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Estimate Antimicrobial activity and Anti-biofilm formation of bark *Cinnamomum zeylanicum* on *Klebsiella pneumoniae* isolated from Urinary Tract Infections

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Abstract

Five isolates (25%) of *Klebsiella pneumoniae* were isolated from urine samples. In addition also isolated bacteria were (10) 50% *Escherichia coli*, while (3)15% *Proteus spp.*, (2)10% *Pseudomonas aeruginosa*. The ethanolic extract of *Cinnamomum zeylanicum* bark were tested against *Klebsiella pneumoniae* by using the well agar diffusion test, the alcoholic bark extract from (200 -12.5) mg/ml possessed antimicrobial activity against tested microorganism. At 200 mg/ml, and 100 mg/ml concentrations was diameter of inhibition zone rang from (18-26mm), (14-16mm) respectively, and these results compared to antibiotics Norfloxacin(10µg) inhibition zone (24-30mm), and Cefotaxim (10 µg) (26-27mm) as control. Antibiotics sensitivity were used ten antibiotics with disc diffusion method against *K. pneumoniae*, they were sensitive to Cefotaxime, Imipenem /Cilactin, Sparfloxacin, and Norfloxacin antibiotics only, and were resisted to the others antibiotics. Minimum inhibitory concentration was tested for plant extract, and was ranged versus *Klebsiella pneumoniae* (12.5-25) mg/ml. It could be concluded that alcoholic extract of *Cinnamomum zeylanicum* had a good antimicrobial effects and anti-biofilm on *Klebsiella pneumoniae* with significant difference ($p < 0.05$). This caused a decrease in the biofilm of the tested bacteria. Hence, it may be used instead of antibiotics to treat UTIs caused by bacteria used in current study.

Keywords: *K. pneumoniae*, UTIs, biofilm formation, Antimicrobial activity

تقييم النشاط ضد مايكروبي وضد تكوين الغشاء الحيوي للحاء القرفة *Cinnamomum zeylanicum* على بكتريا *Klebsiella pneumoniae* المعزولة من التهابات المسالك البولية

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الخلاصة

خمس عزلات (25%) من بكتريا الكليبيسيلا الرئوية تم عزلها من التهابات المسالك البولية. بالإضافة لذلك كانت البكتريا المعزولة (10) 50% *Escherichia coli*، بينما (3) 15% *Proteus spp.*، و (2) 10% *Pseudomonas aeruginosa*. تم اختبار المستخلص الإيثانولي للحاء نبات القرفة *Cinnamomum zeylanicum* ضد *Klebsiella pneumoniae*. باستخدام طريقة الانتشار بالحفر، أظهر مستخلص الحاء الكحولي نشاط مضاد للميكروبات (12.5-200) ملغم/مل المستخدمة في الدراسة. وعند التراكيز 200 ملغم/مل، و 100 ملغم/مل كان قطر منطقة التثبيط يتراوح بين (18-26) ملم و (14-16) ملم على التوالي.

التوالي، و هذه النتائج تم مقارنتها بالمضادات الحيوية القياسية نورفلوكساسين حيث كان قطر منطقة التثبيط بين (٢٤-٣٠ملم)، بينما كانت منطقة التثبيط لمضاد السيفوتاكسيم (٢٦-٢٧) ملغم/مل. واستخدمت بالدراسة عشرة مضادات حيوية بطريقة الانتشار بالاقراص ضد *Klebsiella pneumoniae*، وكانت حساسة للسيفوتاكسيم، امبيبينيم/سيلاستين، سبارفلوكساسين ونورفلوكساسين فقط و مقاومة لبقية المضادات الحيوية. وتم تحديد التركيز المثبط الأدنى (MIC) للمستخلص الكحولي ضد البكتريا *Klebsiella pneumoniae*، إذ يتراوح بين التراكيز (١٢.٥-٢٥) ملغم/مل. ويمكن ان نستنتج ان المستخلص الكحولي للقرفة له تأثير مضاد لبكتريا *Klebsiella pneumoniae*، وكذلك لتكوينها الغشاء الحيوي بوجود فروق معنوية ($P < 0.05$)، فقد تسبب في تقليل تكوين الغشاء الحيوي في تلك البكتريا. وبالتالي يمكن استخدامه بدلا من المضادات الحيوية لعلاج التهابات المجاري البولية المسببة عن البكتريا قيد الدراسة.

Introduction

For the first time *Klebsiella pneumoniae* was isolated from the lungs of patients, who died with infection of pneumonia. These encapsulated bacteria, were described as a saprophyte microorganisms, not only infected the human gastrointestinal tract, skin and nasopharynx, also were caused urinary and biliary tract infections, osteomyelitis, and bacteremia [1]. New stains of *K. pneumoniae* are being acknowledged resistant to antibiotics. *Klebsiella* conceded in the second rank of *E. coli* in urinary tract infections in older persons [2].

The *Klebsiella* infection tends to occur in people with a destabilized immunity. Many of these infections are nosocomial infections [3]. The choice of a specific antimicrobial agent depends on local sensitivity patterns. The performances of invasive procedures are risk factors for the occupation of these strains [4], [5].

K. pneumoniae possesses a multidrug resistance plasmids that encoded for resistance to aminoglycosides, and later, encoding extended-spectrum β -lactamases (ESBLs), mostly (TEMs) and (SHVs) active against last generation cephalosporins, also a variety of genes conferring resistance to drugs other than β -lactams [6]. Formation of biofilms is considered as an influential virulence factor for *K. pneumoniae*. Biofilms are currently known as closed bacterial communities in an exopolysaccharide matrix and adherent to non-biological or biological surfaces [7].

Newly, *C. zeylanicum* have attracted a great important in their useful as an antimicrobial activity. Cinnamon extracts have been shown to exert in vitro activity against some common human pathogens, by inhibition of bacterial endotoxin has been demonstrated by an unidentified component in cinnamon bark [8]. The goals of current study were estimated of antimicrobial effect of *C. zeylanicum* against *K. pneumoniae*, and estimation its role as anti-biofilm formation in *K. pneumoniae* isolated from UT

Materials and Methods

Isolation and identification of bacteria

Fifty samples of urine were collected from Al-yarmuk hospital from patients with UTIs. Only twenty samples were gave growth on plates. The isolated bacteria were identified with morphological characters and biochemical tests. The MacConkey agar (Himedia/India) was used for primary identification of *K. pneumoniae* and other gram negative bacteria; Blood agar was used in the identification of bacteria, and for the detection of hemolytic activity and the kind of hemolysis. Indole and citrate tests were employed to identify *K. pneumoniae* from other Enterbacteriaceae. And oxidase test to identify *Pseudomonas aeruginosa* [9]. The diagnosis of the clinical isolates was confirmed by VITEK 2 compact system for *K. pneumoniae* isolates.

Preparations of plant extract

The *C. zeylanicum* bark used in this study was bought from stores of plants in Baghdad City. The bark was washed with water and then dried in an air oven at 380 C°. Then they were grinded and stored until they used. 10 g of each plant powder was extracted with 200 ml of 80% (v/v) aqueous ethanol by Soxhlet to be used for 8 hrs, then the extracts were dried in oven with 40 C°, and the resulting powder kept in a glass container in refrigerator to be ready use in the preparation different concentrations [10].

Antibiotics sensitivity test

In vitro sensitivity test was done according to Bauer-Kirby [11], previously prepared bacterial suspensions with turbidity of no. 0.5 McFarland standards to get dilution (1.5×10^8) CFU /ml of organisms, after that were inoculated on Mueller Hinton agar (MHA) (Himedia /India).the plates inoculated by sterile swabs and antibiotics discs were placed on media then overnight incubation at 37°C. Ten antibiotics discs were used in this study, all of them manufactured by (Bioanalyse/Turkey) these are: Cefoxitin (30µg), Cefotaxim (10µg), Imipenem/Cilactin(10/10µg), Trimthoprim(30µg), Amoxicillin(10µg), Ciprofloxacin(5µg), Ticarcillin/Clavulanic acid (75/10µg), Cefradine (25µg), Sparfloxacin(5µg), and Norfloxacin (10 µg). The results were compared with CLSI data (2016) [12].

Antimicrobial activity of bark *Cinnamomum zeylanicum*

Antimicrobial activity of *C. zeylanicum* bark ethanol extract was tested with agar well diffusion method by [13], with using the plates of Mueller Hinton Agar (MHA) .The pathogenic strains *K. pneumoniae* were spreading on the surface of agar. Wells of 8 mm in diameter were perforated into agar plates and extract were prepared at (200, 100, 50, 25, 12.5, 6.125 mg/ml) concentrations then placed into each well. The plates were incubated at 37°C for 24 hrs. , and antibacterial activity was evaluated by measuring the diameter of the inhibition zone (IZ) around the wells in millimeter by ruler.

Minimum inhibitory concentrations (MIC) of *Cinnamomum zeylanicum*

The MIC of crude extracts was determined by broth macro-dilution assay. A set of test tubes with concentrations of plant extract (200, 100, 50, 25, 12.5, and 6.25) mg/ml. Tubes were inoculated with diluted bacteria equal to no. 0.5 McFarland tube. After incubation, tubes were examined for changes in turbidity as an indicator of growth. The first test tube that appeared clear was considered as MIC of *Cinnamomum zeylanicum* bark extract against *K. pneumoniae* [14], with used positive and negative control for Comparison.

Biofilm Formation Assay of bacteria

The method described by [15] was followed as standard test for the detection of biofilm formation. Briefly, the diluents bacterial cultures (200 µL) only, and another with 50 µL of *C. zeylanicum* bark extracted at final concentration (12.5mg/ml) were added to 150 µL a micro titer plate. Then added 200 µL of Brain heart infusion broth eight wells without any other additions was a negative control. The micro titer plate was incubated at 37°C for 18-24hrs. After that, the plate was washed five times with distilled water and air dried for 15 min. The plate was stained with 200 µL of 0.1% Crystal Violet for 15 min, and washed five times with distilled water. Afterwards, 200µl of 95% methanol was added to each well for 10 minutes. The amount of crystal violet extracted by the ethanol in each well was quantified by measuring the OD 580nm by using an ELISA reader. Statistical Analysis is expressed with Excel program as Mean \pm SD between the control and each of bacteria. The P value < 0.05 was considered as significant result.

Results and Discussion

Isolation and identification of bacteria

Fifty urine samples were collected from Al-yarmuk hospital from patients with UTIs. Only twenty were gave growth for bacteria (10) 50% belong to *E. coli*, (5) 25% *K. pneumoniae*, (3)15% *Proteus spp.*, (2)10% *Pseudomonas aeruginosa*. And current results disagree with the study that improved Gram-negative rods (90%) were isolated and major pathogens were *K. pneumoniae* (40%) and *Escherichia coli* (33%) [16]. But for detection of the bacteria, we were cultured the samples on MacConkey agar, the bacteria gram negative were appeared pink colonies because lactose ferments or pale if they lactose non- fermented. Indole test was negative for *K. pneumoniae* , while positive for *E. coli*, and *Proteus spp.* And on Cimmon Citrate the bacteria can utilize the citrate; therefore the media was blue with growth of bacteria for *K. pneumonia* and negative for *E. coli*. And on blood agar the types of haemolysis was gamma haemolysis, and positive oxidase for *Pseudomonas aeruginosa*, and identification with VITEK 2 compact system were confirmed that these isolates were *K. pneumoniae*.

Sensitivity test for antibiotics and comparing with inhibitory activity of *Cinnamomum zeylanicum*

The sensitivity test of isolates of *K. pneumoniae* was tested to a number of antibiotics used to treat some of the infections caused by this species in humans.100% of isolates were sensitive to Norfloxacin, Cefotaxime, Imipenem/ Cilactin, and Sparfloxacin, and all isolates resistance to

Cefoxitin, Amoxicillin, Ticarcillin / Clavulanic Acid, Cefitrixon, Trimthoprim, and Cefradine . The increasingly frequent obsession of antibiotic resistance by *K. pneumoniae* strains has a certain highest to a global spread of this multidrug-resistant pathogen, mostly at the hospital level. This case is aggravated when *K. pneumoniae* strains grow as a biofilm [17].

The purpose of this test was to compare the inhibitory effect of some of these antibiotics with the inhibitory effect of the extract used. Figure-1 shows the bacteria used in test were sensitive to Norfloxacin with inhibition zone diameter was (24-30) mm, while the isolates that were sensitive to Cefotaxim showed inhibition zone (IZ) in diameter of (26-27) mm, it was also found that *K. pneumoniae* was sensitive to Imipenem/Cilactin inhibition zone, when the diameter was (27-30) mm, but the bacteria were resistant to Sparfloxacin in diameter (25- 26) mm; and the bacteria were resisted to other antibiotics used in this study. In present study was showed that the five isolates of *K. pneumoniae*, which were isolated from UTIs resistant to most antibiotics used in this study. This agrees with several other studies, in which *K. pneumoniae* were resistant to commonly used antibiotics. Because of increase in drug resistant of strains of *K. pneumoniae* especially which isolated from UTI, their formation of biofilm, and possesses a multidrug resistance plasmids that encoded for resistance to antibiotics, and later, encoding extended-spectrum β -lactamases (ESBLs), mostly (TEMs) and (SHVs) active against last generation cephalosporins, also a variety of genes conferring resistance to drugs other than β -lactams [6], [18].

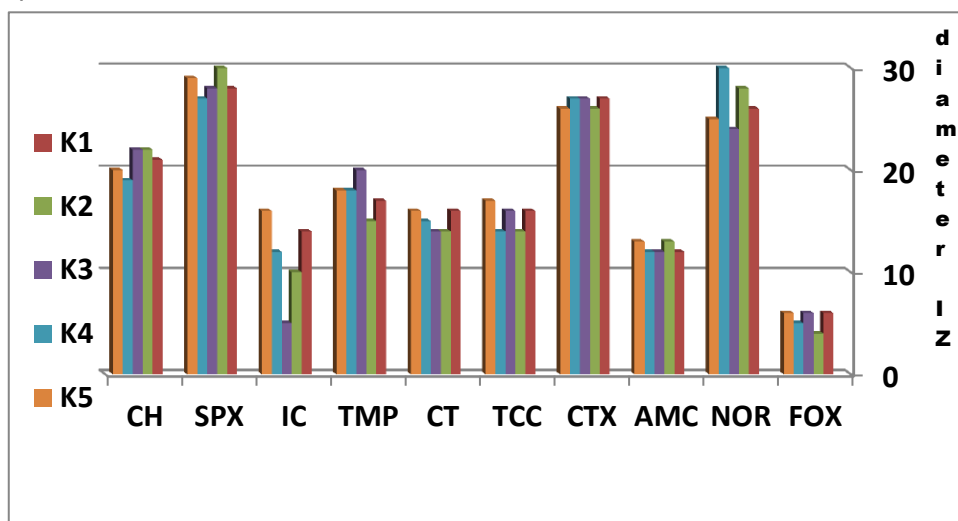


Figure 1- Antibiotics sensitivity test with antibiotics discs against *K. pneumoniae*: FOX:Cefoxitin,NOR:Norfloxacin,AMC:Amoxicillin,CTX:Cefotaxim,TCC:Ticarcillin/Clavulanic acid ,CT:Cefitrixon,TMP:Trimthoprim,IC:Imipenem/ Cilactin,CH:Cefradine, SPX:Sparfloxacin ; K1, K2, K3, K4, K5: isolates of *K. pneumoniae*; (IZ): Inhibition Zone

Antimicrobial activity of *Cinnamomum zeylanicum*:

The results showed a clear inhibitory effect of *C. zeylanicum* bark extracted at a concentration of 200 mg/ml (18-26mm), in concentration 100 mg/ml (14-16mm) against bacteria, while the other concentrations 50 mg/ml revealed inhibitory activity against the isolates of *K. pneumoniae* (11-12mm), and 25mg/ml (9-11mm).While in concentration 12.5 mg/ml the diameter of inhibition zone was ranged(8-10mm).and according to these results was found the effect of plant extract decreases as its concentrations decreases, Table-1.

Table 1-The diameter range of inhibition zone of *Cinnamomum zeylanicum* on *K. pneumoniae* isolates:

Concentrations of plant extract (mg/ml)	200	100	50	25	12.5	6.25	C
diameter range of inhibition zone(millimeter)	18-26	14-16	11-12	9-11	8-10	-	-

C: Control

And these results agree with the results of Mandal *et al.* [19], who were improved the antibacterial activity was determined with diameters of inhibition and MIC values at different times of incubation. Another study of Keskin and Toroglu [20] showed that the antibacterial activities of *C. zeylanicum* bark extracts, extracted with used different organic solvents, as ethyl acetate, acetone and methanol, were tested in vitro against (*Klebsiella pneumoniae*, *Bacillus megaterium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter cloacae*, *Corynebacterium xerosis*, *Streptococcus faecalis*) by the discs diffusion method. The results showed that the antibacterial activity, appear as inhibition zone, ranges from (7–18) mm in concentration 30 μ L, the author suggested a high antibacterial activity [20]. Also results showed that the antibacterial activity of the plant extracts against Gram negative bacteria was more than of Gram positive bacteria and this might be relevant because the differences in the chemical structure of the bacterial cell wall of these bacteria [21].

When comparing the inhibitory effect of antibiotics with the inhibitory effect of the *C. zeylanicum* extract in Table-1 and Figure-1, it was observed that extract of *C. zeylanicum* at higher concentration were near to potent the efficacy appear antibiotics such as Norfloxacin, but low concentration was appeared less potent than the efficacy of antibiotics such as Norfloxacin, and according to current results may can use *C. zeylanicum* bark to treat UTI, which caused by *K. pneumoniae* because it had a good antimicrobial activity against this bacteria with less side effects. Because of using the antibiotics in treatment of infections the bacteria were showed higher resistance to antibiotics; therefore, the people turning to medical plants.

Minimum inhibitory concentrations (MIC) of *Cinnamomum zeylanicum*

The MIC value for bark extract of *C. zeylanicum* was (12.5-25) mg/ml for *Klebsiella pneumoniae*. The results also agree with the results of Alsalim *et al.* [22]. The laboratory tests of antibacterial activity showed that *Staphylococcus aureus* was the most affected by the extracts under study, followed by *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Escherichia coli* and *Klebsiella pneumoniae* respectively. Aqueous extract showed highest values in MIC (400 μ g/ml) for *S aureus*, followed by chloroform (350 μ g/ml, MIC), then methanol (335 μ g/ml, MIC), while the lowest values were recorded for *K. pneumoniae* (MIC 175, 200 and 250 μ g/ml, respectively), other study were studied the antibacterial activity of *C. zeylanicum*, against multidrug resistant Gram-negative bacteria with over expressing active efflux pumps, which make bacteria resistant to antibiotic treatment. The results revealed that cinnamon is able to inhibit bacterial growth, with different MIC values, ranging from (64–1024) μ g/ml, depending on the strains [18].

Anti-Biofilm Formation Assay of *C. zeylanicum* against bacteria

The current study supposes that bark extract of *C. zeylanicum* had antibiofilm activity against *K. pneumoniae* bacteria isolated from UTIs. The results showed that the bacteria used in this study were strong biofilm production 0.251 ± 0.046 , 0.345 ± 0.105 , 0.308 ± 0.131 , 0.338 ± 0.167 , and 0.532 ± 0.137 respectively for all isolates ($p < 0.05$). The statistical tests showed that extract of *C. zeylanicum* has a good activity against biofilm production in *K. pneumoniae* with significant differences ($p < 0.05$). Therefore the plant extract decreased biofilm formation of *K. pneumoniae*, and this result agrees with the researchers were concluded that the antibacterial activity of that cinnamon methanolic bark extract could be used in the treatment of infectious diseases induced by bacteria [20]. Amalaradjou *et al.* were showed the ability of trans-cinnamaldehyde to inhibit UPEC biofilm formation isolated from catheters. In another study that conducted by the same researchers in 2010 demonstrated trans-cinnamaldehyde prevented virulence factors of uro-epithelial cells by down regulating genes in the pathogen. These results showed the trans-cinnamaldehyde can be use as an antibacterial for UTIs. [23], [24]. On the other hand, the results of this study disagree with the results of AL Shahwany *et al.* [25], who reported that cinnamon phenolic extract did not have antibiofilm activity against *K. pneumoniae* and *S. aureus*, this can be attributed to the fact that AL Shahwany *et al.* used concentrations of plant extract that are lower than the ones used in the present study, or may be because they used another solvent which led to different results. The type of solvent used to extract herbs and spices appeared to have a major impact on their antimicrobial activity. This is probably due to the fact that most of the components with antimicrobial properties are aromatic or saturated organic compounds which are generally more soluble in solvents such as ethanol or methanol [26].

Conclusions

The *C. zeylanicum* has a good antimicrobial effect on *K. pneumoniae* like antibiotics. And has also anti-biofilm activity in low concentration against tested bacteria. Therefore, it is important to replace antibiotics with plant extracts, because it more effective in the removal of pathogens with less side effects.

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