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Molecular Identification of *Klebsiella pneumoniae* Isolated from UTI Patients in Al-Anbar Governorate and Study Its Antibiotic Resistance and determination of Antimicrobial Activity of Flax seed Oil

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Abstract: Two hundred and ten bacterial isolates were taken from the mid-stream urine of UTI patients attending Al-Falluja Teaching Hospital and Al-Ramadi Teaching Hospital for Maternity and Children. The isolates were grown on MaCconkey agar, eosin methylene blue agar, blood agar and biochemically tested to confirm the diagnosis. All bacterial isolates assured identified by VITEK® 2 Compact system. To sum up 57 (27,1%) out of 210 (100%) UTI samples showed growth of *Klebsiella pneumoniae* whereas 43 (20.5%) out of 210 (100%) UTI samples showed no growth of bacteria. Also, 110 (52.3 %) out of 210 (100%) UTI samples showed growth of different types of bacteria such as *staphylococci*, *streptococcus*, *proteus*, *Escherichia coli*, and *Acinetobacter*. The antimicrobial susceptibility test performed by VITEK® 2 Compact system and disk diffusion method demonstrated that the isolates showed a high rate of resistance to the third generation cephalosporins. The presence of antibiotic-resistant genes was determined by polymerase chain reaction (PCR). The antimicrobial activity of flax (*Linum usitatissimum*) seed oil has been studied and showed significant antimicrobial activity against all tested bacterial isolates of *K. pneumoniae.*

Keywords: urinary tract infection (UTI)**,** *Klebsiella pneumoniae*, *bla*TEM, flaxseed oil.

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Introduction

The enterobacteriaceae family includes the encapsulated Gram-negative bacillus known as *Klebsiella pneumoniae*.

K. pneumoniae is a bacterium that can infect people and cause a variety of illnesses, including sepsis, soft tissue infections, respiratory tract infections, and urinary tract infections (1).

K. pneumoniae is one of the top three pathogens of global concern listed in the World Health Organization's (WHO),It is the second most frequent etiological agent involved in community-acquired (CA) urinary tract infections (UTIs) (2, 3).

K. pneumoniae is showing high resistance to a broad spectrum of drugs including beta-lactam antibiotics, fluoroquinolones, and aminoglycosides (4,5). This resistance is resulting in a growing worldwide problem regarding the choice of effective antibiotic treatment for hospital-acquired infections (6).

The need for antibacterial medicines based on antimicrobial sensitivity tests is increased due to an increase in multidrug-resistant bacterial infections around the world that are closely linked to limited medication treatments (7, 8).

Studies have shown outbreaks of *K. pneumoniae* in hospitals that are resistant to third-generation quinolones, aminoglycosides, and cephalosporins (4, 5, 7, 9).

Infectious diseases caused by bacteria, viruses, and fungi are still serious problems in public health. In Iraq, it is estimated that over 90% of bacterial pathogens are resistant to antibiotics (10, 11, 12).

Scientists throughout the world are working on new antimicrobial agents as a result of the massive and quick spread of several germs that are resistant to various drugs. Researchers from all around the world have discovered a variety of phytochemicals that have inhibitory effects on a variety of bacteria in vitro (13).

By separating proteinaceous components from plant sources, researchers have discovered novel natural chemicals with potential antibacterial properties (14).

Flaxseed is considered an important functional food because it has numerous substances considered beneficial to health (minerals, vitamins, proteins, and fatty acids), but the main substances present are fiber, lignans, and unsaturated fatty acids (15).

Lignans act as antioxidants and phytoestrogens, and the most predominant is the secoisolariciresinol diglucoside (SDG) (16). Due to the presence of these components, flaxseed has been related to the prevention or control of numerous diseases (15, 17).

Much evidence has demonstrated that flaxseed protein and its hydrolysates exhibit many health-promoting benefits, *L. usitatissimum* fixed oil exhibited good antibacterial activity against some microbial strains (18, 19).

This study aimed to isolation and identification of *K. pneumonia* from UTI. Measures the antibiotic susceptibility to different antibiotics using VITEK system, detection of *TEM* gene by PCR technique and measurement the antimicrobial activity of flaxseed oil against the *K. pneumonia*.

Materials and methods

Samples collection

Two hundred and ten samples from patients with urinary tract infection (UTIs) of all ages and both sexes were collected in sterile containers from Fallujah General Hospital, Ramadi Women's Hospital, and Ramadi General Hospital from the beginning of November 2020 until the end of February 2021.

Identification bacterial

All urine sample were cultured on MacConkey, Blood, and Eosin Methylene Blue (EMB) agar incubated at 37°C for 24 hours.

Further identification were done using VITEK2 system (20, 21).

Antimicrobial susceptibility test

According to the Kirby-Bauer standard single disc diffusion method and the clinical laboratory standard institute, the results of the antibiotic sensitivity testing of bacterial isolates were examined on Mueller Hinton Agar plates(22, 23). Fourteen antimicrobial drugs were tested Cefuroxime, Gentamicin, Ampicillin/Sulbactam, Ceftazidime, Ceftizoxime, Cefotaxime, Tetracycline, Aztreonam, Amoxicillin-clavulanic acid, Cefazolin, Cefoperazone, Cephalothin, Co-Trimoxazole, and Vancomycin.

The concentration of antibiotics in the area influenced the size of the area of repressed growth (zone of inhibition), therefore the diameter of the inhibition zone indicates the relative susceptibility to a particular antibiotic. Using industry-standard charts that the makers had provided, the results were interpreted as resistant.

Molecular methods

DNA extraction

Utilizing the Promega kit and following the manufacturer's instructions, genomic DNA was extracted from K. pneumoniae isolates (USA).

A Nanodrop Spectrophotometer was used to determine the DNA concentration of the samples (Thermo Scientific, USA). On a 1% agarose gel, genomic DNA was isolated, stained with ethidium bromide, and photographed under ultraviolet (UV) light (24).

PCR technique

Detection the presence of blaTEM-1 β-lactamases

PCR analysis for the β-lactamase gene of TEM was carried out. Primers were obtained from (Promega, USA). The following primers were used 5´-GAG TAT TCA ACA TTT CCG TGT C -3´and 5´-TAA TCA GTG AGG CAC CTA TCT C -3´ specific for the *bla TEM* gene(25).

A total of 35 cycles of heat denaturation at 95 °C for 1 min, primer annealing at 60 °C for 30 s, DNA extension at 72 °C for 1 min, and a final cycle of exertion at 72 °C for 5 min were used in the PCR. The initial cycle of heat denaturation lasted for 2 min. A 1.5% agarose gel was used to separate the PCR products. The gels were then stained with ethidium bromide (1 g/ml), and the gels were photographed under UV illumination(26).

Extraction of flaxseed oil

The oil was extracted by placing (75) grams of dry vegetable powder in a thimble placed in the extraction device (Soxhlet) using 500 ml of hexane (n-hexane), the extraction process continued for (7 hours). Then distillation was carried out to separate the hexane, the oil has been preserved until use (27).

Identifying the active ingredients in the flaxseed oil:

At the laboratories of the Ministry of Industry in Baghdad, once the oil from flaxseed was extracted, the oil components were identified using gas chromatography-mass spectrometry (GC-MS).

Determination of the minimum inhibitory concentration (MIC):

With a few minor adjustments, the Resazurin Microtiter-plate Assay (REMA) was used to determine the flaxseed oil's MIC. 100 µl of Mueller-Hinton Broth (MHB) was poured into each well of a microtiter plate under aseptic conditions, and 100 µl of the substance test (flaxseed oil, 1%) was then added to the first row of the 96-well plates.

Pipetting 100 µl of the material test from the first row to the other rows in sequentially decreasing concentrations (1/2, 1/4, 1/8, 1/16, 1/32, and 1/64) was used to execute serial dilutions. Each well received 10 µl of a bacterial suspension containing 1 $\times 10^8$ CFU/ml. To prevent bacteria from being dehydrated, they were loosely wrapped with Para-film and incubated for 18–24 hours at 35–2°C. After the initial incubation, each well received 10 µl of the resazurin solution (Alamar blue), and the plate was once again incubated for 24 hours to see the color change.

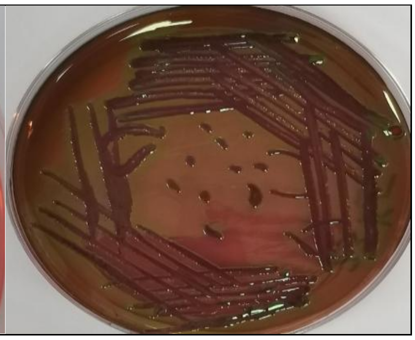
Resazurin's color changes were used to visually examine the data; shifts from purple to pink, red, or colorless were considered favorable changes.

As the MIC value and sub-MIC, the lowest concentration at which the color of the resazurin did not change, was chosen (28, 29).

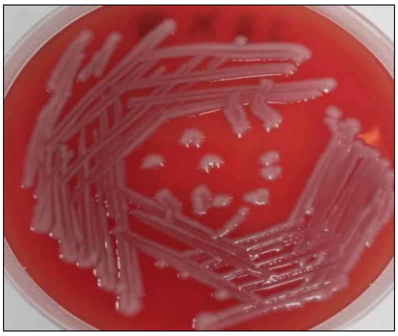
Results and discussion

Isolation and identification of *K. pneumoniae*

Bacterial isolates were initially cultured on MacConkey agar, EMB agar, and Blood agar. On MacConkey agar plates, *K. pneumoniae* isolates appeared mucoid, large, and pink due to lactose fermentation. These results are similar to those of (10) and (30). All the isolates were cultured on EMB and colonies appeared large, purple, and mucoid. *K. pneumoniae* bacteria did not cause blood hemolysis when cultured on blood agar and were transparent glossy. These results are similar to those of (31), figure (1) A, B, and C.

A. Colonies on the MacConkey agar **B. Colonies on the EMB agar.**



C. Colonies on the blood base agar

Figure (1): A, B, C: *K. pneumoniae* on different culture media after 18h of incubation at 37ºC.

For were Further identification all 57 isolates VITEK 2 system was used for the identification of *K. pneumoniae* bacterial isolates with accuracy and reliability of identification. The VITEK 2 system increases the confirmation of the diagnosis and for being the latest device in diagnosis, containing a large number of biochemical tests numbering 64 test results (20, 21), It was found that the results of the culture method are identical to the results of VITEK 2.

Antibiotic susceptibility test

Fifty-seven *K. pneumoniae* strains from the samples showed mostly resistance to Cefuroxime 57(100%), Gentamicin 53(93%), Ampicillin/Sulbactam 51(89.4%), Ceftazidime 57(100%), Ceftizoxime 57(100%), Cefotaxime 57(100%), Tetracycline 57(100%), Aztreonam 55(96.5%), Amoxicillin-clavulanic acid 57(100%), Cefazolin 57(100%), Cefoperazone 55(96.5%), Cephalothin 53(93%), Co-Trimoxazole 51(89.4%), and Vancomycin 55(96.5%), figure (2).

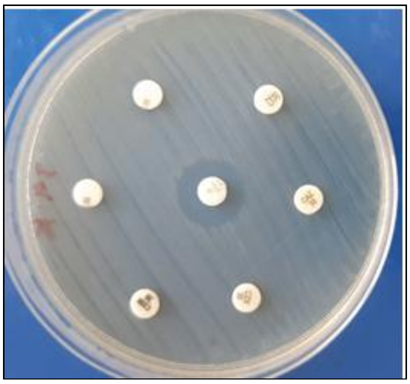


Figure (2): *Klebsiella pneumoniae* resistance to antibiotics

Study results are in accordance with (44). and (45) And also are in accordance with (46).

Molecular methods

Genomic DNA extraction

The DNA was extracted from 57 bacterial isolates and diagnosed using the extraction kit processed by Promega/USA. The concentrations of the DNA ranged between 40-160 ng/µl, and its purity ranged between 1.5-1.9 depending on the reading of the UV absorption using the Nanodrop device. The presence of DNA in the isolates under study was also confirmed by agarose gel preparation and DNA loading before use as a template in the PCR reaction. The isolates were run off on agarose gel at a concentration of 1% at 50 volts for 30 min. followed by gel examination after staining with ethidium bromide under ultraviolet light and gel images as shown in figure (3).

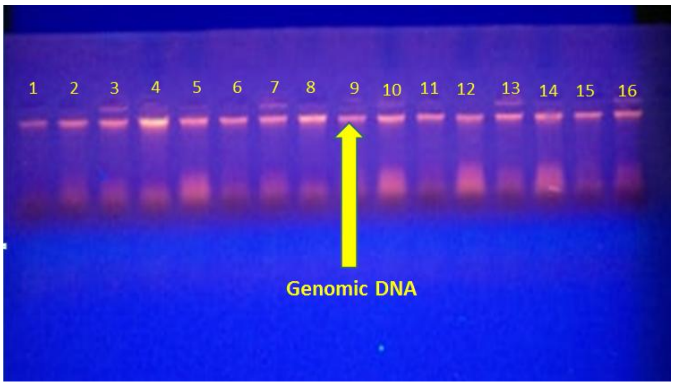


Figure (3): Genomic DNA from bacterial isolates was electrophoresed in 1% agarose gel at 50 V/cm for 30 min, stained with ethidium bromide, and then seen under ultraviolet light.

Detection of ESBL gene by PCR

All ESBL-producing 57 *K. pneumoniae* isolates were subjected to PCR to detect the *blaTEM* gene. The amplified PCR products for the *blaTEM* gene by using the TEM-F and TEM-R primers exhibited a predicted band of 861 bp, figure (4). These results are similar to those of Rodriguez *et al*. (25), Duttaroy and Mehta (35) and Juma *et al.* (36).

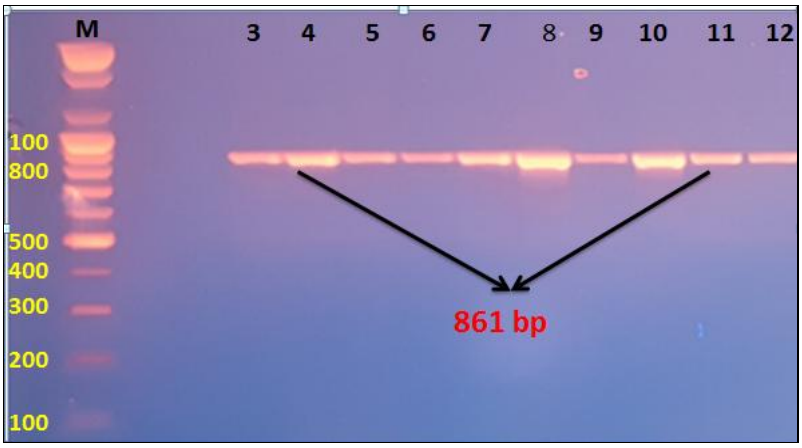


Figure (4): PCR products' gel electrophoresis for the *blaTEM* resistance gene (861 bp). Agarose 1.5 %, 70 V/cm for 120 min, ethidium bromide dye-stained and seen with a UV transilluminator. Lane M: 100bp DNA ladder; lanes 3-12: *K. pneumoniae* isolates.

Flaxseed oil's impact on bacterial isolates:

The flaxseed oil was extracted by the extraction device (Soxhlet) (37), at the laboratories of the Ministry of Industry in Baghdad, the oil components were identified using gas chromatography-mass spectrometry (GC-MS). as shown in figure (5).

The main compounds in flaxseed oil were (Tannins, carbohydrates, Glycosides, Resins, Flavonoids, Saponin, AlKaloid, and Coumarins) this results match with what Joshi *et al*.(38) reached, as shown in table (1).

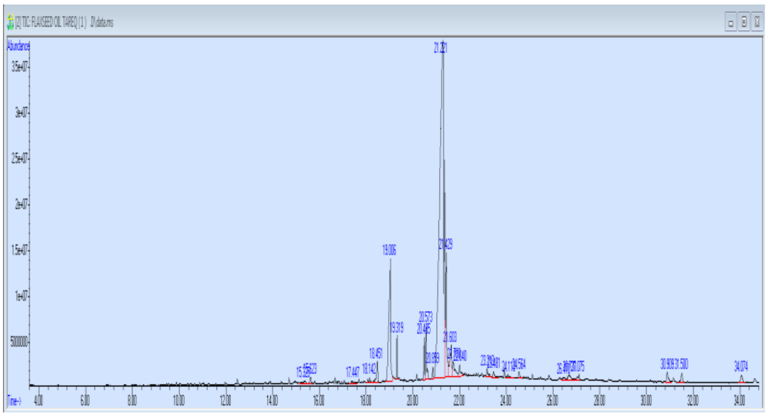


Figure (5): GC-MS analysis of flaxseed oil.

Table (1): Active Compounds Flaxseed oil.

	Compounds
1	Tannins
2	Carbohydrate
3	Glycosides
4	Resins
5	Flavonoids
6	Saponin
7	AlKaloid
8	Coumarins

Linseeds have high antibacterial activity against bacteria, according to Kaithwas *et al*. (32),(33), although it relies on storage conditions, extraction techniques, and concentration.

Mathkhury *et al*. (28) and (34) studied the effects of flaxseed oil's antibacterial and antibiofilm properties on a few bacterial pathogens. Various negative impacts were created for Methicillin resistant *S. aureus* (MRSA), methicillin sensitive *S. aureus* (MSSA), *K. pneuminae* and *S. epidermidis*.

In comparison to Gram positive bacteria, lignan extracts were discovered to be the most efficient antibacterial agents. Gram negative bacteria had MICs that varied from 224 to 366 µg/ml (39).

The inhibitory activity of flaxseed oil on β-lactamase *K. pneumoniae*

Results showed that flaxseed oil had an inhibitory impact against β-Lactamase *K. pneumoniae,* as shown in figure (6).

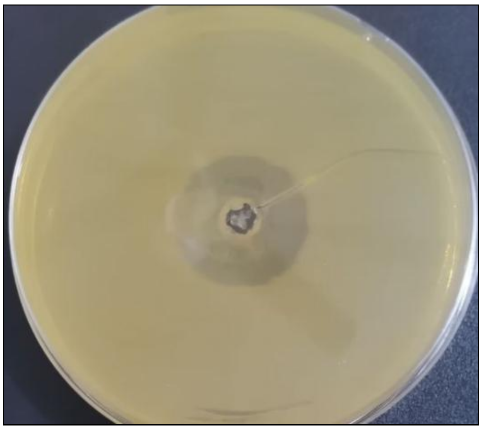


Figure (6): Flaxseed oil resistance of *K. pneumoniae*.

Various essential oils have different antimicrobial activities due to their components. Numerous studies linked the presence of natural polyphenols in general, glycosylated lignans (such SDG or SMG), and aglycones in particular to flaxseed's antibacterial properties (such as SECO or anhydro-SECO) (27, 40).

Determination of minimum inhibitory concentration (MIC) and (sub-MIC) of flaxseed oil by using (REMA) method

Resazurin microtitre-plate assay is distinguished by its simplicity, affordability, quickness, efficiency, and dependability. It is a colorization technique based on the oxidation and reduction of resazurin and used to test the susceptibility of bacteria, medicines, and antibiotics. The blue reduction pigment (Resazurin), which is frequently employed as an indicator to assess bacterial growth in a little amount of solution in microliter-plates without the need for a spectrophotometer, is non-toxic to cells in the media (41).

Table (2) in this study displays the results of the MIC of flaxseed oil for the growth of isolates of *K. pneumoniae* that produce ESBLs.

Figure (7) displayed the MIC values of flaxseed oil. The blue color indicates that the oil inhibited bacterial growth by not lowering resazurin, whereas the pink and red colors were caused by the bacteria's conversion of resazurin to resorufin (42).

Because phenolic chemicals in flaxseed oils disrupt the cytoplasmic membrane, the proton motive force, the flow of electrons, active transport, and the coagulation of cellular contents, flaxseed oils have the power to impede growth (43).

Table (2). The MIC of flaxseed oil for cultivating isolates of *K. pneumoniae* that produce ESBLs.

No. of isolate	Minimum Inhibitory Concentration (MIC) Flaxseed oil (Titer)	Sub (MIC) Flaxseed oil (Titer)
***K. pneumoniae* 23**	2	3
***K. pneumoniae* 25**	2	3
***K. pneumoniae* 28**	1	2

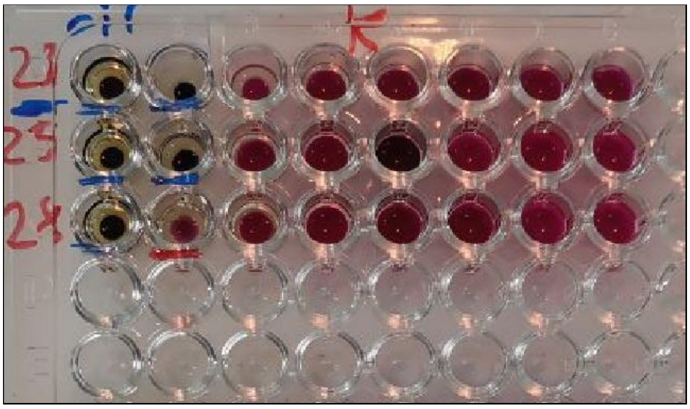


Figure (7): Effect of flaxseed oil on the development of *K. pneumonia*.

The extract of flaxseed oil was evaluated for its antibiofilm activity. It was specifically selected for its antibacterial properties. The flaxseed exhibited antibiofilm activity against all *K. pneumoniae* isolates (28). These results are similar to those of (47).

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