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Detection of (CTX-M) Resistance Genes in Extended Spectrum Beta-lactamases Bacterial Isolates of *Escherichia coli* and *Klebsiella pneumonia* and the Antibacterial Effect of Ethanolic Extract of Neem plant (*Azadirachta indica*) Against these Bacteria.

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Abstract

The production of beta-lactamase enzyme by some Gram-negative bacteria is one of the common mechanisms of resistance to beta-lactam antibiotics. In this study, we isolated *E. coli* and *K. pneumonia* from infected urine samples and then determined the existence of the (*CTX-M*) resistant genes in ESBL-producing bacterial isolates of *E. coli* and *K. pneumonia*, and determined the antibacterial effect of Neem plant (*A. indica*) ethanolic extract against these bacteria. For Phenotype detection of ESBLs we used the hybrid disc method, 12 available commercial antibiotics were used in the Antibiotic susceptibility test and the tested bacterial isolates showed high resistance to these antibiotics. The PCR results confirmed that the ESBL-producing isolates *E. coli* and *K. pneumonia* had the *CTX-M* gene and the *CTX-M-1* gene. The antibacterial activity of ethanolic extract of Neem plant against this bacteria was done by agar well diffusion method at different concentrations (50, 100, 200, and 300mg/ml) and evaluated then the inhibition zone diameters were (2, 4, 5 and 6mm) against *E. coli* (ESBL+) and (0, 2, 5 and 6mm) against *K. pneumonia* (ESBL+) respectively while *E. coli* (ESBL -) was (7, 6, 4 and 3 mm) respectively and with *k. pneumonia* (ESBL-) it gives (7, 6, 3 and 2 mm) respectively. The MIC for *E. coli* (ESBL+) was 125mg/ml, while the MIC for *E. coli* (ESBL-) was 62.5 mg/ml. The MIC for *K. Pneumonia* (ESBL-) was at a concentration of 31.25mg/ml and for *K. Pneumonia* (ESBL+) was at 62.5mg/ml.

Key words: Neem plant extract, antibiotic resistance, Polymerase Chain Reaction, *CTX-M* genes.

1. Introduction

Neem plants *Azadirachta indica* have been considered the Holy Grail for many diseases and natural remedies. The antimicrobial properties of the Neem plant range from antibacterial, antifungal, and antiviral to combating different diseases and infections also it has anti-inflammatory, hepatoprotective, and cardioprotective effects **[1].** *Azadirachta indica* belongs to the family *Meliaceae*, which is known as (Neem). The United States National Academy of Science announced that the Neem plant is able to treat many infections all over the world. About thirty countries cultivate this plant from the African continents to the Asian continents. All parts of the Neem plant including, (fruit, root, leaves, seed, and flowers) are widely used in old medication as a medicine to treat many human diseases because of its secondary metabolites that are biologically active and effective **[2].**

Neem plant consists of many active chemical components such as polyphenol flavonoids, 17-hydroxyazadiradione, 7-desacetyl-7-benzoylgedunin, 7-desacetyl-7-benzoylazadiradione, nimbin, azadirachtin, nimbiol, etc **[3].** The importance of the Neen plant has increased in the medicinal and

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pharmaceutical fields and also has a wide range of biological activities because of its chemical components **[4-5].**

Several studies have reported the role of Extended Spectrum Beta-lactamases ESBL-producing bacteria in the pathogenesis of urinary tract infection (UTI). However, the proposed treatment methods have not had much success due to several factors the most important of which are resistance to an antibiotic, doctors' inability to estimate the optimal time for the treatment, resulting in misprescribing of antibiotics, also not understanding of how UTI function in different groups of people, leading to inappropriate antibiotic treatment of the disease. **[1].**

ESBL-producing bacteria are Gram-negative bacteria that belong to the family *Enterobacteriaceae* **[6]** such as ***E. coli*** and ***K. pneumonia*** and they are responsible for many infections like urinary tract, respiratory, and bloodstream infections. In humans to treat these types of infections, β-lactam antibiotics like penicillin and cephalosporin are widely used. The overuse of these antibiotics leads to a mechanism of resistance of the bacteria to these antibiotics **[7].** For many years the beta-lactam antibiotic used as an effective antibiotic frequently used to treat a lot of infections. Therefore, the resistance of bacteria to this antibiotic was gradually raised because of the transfer of beta-lactamase genes through conjugation between bacteria. **[8-9].**

ESBLs are enzymes that are capable of hydrolysis oxiamino-cephalosporins species **[10].** In recent years, beta-lactamases CTX-M enzymes have been mostly reported. **[11-12]**. In 1989, this was the first time that the *CTX-M* gene was reported from ESBLs in Germany, later on, these enzymes spread all over the world. The CTX-M enzyme has been reported in *E. coli* and *K. pneumonia*, also it has been founded in many *Enterobacteriaceae* species **[10].** In general, the genes of resistance are found in plasmids, and in some species of Enterobacteriaceae resistant genes are encoded by chromosomes **[13-14].** Previous studies have identified more than 60 types of CTX-M enzymes. **[15].** This study aimed to isolate E. coli and K. pneumonia from infected urine samples then determine the existence of the resistant genes (CTX-M) in ESBL-producing bacterial isolates of E. coli and K. pneumonia, and to determine the antibacterial effect of Neem plant (A. indica) ethanolic extract against this bacteria.

2. Material and methods:

2.1 Bacteria Isolation from Specimens.

In this study, *Enterobacteriaceae* isolates were isolated from infected urine samples, and 10 µL well-mixed urine sample was inoculated into MacConkey agar media and incubated at 37°C for 24 hours. A colony count with at least 100 CFU/ml for a single midstream was taken as a positive urine culture. All the isolates were preliminarily screened by their colony morphology, pigment production, and Gram-staining techniques. Further identifications of isolates were made by the conformation of identified isolates by using 16srRNA gene amplification.

2.2 Collection of Neem plant.

The Neem plant leaves were collected from Jeddah - Saudi Arabia. The collected samples were packed in plastic containers and were transported to the Microbiology Lab, Faculty of Science, King Abdul-Aziz University, Jeddah - Saudi Arabia for the preparation of Neem leaf extract and extensive studies.

2.3 Preparations of Neem leaf extract (Ethanol extraction)

Fifty grams of dried powder of grained leaves were soaked in 500 ml 80% ethanol for 48 hr. and kept in a rocking shaker at 75 rpm at room temperature 25°C the mixture was allowed to stand for 30min, then filtered with filter paper. A Rotatory evaporator was used at 45-55°C to evaporate excess water for 2-3 hours. After complete evaporation the residue obtained was semi-liquid, then decant into Petri dishes to complete dryness for one day then the residue was collected, weighted, and dissolved in Di-Methyl-Sulfoxide (DMSO) 5 % to obtain starting concentration of 500 mg/ml.

2.4 Screening for ESBL+ bacteria.

2.4.1 Phenotype detection of ESBLs using the hybrid disc method.

Antibiotic susceptibility test of bacteria was tested by disc diffusion method **[16]** against 12 commercially available antibiotics such as Ampicillin, Amoxicillin/ (clavulanic acid), Piperacillin / Tazobactam, cefalotin, cefazolin, cefuroxime, cefotetan, cefoxitin, cefixime cefotaxime, ceftazidime, and ceftriaxone (Hi Media, India). The test results were scored as sensitive and resistant according to the interpretation of the area diameter.

2.4.2 PCR for Amplification of ESBL *CTX-M and CTX-M-1* Genes.

2.4.2.1 DNA Extraction.

DNA extraction was performed using the boiling method **[17]**. In this method, three to four colonies of positive phenotypic strains were suspended in microtubes with 500 µL of sterile distilled water. They were placed in a thermo black machine for ten minutes at 100°C. Then, the samples were centrifuged for 10 minutes at 4°C and 12000 rpm. In addition, 200 µL of the surface fluid was separated in a sterile condition and was stored in Eppendorf microtubes at -20°C. The

concentration of the extracted DNA was measured using a Bio Photometer.

2.4.2.2. PCR screening for the *CTX-M* and *CTX-M-1* Genes

The PCR reactions were carried out using two pairs of primers. As in (**Table 1) [18-19].** The best conditions for the PCR reaction were recognized in a pilot method by performing multiple repetitions of the test and concentration changes and the temperature gradient in a Master Cycler (Eppendorf, Germany).

Table.1: Primer Sequences of the ESBL *CTX-M* Genes Amplified By PCR:

Primer	Sequence (5' to 3')	Size
bla $_{CTX-M}$ **F**	TTT GCG ATG TGC AGT ACC AGT AA	**590 bp**
bla $_{CTX-M}$ ***R***	CGA TAT CGT TGG TGG TGC CAT	
bl a $_{CTX-M-1}$ **F**	GAC GAT GTC ACT GGC TGA GC	**499 bp**
bla $_{CTX-M-1}$ **R**	AGC CGC CGA CGC TAA TAC	

2.4.2.3 Gel Electrophoresis.

The PCR product was evaluated by using 1.2% agarose gel with the addition of 0.5µg/mL of ethidium bromide (Cina- Gen Co., Iran). Relevant gel photography was performed using Gel Documentation.

2.5 Detection of the antibacterial effect of the ethanolic extracts of Neem plants on isolated bacteria.

2.5.1 Agar well diffusion method (determination of inhibition zone diameter)

Molten Mueller Hinton agar media was poured into each Petri plate and allowed to solidify, bacterial strains were spread with 0.5 McFarland using a sterile cotton swab and allowed to stand for 10 min, then four wells were made in each plate with a sterile cork borer, then add 100 µl of the ethanolic plant extracts in each well from different concentration 300,200,100,50mg/ml and leave it for 24 hours at 37 °c then take the result and measure the diameter of inhibition zone.

2.5.2 Determination of Minimal inhibitory concentration (MIC) and Minimal bacterial concentration (MBC) of the plant extract against *E. coli* and *K. pneumonia.*

Microplate (96 polystyrene well) was used for the preparation of different concentrations of Neem extract by serial dilution. Bacterial strains were taken after 24 hr. incubation at 35°C. The turbidity of bacteria suspension was adjusted to 0.5 McFarland equivalent to a concentration of (1-2 X 10^8) CFU /ml 10 µl of bacterial suspension in supplemented MH broth .100 µl of the medium was dispensed in each well that started from concentration 500mg/ml of plant extract. Row 2 was used as a negative control with 200 µl of media, and Row1was used as a positive control with 100 µl of media and 100 µl of bacteria suspension; the micro-plates were sealed in plastic bags and were incubated for 24 hr. at 37°C. MIC was determined as the lowest concentration of each plant extract that inhibited the bacteria growth, the minimal bacterial concentration (MBC) was obtained by subculturing from each well of microplate onto a nutrient agar plate. The well that failed to show growth on subculture was considered as MBC for that test strain. Phenol red was used as an indicator **[20-21].**

3. Results

3.1 Bacteria Isolation from infected urine samples.

After growing into MacConkey agar, we isolate four *Enterobacteriaceae* isolates. Two of them are ***E. coli*** and two isolates are ***K. pneumonia*** then we confirm the bacterial isolates by PCR amplification of 16s r RNA genes as shown in (**fig. 1).**

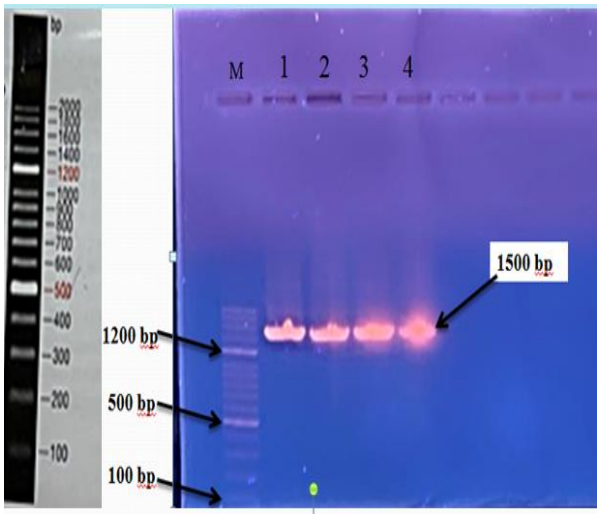

Fig. 1: Gel electrophoresis for 16s rRNA genes. The band 1500 bp is related to 16s rRNA genes. Lane M is (DNA marker) and Lanes 1– 2 are positive *E.coli* isolates and Lanes 3– 4 are positive *K. pneumonia* isolates.

3.2 Phenotypic Detection of ESBLs with combination disc diffusion method

Antibiotic susceptibility test for isolated bacteria was done by using the disc diffusion method against 12 commercially available antibiotics. From the result we obtained 2 resistant isolates **ESBLs+** (***E.coli*** isolate and K***. pneumonia*** isolate.) also obtained 2 sensitive isolates **ESBLs-** (***E.coli*** isolate and K***. pneumonia*** isolate.) as shown in (**fig. 2).** The effects of 12 antibiotics on the different isolates are shown in (**Table 2).** Examination of the phenotype of ESBLs in the four isolates showed that two of the isolates were ESBL-producing and two isolates were not ESBL-producing isolates.

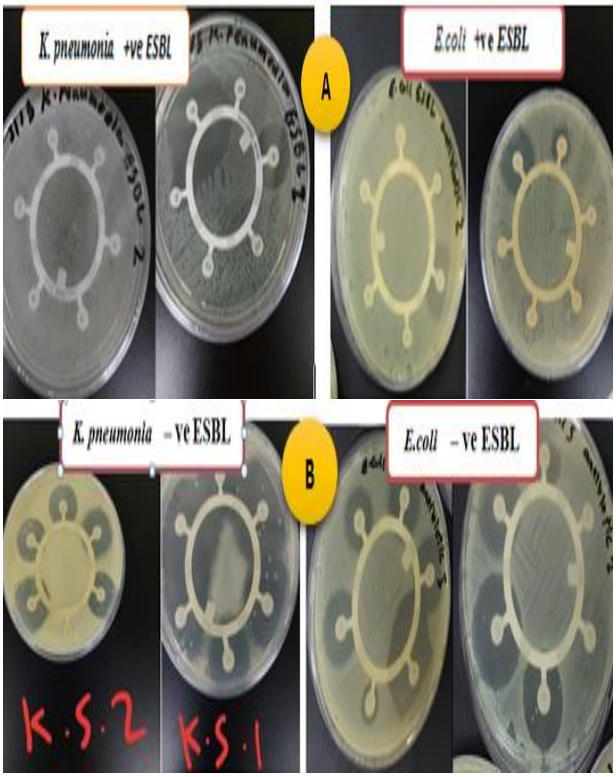

Fig.2: Disc diffusion method against 12 commercially available antibiotics
A: 2 resistant isolates **ESBLs+v**
B: 2 sensitive isolates **ESBLs**-ve

Table 2: Frequency of Phenotypic ESBL+ and ESBL-*Enterobacteriaceae* isolated against 12 antibiotics.

	Antibiotics	Sensitivity against antibiotics			
		K. pneumonia		*E.coli*	
		Isolate 1	*Isolate 2*	*Isolate 1*	*Isolate 2*
1	Ampicillin	R	R	R	R
2	(clavulanic acid)/Amoxicillin	S	S	S	S
3	Tazobactam/Piperacillin	R	S	S	R
4	Cefalotin	R	S	S	R
5	+ cefazolin	R	S	R	R
6	+cefuroxime	R	S	S	R
7	+cefotetan	R	S	S	S
8	Cefoxitin	R	S	S	R
9	+cefixime	S	S	S	S
10	Cefotaxime	S	S	S	R
11	Ceftazidime	R	S	S	R
12	Ceftriaxone	R	S	S	R
ESLB probability		+ ve **ESLB** 93% probability	-ve **ESLB** sensitive	-ve **ESLB** sensitive	+ ve **ESLB** 98%probability

+ ve ESLB: ESBL-Positive **-ve ESLB**: ESBL-Negative **R**: Resistant **S**: Sensitive

3.3 Confirmation for the ESBL-producing isolates by PCR Amplification of ESBL *CTX-M and CTX-M-1* Genes

The PCR results confirmed that the two isolates which showed antibiotic resistance by phenotypic detection are ESBL-producing isolates and these isolates have the *CTX-M* and the *CTX-M-1* genes. The Agarose gel electrophoresis of the PCR products for the *CTX-M* and *CTX-M-1* genes are presented in (**fig. 3)**

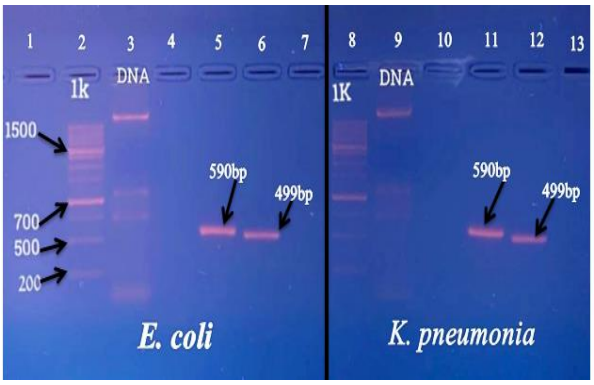

Fig. 3: Gel electrophoresis in 1x TAE buffer, the band 590bp is related to the *CTX-M* gene, and the band 499bp is related to the *CTX-M-1* gene. lanes 2 & 8: DNA marker; lanes 4&10: *CTX-M* and *CTX-M-1* negative control respectively; lanes 5&11: positive ***E.coli*** and ***K. pneumonia*** possessing the *CTX-M* gene respectively; lanes 6&12: positive ***E.coli*** and ***K. pneumonia*** possessing the *CTX-M-1* gene respectively; lanes 3&9: DNA of ***E.coli*** and ***K. pneumonia*** respectively.

3.4 Agar well diffusion method

The Neem plant extract was tested against ESBL- and ESBL+ isolates of *E. coli* and *K. pneumonia*. For ***E.coli*** (ESBL-) Maximal inhibition zone was (7 mm) obtained at the highest concentration of 300 mg/ml and the minimal inhibition zone was (3 mm) obtained at the lowest concentration of 50 mg/ml. For ***E.coli*** (ESBL+) Maximum inhibition zone was (6 mm) obtained at the highest concentration of 300 mg/ml and the minimum inhibition zone was (2 mm) obtained at the lowest concentration of 50 mg/ml.
For ***K. pneumonia*** (ESBL-) Maximum inhibition zone was (7 mm) obtained at the highest concentration of 300 mg/ml and the minimum inhibition zone was (2 mm) which was obtained at a concentration of 100 mg/ml while at the lowest concentration of 50 mg/ml there was no inhibition zone. For ***K. pneumonia*** (ESBL+) Maximal inhibition zone was (6 mm) obtained at the highest concentration of 300 mg/ml and the minimal zone of inhibition (2 mm) was obtained at a concentration100 mg/ml while at the lowest concentration of 50 mg/ml, there was no inhibition zone(**fig .4, fig .5, fig.6 &Table 3).**

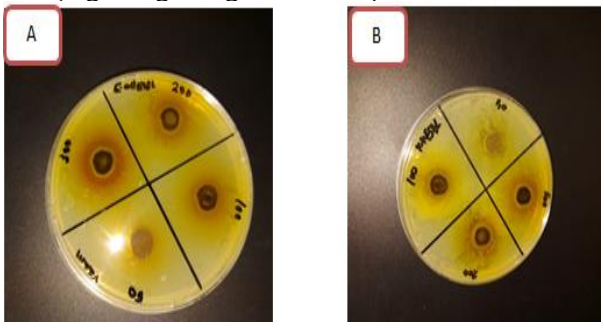

Fig. 4: (Agar well diffusion method) Effect of Neem plant extract at different concentrations 300, 200,100, and 50 mg/ml on: **A:** *E.coli* (ESBL+) **B:** *k. pneumonia* (ESBL+).

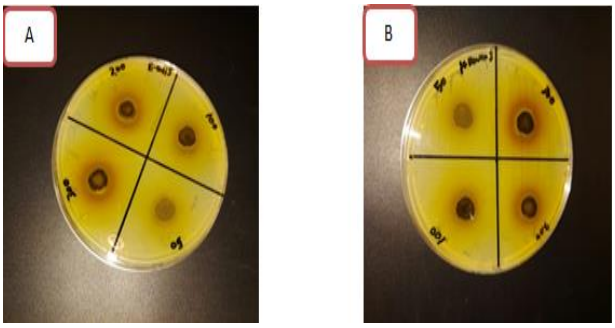

Fig. 5: (Agar well diffusion method) Effect of Neem plant extract at different concentrations 300, 200,100, and 50 mg/ml on: **A:** *E.coli* (ESBL-) **B:** *k. pneumonia* (ESBL-)

Table 3: Effect of Neem plant (*A. indica)* extract on the bacterial isolates.

Bacteria isolates	Inhibition Zone in mm at different concentrations (mg/ml)			
	300 mg/ml	200 mg/ml	100 mg/ml	50 mg/ml
E.COLI (ESBL-)	7mm	6mm	4mm	3mm
E.COLI (ESBL+)	6mm	5mm	4mm	2mm
K. pneumonia (ESBL-)	7mm	6mm	3mm	2mm
K .pneumonia (ESBL+)	6mm	5mm	2mm	-ve

ESBL: Extended‑spectrum beta‑lactamase.

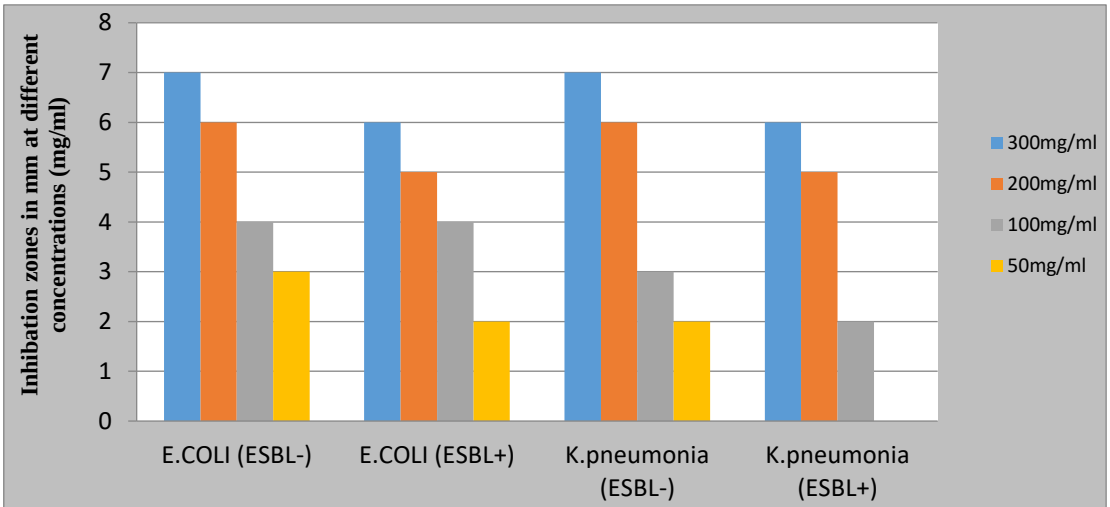

Fig. 6 :Effect of Neem plant (*A. indica)* extract against different bacterial isolates

3.5 The MIC and MBC of Neem plant extract against the bacterial isolates:

In this study, the ethanolic extract of the Neem plant was subjected to antibacterial activity against ***E. coli*** *and* ***K. Pneumonia*** (ESBL+) and (ESBL-). The plant extract at 500 mg/ml concentrations completely inhibited the growth of all isolated bacteria. The **MIC** of the extract against ***E. coli*** (ESBL+) was 125mg/ml, while the **MIC** against ***E. coli*** (ESBL-) was 62.5 mg/ml. the **MIC** against ***K. Pneumonia*** (ESBL-) was at a concentration of 31.25 mg/ml and ***K. Pneumonia*** (ESBL+) was at 62.5mg/ml. This indicates that Neem plant extract has antibacterial activity against ***E. coli*** *and* ***K. Pneumonia*** as shown in (**fig. 7).**

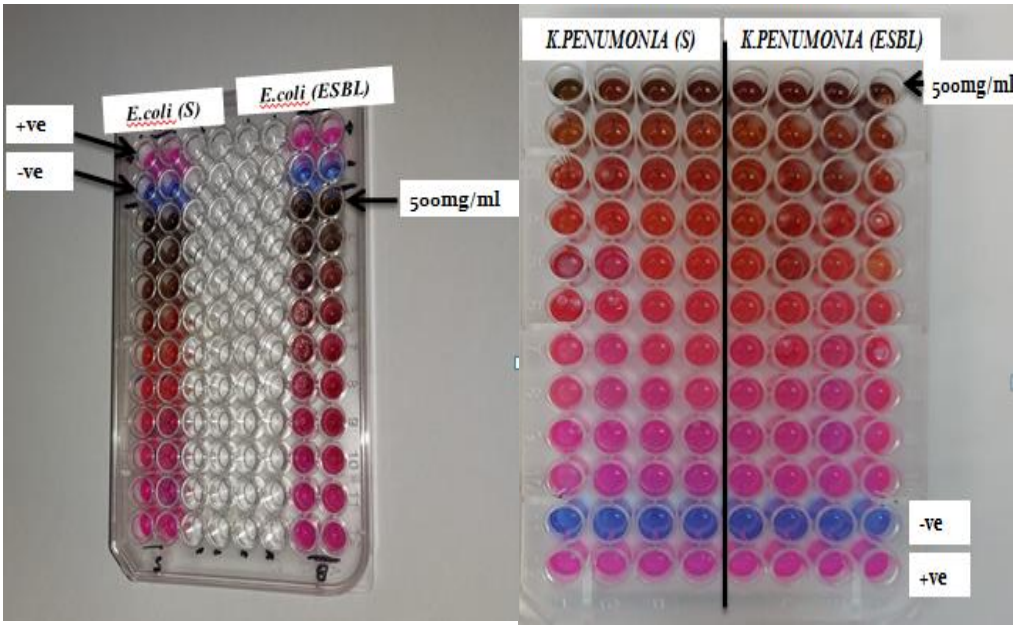

Fig. 7: MIC micro-plate starting from concentration **500 mg/ml** and it decreases descendingly to half at each well. **-ve** (Negative control) and **+ve** (Positive control). (**S**): Sensitive bacteria (ESBL-).

The (MBC) was obtained by subculturing the content of the microplate wells on a nutrient agar media. The well that did not show any growth on the media was considered as MBC for the isolated strains, all the result is shown in **(fig.8 &Table 4)**

Table 4: MIC and MBC of *A. indica* against the isolates.

Bacteria isolates	MIC (mg/ml)	MBC (mg/ml)
E.COLI (ESBL-)	62.5	125
E.COLI (ESBL+)	125	250
K. Pneumonia (ESBL-)	31.25	62.5
k. Pneumonia (ESBL+)	62.5	125

MIC: Minimum inhibitory concentration,
MBC: Minimum bacterial concentration, ESBL: Extended‑spectrum beta‑lactamase.

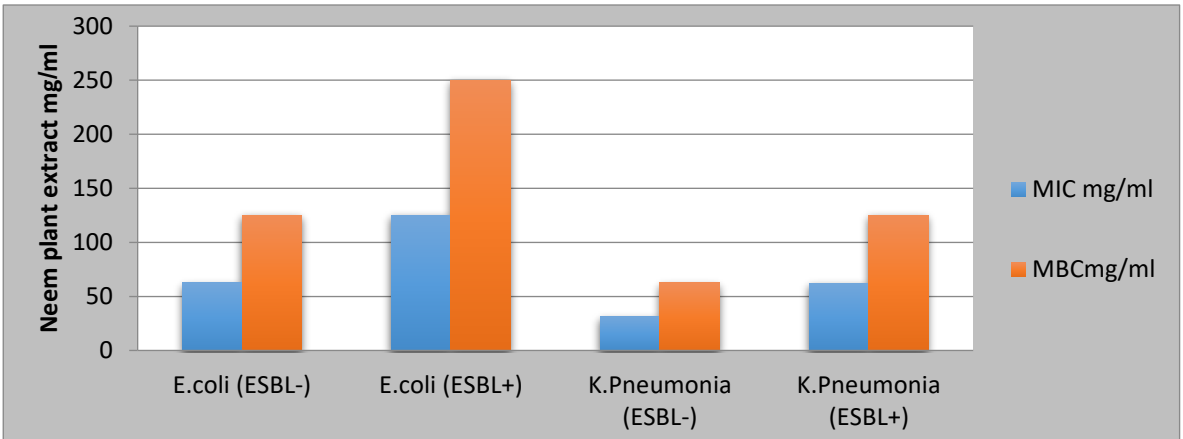

Fig. 8: MIC and MBC of *A. indica* against the bacterial isolates and its distribution

4. Discussion

WHO has released a severe warning that the world will enter "the post-antibiotic era" when most of the commonly used antibiotics will no longer be effective. **[22].** Antimicrobial resistance to antibiotics poses a major threat to all medicine branches and public health **[23].** It puts us at risk in the health field by raising mortality and morbidity rates and causing an enormous economic burden. **[24-26]** Furthermore, most new antibiotics are expensive and run the risk of an adverse effect of drug reaction, so the plant sources alternatives are more safe and effective than modern antibiotics. **[27-28]** and are used in medicine. Studies have indicated that medicinal plants will be an alternative source for treatment the of infectious microorganisms **[29- 30].**

Previous studies have proven that the extracts from the Neem leaves (20µg/disk) have an antibacterial effect on *E. coli* **[31]** and this agreed with our results. In our study the extract of Neem plant was subjected to antimicrobial activity against ***E.coli*** *and* ***K. Pneumonia*** *ESBL + and ESBL.*

The Neem leaves extract at 500 mg/ml concentrations completely inhibited the growth of ***E.coli*** (ESBL+), ***E.coli*** (*ESBL*-), ***K. Pneumonia*** (ESBL-), ***and K. Pneumonia*** (ESBL+). The **MIC** of the extract against ***E.coli*** (ESBL+) was 125mg/ml, while the **MIC** against ***E.coli*** (ESBL-) was 62.5 mg/ml. the **MIC** against ***K. Pneumonia*** (ESBL-) was at a concentration of 31.25mg/ml and ***K. Pneumonia*** (ESBL+) was at 62.5mg/ml. This indicates that that extract has antimicrobial activity against ***E.coli*** *and* ***K .pneumonia.***

Studies investigated that the ethanolic Neem leaf extract has an antimicrobial effect on ***K. pneumonia***, and it has an inhibitory effect on ***K. Pneumonia*** at a concentration of 12.5 mg/mL with an inhibition zone

diameter of 18mm **[32].** The finding somewhat agreed with our result where the **MIC** against ***K. Pneumonia*** (ESBL-) was at a concentration of 31.25mg/ml and for ***K. Pneumonia*** (ESBL+) was at 62.5mg/ml but it was not consistent with our result where the diameter of inhibition zone was 7mmand 6mm for both ***K. Pneumonia*** (ESBL-) and ***K. Pneumonia*** (ESBL+) respectively. In our study at a concentration of 50 mg/mL, the Neem extract has no inhibitory effect on the growth of ***K. pneumonia*** (ESBL+).

The results obtained in the agar diffusion method are followed by what we obtained in the MIC test. For ***E. coli*** (ESBL-) Maximal inhibition zone was (7 mm) obtained at 300 mg/ml at the highest concentration and the minimal inhibition zone was (3 mm) obtained at the lowest concentration at 50 mg/ml. For ***E. coli*** (ESBL+) Maximum inhibition zone was (6 mm) obtained at the highest concentration of 300 mg/ml and the minimal inhibition zone was (2 mm) obtained at the lowest concentration of 50 mg/ml. For ***K. pneumonia*** (ESBL-) Maximum inhibition zone diameter was (7 mm) obtained at the highest concentration of 300 mg/ml and the minimum inhibition zone diameter was (2mm) obtained at a concentration100 mg/ml while at the lowest concentration50 mg/ml, there was no inhibition zone.

For ***K. pneumonia*** (ESBL+) Maximum inhibition zone was (6 mm) obtained at the highest concentration of 300 mg/ml and the minimal zone of inhibition was (2 mm) obtained at a concentration100 mg/ml while at the lowest concentration of 50 mg/ml, there was no inhibition zone. These results are similar to previous studies done by **Odunbaku** [**33]** who stated that the ethanolic extract of Neem plant showed antibacterial activity against ***E. coli*** and ***K. pneumonia*** and a few other bacteria. Other investigators have made similar observations **[34- 35]. Jahan [36]** in her study found that Neem oil has a growth inhibitory effect on ***E. coli***.

5. Conclusion

The results of our study are phenotypically and genotypically confirming the ESBL-producing strains among *Enterobacteriaceae* isolates ***E. coli*** (ESBL+) and ***K. Pneumonia*** (ESBL+) by the *CTX-M* and *CTX-M-1* genes. Therefore, we recommend antibiotic susceptibility tests for the bacteria before prescribing any antibiotics. Doing these tests can help in the prevention of the spreading of resistant strains. In this study, the ethanolic extract of Neem leaf has antibacterial activity against ***E. coli*** (ESBL) and ***K. Pneumonia*** (ESBL) bacterial isolates. And it is a good and safe alternative source to treat the resistant bacteria that are drug resistant.

6. Acknowledgment

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