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Antibacterial Activity of Some Medicinal Plant Extracts Against Biofilm and Non-Biofilm of *klebsiella pneumoniae*

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Abstract: The main objective of this study was to study the antibacterial activity of some medicinal plant extracts against resistant planktonic and biofilm forming *K.pneumoniae* isolated from patients admitted to Mansoura University Hospitals. In addition, this study was extended to investigate the effect of some natural plants extracts as abioagent against biofilm and non-biofilm producer *K. pneumoniae*. Forty clinical *K.pneumoniae* isolates were collected from patients admitted in different intensive care units (ICU) of Mansoura University Hospitals. Antibiotic susceptibilities of *K.pneumoniae* pattern was tested by the disk diffusion method .Ethanolic plant extracts were tested against biofilm and non-biofilm producer *K. pneumoniae* isolates by using agar well diffusion method. High rate of antibiotic-resistance among clinical *K. pneumoniae* isolates was detected and this necessitates monitoring the microbial trends and resistance patterns. Plants may be used as natural antibiotics in the treatments of antibiotic resistant *K. pneumoniae* infections **.**

keywords: *Drug Resistance, , Bacterial, klebsiella pneumoniae,Biofilm formation.*

1.Introduction

The pathogenic success of *K.pneumoniae* in causing urinary and respiratory tract infections may be attributed to its ability to form biofilms, particularly on indwelling medical devices **[1]**. The formation of biofilm in *K.pneumoniae* has also been hypothesized to contribute to bacterial persistence, i.e. reduced susceptibility to be killed by the innate defense mechanisms of the host **[2].**

The resistance of biofilm-forming *K.pneumoniae* strains isolated from medical devices to other antibiotics such as gentamicin, cefotaxime and ciprofloxacin have also been reported **[3].** The expression of type 3 fimbriae have been shown to promote biofilm formation on biotic as well as abiotic surfaces **[4].** These fimbriae belong to the chaperone-usher class of fimbriae and are encoded by five genes (mrk ABCDF) arranged in the same transcriptional orientation **[5]**. This operon called mrk comprises the structural genes and those encoding the polypeptides required for assembly of the structure to the surface of the bacterium **[6]**. The MrkD adhesin has been shown to mediate adhesion to collagen structures **[7].**

The scientists developed new drugs from natural sources such as plants, which have been extensively used as an alternative treatment of diseases **[8]** with minimal side effects, being inexpensive and safe compared to the synthetic drug **[9]**.

These plants contain phytochemicals active compounds such as flavonoids, tannins, saponins, alkaloids, terpenes **[10]**, vitamins (A, C, E and K), carotenoids, polyphenols, pigments, enzymes and minerals **[11]** which are responsible for its antimicrobial activities. The extract of these herbal plants are used in the treatment of acne, diarrhea, cold, cough, digestive disorders etc.

This study aimed to detect the resistance of gentamicin, cefotaxime and ciprofloxacin in *K. pneumoniae* isolates from patients admitted to Mansoura University Hospitals and its ability to form biofilm; additionally, to identify certain natural plant extracts that could be used against

resistant biofilm and non-biofilm forming *K.pneumoniae* .

Materials and methods

Collection of samples and identification of *K. pneumonia*

Clinical samples (endotracheal tube and urinary catheter) were collected from patients admitted in different intensive care units (ICU) of Mansoura University Hospitals. These samples were cultured using the standard media Blood agar and MacConkey's agar and incubated aerobically at 37^0C overnight. The identification of *K. pneumoniae* isolates was done by colony morphology, microscopic examination after Gram staining, and biochemical tests including Kligler Iron Agar (KIA), Lysine Iron Agar (LIA), Motility, Indole, Ornithine medium (MIO), Urease and citrate utilization tests.

Antimicrobial susceptibility test

Antibiotic susceptibilities of *K.pneumoniae* isolates was done by Kirby Bauer disc diffusion method **[12].** Using Muller-Hinton agar medium. The tested antibiotics include: Ciprofloxacin, CIP (5 µg); Cefotaxime, CTX (30 µg); Gentamicin, CN (10 µg). The clear zones were measured and compared with the standard recommendation of Clinical Laboratory Standard Institute (CLSI) **[13]**.

Biofilm formation assay

Biofilm was performed in 96 well microtiter plates as described by **[14]** with some modification. Briefly, *K.pneumoniae* isolates were subcultured on brain heart infusion broth over night at 37^0C. 200 µl of bacterial cultures were transferred to each well then incubated for 24h at 37^0C and negative control contained media only. The media was removed and washed three times with phosphate buffer saline then 25µl of crystal violate (1% w/v) was added to the wells for 15 min at room temperature. Crystal violate was then removed

and washed three times with water. The crystal violate inside the cells was dissolved by 30% acetic acid and the absorbance was measured by Readwell Touch Elisa Plate Analyser at OD=550. The isolates were classified according to OD value as follows: **<0.120**weak biofilm,**0.120-0.240** moderate and **0.240**strong biofilm **[15].**

Preparation of plant extracts

Plant materials of four plant species included in this study **Table (1)** were collected from herbalists and markets in Mansoura, Egypt. The collected herbal plants was dried and pulverized into a fine powder. The powdered material was stored in air tight sterile containers and protected from sunlight until required. 10 g of every dried powdered plant material were mixed with 100 ml of 95% ethanol solvent in a sterile conical flask, which was covered with foil paper and placed on a rotatory shaker for 24 hrs, then filtered through Whitman filter paper (No 1). The supernatant was collected and concentrated in vacuum for 15 min at 37°C using a Rotatory evaporator to make the final volume half of the original volume (stock solution). The concentration was then dissolved in 10 ml of 1% dimethylsulfoxide (DMSO). All extracts were sterilized by filtration through a bacterial filter of pore size 0.45µm using positive pressure, then the filtrate was kept at 4°C in the refrigerator till use.

Antibacterial activity of herbal plant extracts

Agar well diffusion method was used to evaluate the antimicrobial activity of each plant extract. Muller Hinton Agar medium was prepared and inoculated with biofilm and non-biofilm forming *K.pneumoniae* suspension by streaking the sterile nontoxic cotton swab in three directions over the entire surface of the agar plates to obtain a uniform inoculum. The density of the *K.pneumoniae* suspension was equivalent to that of 0.5 MacFarland standard (1.5×10^8 CFU/mL) .Sterile cork borer was used to make wells of 6mm in diameter in the agar plate. 150 µl of Plant extracts were introduced into each well using sterile Pasteur pipette and allowed to stand for 1 hour at room temperature to diffuse the plant extracts into the medium. The DMSO was used in the same manner as a negative control. The plates were then incubated at 37ºC for 18-24 hours. After incubation the entire diameter of the inhibition zone was measured in three different directions on all 3 replicates and the average value was tabulated then subtracting the diameter of the well.

Table (1): Family, scientific, English, Arabic names and parts used from each plant in preparing extracts

Family	Scientific name	English name	Arabic name	Used part
Myrtaceae	*Syzygium spp*	Clove	القرنفل	Flowers puds
Liliaceae	*Allium spp*	Garlic	الثوم	Bulb
Theaceae	*Camellia spp*	Green tea	الشاي الأخضر	Leaf
Lauraceae	*Cinnamomum spp*	Cinnamon	القرفه	Steam

Sterile cork borer was used to make wells of 6mm in diameter in the agar plate. 150 µl of

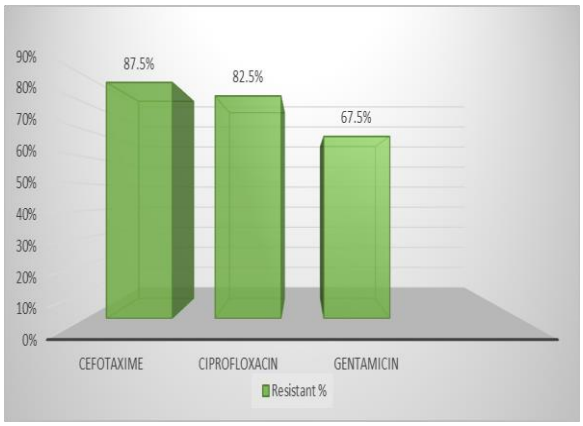


Fig. (1): Resistance of *K.pneumoniae* isolates against antibiotics

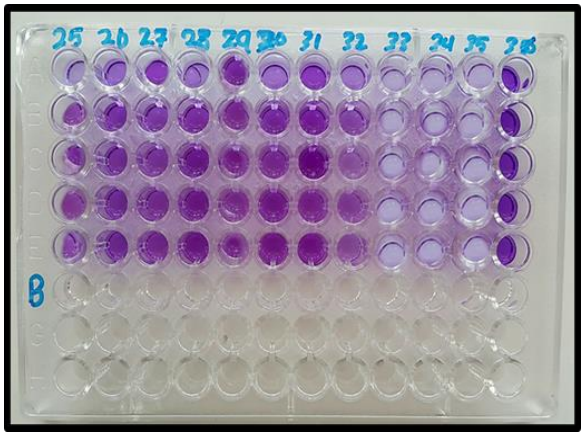


Photo (1): *K.pneumoniae* no.33, 34, 35 are non/weak biofilm forming according to OD was less than 0.120 at 550

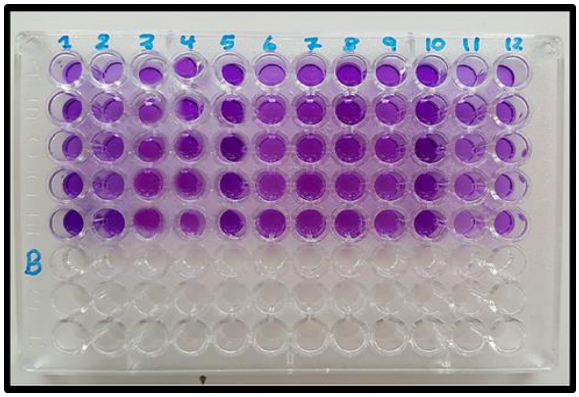


Photo (2): *K.pneumoniae* no.10 is moderate biofilm forming according to OD at 550 was between 0.120-0.240 the rest of isolates are high biofilm forming OD at550 was more than 0.24

Results

Antimicrobial susceptibility

Forty *K.pneumoniae* isolates were tested for their resistance to deferent antibiotic categories. The *K.pneumoniae* isolates showed high resistance to Cefotaxime (87.5%) followed by ciprofloxacin (82%) and Gentamicin (67.5%). (Fig. 1).

Biofilm formation assay

Forty clinical *K.pneumoniae* strains isolated in our study were tested for their ability to form biofilms by tissue culture plate method (TCP) the results were interpreted according to that described by **[15],** the data obtained was used to classify the OD value less than 0.120 was considered as non- biofilm producers, (0.120-0.240) as moderate biofilm producers, and more than 0.240 as strong biofilm producers. **Photo (1,2).**

The *K.pneumoniae* isolates showed high biofilm formation (65%) moderate (22.5%) and weak /non (12.5%) this explains most of isolates were resistance to antibiotics.

Antibacterial activity of herbal plant extracts

Four plant species were investigated to evaluate their antibacterial activity against biofilm and non-biofilm forming *K.pneumoniae* using agar well diffusion method. Evaluation of antibacterial activity of these plant extracts was recorded in **Tables (2,3)** and illustrated in **Photo (4,5)**. The ethanolic extract of Green tea was the most active one with inhibition zones diameter ranged between 7mm-18mm and Clove caused inhibition zones diameter ranged between 9mm-16mm. followed by Cinnamon and without any effect of Garlic.

Table (2): Antimicrobial activity of ethanolic plant extracts against biofilm former *K.pneumoniae:*

	Inhibition zones diameter (mm)			
Strains	**Green tee**	**cloves**	**Cinnamn**	**Garlic**
Kp1	12mm	10 mm	0	0
Kp2	12mm	11 mm	0	0
Kp10	0	12 mm	0	0
Kp36	0	12 mm	11 mm	0
Kp39	7mm	9 mm	0	0

Table (3): Antimicrobial activity of ethanolic plant extracts against non-biofilm former *K.pneumoniae*:

	Inhibition zones diameter (mm)			
strains	**Greentee**	**cloves**	**Cinnamn**	**Garlic**
Kp33	14mm	13mm	0	0
Kp34	13mm	14mm	0	0
Kp35	18mm	16mm	0	0
Kp37	12mm	10mm	0	0
Kp38	14mm	15mm	10mm	0

Photo (4): Inhibition zones of different ethanolic plant extracts against biofilm former *K.pneumoniae*

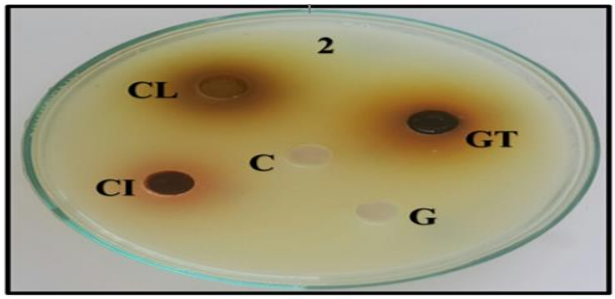
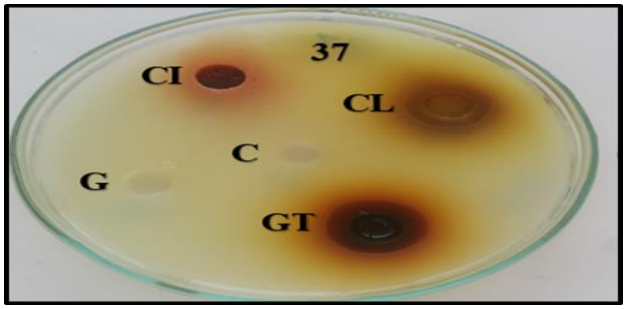


Photo (4): Inhibition zones of different ethanolic plant extracts against Non- biofilm former *K.pneumoniae*



GT = Green tea,CL = Clove,G = Ginger,CI = Cinnamon,C = Control (Dimethylsulfoxide (DMSO))

Discussion

The main objective of this study was to evaluate the antibacterial activity of some medicinal plant extracts against resistant planktonic and biofilm forming *K.pneumoniae* isolated from patients admitted in Mansoura University Forty pure *K.pneumoniae* were isolated from endotracheal tube and urinary catheter from patients admitted to ICU of different Mansoura University Hospitals.

In this study, the antibiotic susceptibility of *K.pneumoniae* to deferent antibiotic categories, showed that the tested isolates were highly resistant to Cefotaxime (87.5%) followed by Ciprofloxacin and Gentamicin with 82.5% and 67.5% resistance, respectively.

This result was in agreement with **[16]** in Pakistan who noticed that the percentage of resistance to cefotaxime were 82.5 %. Resistance of *K.pneumoniae* to the cephalosporin group may be related to possessing of β-lactamase enzymes (cephalosporinase) which are able to the inactivate cephalosprins through cleavage of β-lactam ring of the drug **[17].**

Biofilm assembly plays a role in the increasing resistance of *K.pneumoniae* strains to antibiotics. In this study 40 *K.pneumoniae* isolates screened for biofilm production observed that 77.5% were positive and 12.5% were negative for biofilm production. This result was supported by **[18]** who found that about 63% of *K.pneumoniae* isolates from urine samples of catheterized patients suffering from UTIs were positive for *in vitro* biofilm production.

Nowadays; pharmaceutical companies are developing new antibiotics to replace those that are no longer effective **[19].**Healing with medicinal plants is as old as mankind itself.

Two out of four plant extracts tested (green tea extract and clove) showed a wide variation of antibacterial activity against the growth of biofilm and non-biofilm forming *K.pneumoniae*, green tea was the most active one with inhibition zones diameter ranged between 7mm-18mm and clove caused inhibition zones diameter ranged between 9mm-16mm. followed by Cinnamon and without any effect of Garlic.

These results are supported by **[20,21]** who reported that Clove extracts had potent antimicrobial activity against *K.pneumoniae* with inhibition zones diameter ranged between 9mm-17mm**.** Moreover, **[22]** demonstrated that alcoholic extracts of green tea was effective plant extracts against *K.pneumoniae* with average zone 27mm. On the other hand, the garlic tested extract showed no evidence for

antibacterial activity against *K.pneumoniae.*It was interesting to note that crude extracts of tested herbal plants showed good activity against non-biofilm formation *K.pneumoniae* compared to biofilm formation *K.pneumoniae.*

Conclusion

Antibiotics resistance becoming a global problem for public health which threatens the lives of hospitalized individuals as well as health care cost and long-time treatment. The uncontrolled use of antibiotics such as fluoroquinolones share significantly to their resistance among *K.pneumoniae* isolates with produce biofilm representing an important factor of resistance. Therefore it is important issue to be addressed by policy makers to formulate a strict antibiotics prescription policy for bacterial infections in our country. Contrary to the synthetic drugs, antimicrobial of plant extracts are not associated with many side effects and have a great therapeutic potential to treatment of infectious caused by resistant microbes. Scientists have realized an immense potential in natural products from medicinal herbal plants to serve as alternative source of combating infections in human being which may also be of lower cost and less toxicity. However, further studies are needed to better evaluate the potential effectiveness of the crude extracts as the antimicrobial agents.

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