichia (3). *K. pneumoniae* bacilli form, an encapsulated non-motile, nonferment lactose sugar, and one of the primary causes of bacterial Infections in hospitals (4) most common clinical symptoms include bacteremia, urinary tract infections, and respiratory infections (5). *K.pneumonia* is known to infect people with impaired immune systems due to its opportunistic nature (6). Various virulence factors, including iron transport systems, efflux pumps, outer membrane proteins, lipopolysaccharides, and purines, each plays a specific role in *k.pneumonia* infections (7).Bacterial resistance to most antibiotics appears to have grown in recent years (8). *K. pneumoniae* is showing high resistance to a

broad spectrum of drugs including beta-lactam antibiotics, fluoroquinolones, and aminoglycosides (9). Bacterial resistance can also take the form of the development of biofilms, a community of microorganisms are affixed to an inert or living surface through a self-produced polymeric outer matrix that contains proteins, extracellular DNA (exDNA) and polysaccharides. Biofilms restrict antibiotic infiltration, facilitate gene exchange and slow bacterial growth promote the formation of persistent cell. Biofilm formation among clinical strains of *K. pneumoniae* has been associated with a large number of genes (10). Biofilm-associated *K. pneumoniae* infections have received little attention, and their role in countering the effects of antimicrobial remedy is not entirely known (11). *K.pneumoniae* generates biofilms, which are multicellular formations that are formed by single-celled microorganisms that adhere to inert surfaces. Pathogenic bacteria can develop as biofilms on the inert surfaces of implanted devices, such as prosthetic heart valves, joint replacements, and catheters. Extracellular polymeric substance (EPS), nucleic acids, proteins, and polysaccharides are the main components of biofilms, which are complex structures that encapsulate one or more species of microbes Because of their capacity to endure external pressures, including pharmaceuticals and host defense mechanisms, bacterial biofilms are a significant global health concern. As such, their existence plays a role in the emergence of chronic infections that are persistent (10). As antibiotic resistance rises, *K. pneumoniae* infection becomes a major public health concern hindering the development of effective treatments (12).

Materials and Methods

Bacterial isolates

Fifty sample were collected from hospitals in Baghdad, such as Ghazi Al-Hariri and Al-Karama Hospital, from patients suffering from burns and wounds, including 50 sample. The isolates were inoculated into selective media such as (CHROMagar, blood agar and MacConkey agar for colony characterization and staining with Gram stain. Following this they were subjected to a set of biochemical tests. They were save it at 37oC for 24 h.Ultimately, the isolates were genetically identified using *K. pneumoniae* 16S rRNA gene.

Detection of *Klebsiella pneumonia* by16SrRNA Selecting identification genes

The present investigation employed a conventional (PCR) polymerase chain reaction assay to detect the gene 16S rRNA, which is utilized to identify the subspecies of K. pneumoniae. 16S rRNA [rrsE gene] were generated with primersF-TTGAC GTTACCCGCAGAAGAA and R-5TCTACAAGACTCTAG CCTGCCA (13). the final volume of 25 µl (The DNA template was amplified in 5 µl using 12.5 µl of Taq green master mix and 1.25 µl of each primer at a concentration of 10 pmol/µL. and then added nucleases free water. The program was developed for PCR amplification in the current study. The program was composed of the following of 45 cycles, each lasting 20 seconds at 95°C, 20 seconds at 60°C, and 30 seconds at 72°C. A 1% agarose gel was stained with a red reagent to visualise PCR products (14, 15).

Antibiotics susceptibility test

All isolates were tested for antibiotic susceptibility using (6 group) were conducted bythe Kirby-Bauer way on Muller-Hinton agar described by clinical research laboratory standard indicated CLSI 2023 the discs were Ciprofloxacin, Ceftriaxone, Imipenem, Nalidixic acid, Meropenem, Tetracycline, Levofloxaccin, ceftazidim, Chloramphenicol, Ampicillin,Norfloxacin,Cefepime,Ofloxacin,Amikacinand Tazobactam (16).

Preparation of *Punnica granatum L.peel extract.*

Pomegranate peels were obtained from local markets and left to dry for a week, then the air-dried powdered plant material (150 g from the sample) was extracted for 8 hours under Soxhlet on a water bath with the solvent methanol (80 %) 500 ml. The extracts were concentrated and dried using a rotary evaporator (17).

Antibacterial activity of Punica *granatum* peel extract on *Klebsiella pneumonia*

The Kirby Bauer method was used to determine the effectiveness of pomegranate peel extract concentration by taking the concentrated solid extract and diluting it. When 100 µl of test bacteria inoculum was introduced onto the Petri dish, 15 ml of MHA medium was poured, mixed, and allowed to solidify. The positive control was an antibiotic cefepime tablet. To ensure the safety of its action as an antibacterial inhibitor, it was relied upon to measure the diameters of different concentrations of the plant extract.The dishes were drilled with a 9-hole cork drill, 8 holes of which are placed in different concentrations of the plant extract, which are (50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39) mg/ml An antibiotic is placed in the ninth hole.Petri dishes were incubated at 37 °C for 24 h to determine the diameter of the inhibitory zone produced. A digital caliper was used to measure the inhibitory zone's diameter (mm) (18,19).

Results

Culturing examination

The colonies showed up on MacConkey agar as pink, lactose-fermenting, mucous colonies, and on CHROM agar as blue, mucous, metallic colonies. They showed up as non-hemolytic, grayish-white colonies on blood agar (20). The bacterial enzymes broke down the synthetic chromogenic substrates (chromogens) in the media during metabolism (21).

Identification of Biochemical

A set of biochemical tests were conducted on all isolates and the results appeared as in the table (1) and were consistent with what was mentioned by (22).

Method of molecular identification for isolates

All isolates were identified by polymerase chain reaction (PCR) technique to determine their presence of the 16S rRNA gene. All samples appeared positive (100%) for the 16S rRNA gene as shown in Figure (1).

Table 1: Diagnosis characteristics of *Klebsiella pneumonia*

NO	Biochemical Test	Result
1	Gram stain	**-**
2	Oxidase test	**-**
3	Catalase formation	+
4	Indole production	**-**
5	Lactose fermentation	+
6	Urease production	+
7	Motility examination	**-**

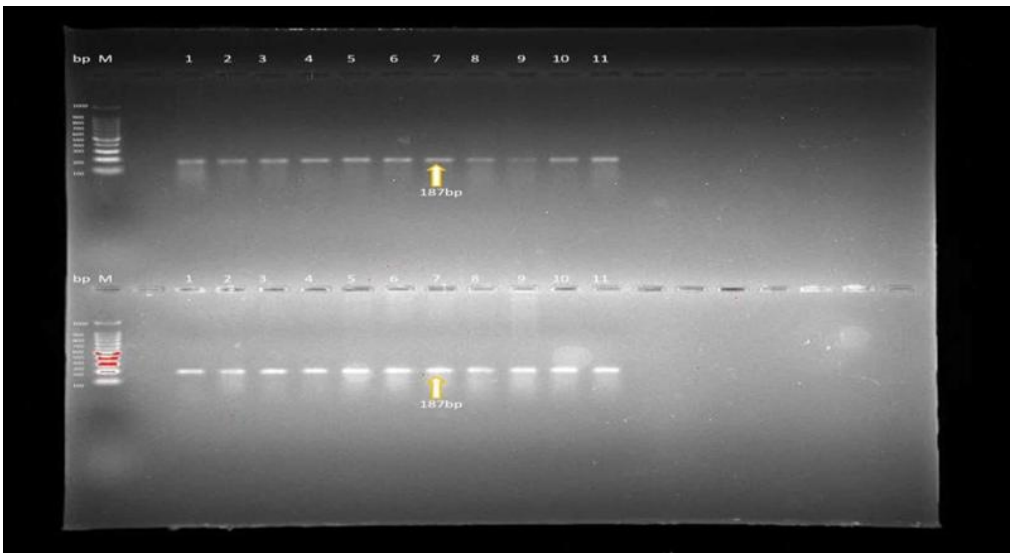

Figure 1: PCR product the band size 187 bp. The product was electrophoresis on 1.5% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. M: DNA ladder (100)

Antibiotics susceptibility test

In accordance with the protocols, the susceptibility of antibiotic of *K. pneumonia* isolates was ascertained via disk spreading (15). A susceptibility check was performed on 22 isolates against fifteen antibiotics. The findings indicated that frequently, isolates exhibited a significantly high degree of resistance to the antibiotics utilized in this investigation, as shown in figure (2). The susceptibility of antibiotic test results for *K. pneumonia* indicates that the susceptibility of isolates to the antibiotics varied as shown in figure (3). Multidrug resistance (MDR) was observed in the majority of the isolates in the current investigation, as determined by the standard disk diffusion technique. Among *22 K. pneumonia tested isolates* ,22 isolates tested, 21 isolates 95.45% were resistant to Tetracycline and Amoxicillin,15 isolates 90.90% resistant to ciprofloxacin, As for the results below, the isolates are resistant at a rate of 50%, whereas 6 isolates 27.27% intermediate to Amikacin, 12 isolates 54.54% were sensitive to chloramphenicol and meropenem. From the results the aforementioned *K. pneumonia* grow more resistance to*β*-lactam and tetracyclines combination class agents with 96% resistance, up to 70% of the isolates confer resistance to 3rd compeers cephalosporines, and the less resistant was to phenicol agents with 36%. Aminoglycoside class was intermediate action to *K. pneumonia* with 27.27% at maximum, and the minimal value in the hundreds of intermediate susceptibilities to agents of the quinolones, B-lactam, and fluoroquinolone classes that 9.09%. Carbapenems exhibited the greatest sensitivity among *K. pneumonia* isolates, with a maximum sensitivity of 54.54%.

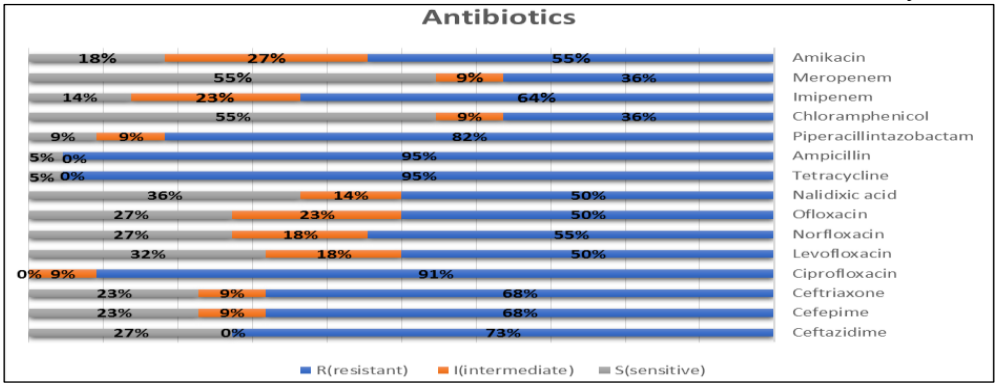

Figure 2: Percentage of Susceptibility of *Klebsiella pneumonia* isolates to 15 antibiotics

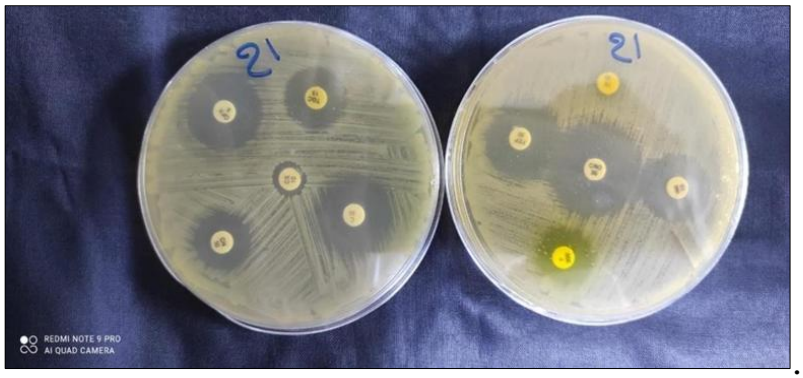

Figure 3 Antibiotics susceptibility for *K.pneumoniae*

Antibacterial activity of Punica *granatum* peel extract on *Klebsiella pneumonia.*

The plant extracts have different antimicrobial activities, as shown in our current results (Figure 4).

The effectiveness of pomegranate peel extract against nine isolates of *K.pneumoniae* that are resistant to multiple antibiotics was evaluated, as shown in Table (2). The study revealed that the extract at concentrations (50, 25, 12.5, 6.25, 3.12 and 1.56) mg/ml showed significant activity against bacterial isolates. The zones of inhibition ranged from (12 to 36) mm. The results indicate that the isolate (6) has greater resistance. Compared to isolation (19 and20) .

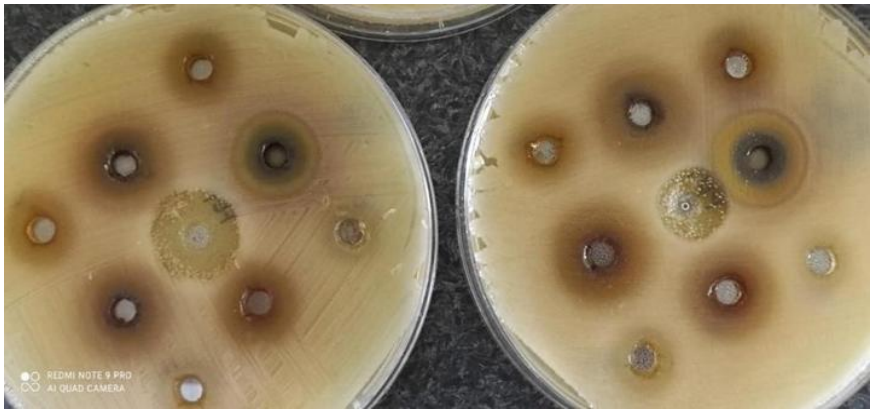

Figure 4: Inhibition zone of *Punnica.granatum* peel extract on *Klebsiella pneumonia*

Table 2: Inhibition zone (mm) of *Punnica.granatum* peel extract on multidrug Resistance (MDR) *Klebsiella pneumonia*

Isolation	Concentration(mg/ml)							
	50	**25**	**12.5**	**6.25**	**3.12**	**1.56**	**0.78**	**0.39**
2	33	30	27	25	23	20	18	15
3	33	30	27	25	20	17	15	12
6	36	27	25	23	22	20	18	15
7	35	30	27	25	23	20	17	15
8	35	30	27	25	23	20	16	15
14	30	25	25	23	23	21	18	13
19	25	24	23	20	20	20	17	15
20	25	23	23	20	20	18	15	15
22	30	27	25	23	20	18	16	16

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Discussion

The current results are consistent with the results of (14). The findings presented here were consistent with those of (23) who discovered that the majority of *K. pneumoniae* isolates in his study are multidrug-resistant organisms, which restricts the available treatment options. These results are consistent with (24) that the bacteria are resistant to the antibiotic ciprofloxacin at a high rate of 90.90%, and differ with (25) as the results of his research showed that the antibiotic levofloxacin is resistant to all isolates. these results approved with the findings of (26) who identified a significant resistance level of 95% among strains of *K. pneumonia* to agents of the β-lactam grouping class and demonstrated a high degree of sensitivity to causes of the carbapenem class. Additionally, (27). The results differ from (25), as his results show that 84% of the isolates are sensitive to the antibiotic imipenem, while the results below are resistant to the antibiotic. This means that the bacteria were sensitive to the antibiotic, but they occurred and became resistant due to misuse of this antibiotic. Additionally, there are some shared characteristics of multidrug resistance in Gram-negative bacteria, for instance, the production of efflux pumps and antibiotic-inactivating enzymes that actively expel antibiotics from bacterial cells, thereby reducing drug concentrations and promoting drug resistance. Inappropriate use of antibiotics is one of the important causes of the rise of multidrug resistance (MDR). This mechanism is through the transfer of resistance genes by plasmids for example or the occurrence of various mutations (28) The results showed that the effectiveness of plant extracts at different concentrations is effective against multiple antibiotic-resistant bacteria, and these results are also consistent with (29,30). where methanol was used *Punica granatum* extracts to test their Activity of antibacterial against different strains of *Escherichia coli.* Alcohol extract from *Punica granatum* it was very effective against gram-negative.

Conclusion

This research found that different concentrations of pomegranate peel extract are bacteriostatic for bacteria resistant to multiple antibiotics.

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