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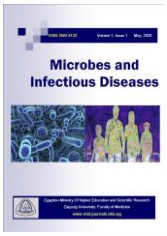

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Original article

Effect of Cinnamon verum extract on growth and antibiofilm activity of *Klebsiella pneumoniae*

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ABSTRACT

Background: Pathogenic bacterial species, particularly *Klebsiella pneumoniae*, are significant contributors to infections in patients. These bacteria are commonly isolated from infection environments such as sputum, urine, and urinary catheters. The ability of *K. pneumoniae* to form biofilms is a major factor contributing to its pathogenicity, as biofilms impede the efficacy of antimicrobial treatments. **Aim of the Study**; This study aimed to investigate the biofilm-forming ability of *K. pneumoniae* isolates and evaluate the antimicrobial and antibiofilm effects of cinnamon extract as a potential therapeutic agent. **Methodology**: Samples were collected from different hospitals in Baghdad, Iraq, between September and December 2022. Fifty isolates of *K. pneumoniae* were identified and subjected to antimicrobial susceptibility testing using the Kirby-Bauer method with ten antibiotics. The ability of isolates to form biofilms was determined and categorized into strong, moderate, and weak biofilm-forming groups. The effect of cinnamon extract on bacterial growth and biofilm formation was evaluated at various concentrations (128 µg/ml to 1024 µg/ml) by measuring zones of inhibition and biofilm reduction. **Results**: The antimicrobial susceptibility test revealed that 98% of the isolates were resistant to amoxicillin, while 94% were sensitive to imipenem. Of the 50 isolates, 32 demonstrated biofilm-forming ability, with 15 showing strong biofilm formation. Cinnamon extract exhibited significant antibacterial activity, with inhibition zones ranging from 10.8±1.3 mm to 22.8±0.3 mm at increasing concentrations. Biofilm formation in strong biofilm-producing isolates was significantly reduced, with a reduction from 0.0604 ± 0.056 to 0.114 ± 0.028. **Conclusion**: Cinnamon extract demonstrated a considerable antibacterial and antibiofilm effect against *K. pneumoniae*, highlighting its potential as an alternative therapeutic agent to combat biofilm-associated infections.

Introduction

Klebsiella pneumoniae is a bacillus characterized by its rod-shaped morphology, Gram-negative staining, and lactose fermentation capability [1]. Its unusual capsule is very noticeable. This bacterium species inhabits certain habitats, including the oral cavities, dermis, and gastrointestinal tract, anatomical sites that also harbour prevalent opportunistic infections like *K. pneumoniae*, in addition to medical equipment and healthcare surroundings [2]. These bacteria can swiftly acquire resistance mechanisms that are effective against many medicines [3,4]. They induce

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infections by multidrug-resistant *K. pneumoniae*, for which carbapenem-class medicines have been evaluated and chosen. Microbial biofilms are structured communities of microbial cellular components that promote bacterial adhesion, and the integration of extracellular polymeric materials incorporated in a polymer matrix of microbial origin [5]. The extracellular matrix of biofilms comprises for 20% to 30% of the biofilm mass and is predominantly made of polysaccharides (glucans and fructans) of microbial origin [6]. Bacterial species, such as *K. pneumoniae*, exhibit resistance to the majority of antibiotics, necessitating the development of therapies for infections caused by these organisms [7]. One of the safe uses of antimicrobial growth control is to employ active components from medicinal plants (e.g., essential oils) as inhibitors of bacterial growth [8]. Cinnamon is a prevalent spice utilized in several home-cooked foods. In addition to the bark of the cinnamon tree, its roots, leaves, fruit, and flowers have long been utilised in traditional medicine in many cultures. Cinnamon is utilized in culinary applications and many food items[9]. Given the significance of safe and reliable methods for assessing the efficacy of active compounds in the cinnamon plant, numerous bioactive constituents derived from cinnamon have demonstrated therapeutic effects against various ailments, including diabetes, cancer, oxidative stress, cardiovascular disease, and fungal and bacterial infections [10,11]. Multiple clinical investigations have examined the efficacy of cinnamon in mitigating pathogenic consequences, demonstrating its ability to treat symptoms of nausea and diarrhea. Numerous clinical investigations indicate that cinnamon supplementation significantly impacts body mass index (BMI), hormonal equilibrium, oxidative stress, fertility, and glucose homeostasis in individuals with polycystic ovarian syndrome (PCOS) to some degree [12-14]. In this scientific investigation, different doses of cinnamon plant extract were evaluated to establish the degree of its bioactivity in suppressing biofilm formation of *K. pneumoniae*.

Methods

Bacterial isolation

Samples were collected in the field between September and December of 2022. 350 samples of urine and 76 samples of sputum were collected from patients in medical institutions in Baghdad. Samples included body fluids and microorganisms. The molecular testing system was the VITEK® 2 system (bioMérieux, Hazelwood, MO, USA) and Gram staining was employed to differentiate other bacteria. [15,16].

Antibacterial sensitivity testing

The experiment was performed on 50 different strains of *K. pneumoniae* and 10 different antibiotics. The antibiotics comprised ciprofloxacin (10 μg), cefotaxime (30 μg), penicillin (10 μg), amoxicillin (AX) (10 μg), PCN, streptomycin (25 μg), and nalidixic acid (30 μg). Additionally, gentamicin (10 μg) was included. A new version of the Kirby-Bauer method of disk propagation was employed on Muller-Hinton agar plates (MHA: HiMedia Laboratories LLC, PA, USA). According to the CLSI guidelines (Clinical and Laboratory Standards Institute, 2016) [15], the isolates were classified into three categories: "R" for "resistant", "I" for "intermediate", and "S" for "susceptible". This is accomplished by determining the size of the zone of inhibition (ZOI) of the antibiotic disks. Turkish markets for biological analysis have no limits to their purchases of antibiotics.

Preparation of cinnamon extract

The Cinnamon bark was purchased from a local store and placed in an oven that was set to 60 degrees Celsius and held there for a consistent weight. The dried bark was then blended using a powerful blender (Hanil, Gwangju, South Korea) that produced a powder that was sized at 500 micrometers. This was used to obtain a fine powder of cinnamon. The resulting cinnamon powder (CP) was kept at −20°C for future use.

Cinnamon ethanol extract

Around 20 g of CP was incorporated into 100 ml of ethanol (70) (1 g: 5 ml). The sample was then stirred for 12 hours at room temperature in the presence of a solvent that completely dissolves the particles. The extract that was in liquid form was passed through a filter that was made of paper (Whatman No.1). After ten minutes of centrifugation, the ethanol extract was sealed in a freezer that was -70 degrees Celsius. The extract was then transferred to a fresh, sterile petri dish and allowed to dry at a temperature of 40°C for between two and three days.

Antibacterial activity of Cinnamon extract against *K. pneumoniae* isolates

Bacterial isolates were cultured in nutrient broth and incubated at 37°C for 18-24 hours. After

the incubation period, 0.1 ml of each bacterial suspension was transferred to nutrient agar plates and incubated at 37°C for 24 hours. Next, individual colonies were transferred to test tubes that were filled with 5 ml of saltwater, this produced a suspension with a turbidity of approximately 1.5×10^8 CFU/ml, which was similar to the standard concentration. The bacterial suspension was spread uniformly across the surface of the Mueller-Hinton agar using a sterilized ball of cotton and holding it there for 10 minutes. Three wells were dug in the agar layer, each having a diameter of 5 mm. With a micropipette, 50 µl of the cinnamon extract at a concentration of 128 µg/ml or 1024 µg/ml was added to each well. The central pit was considered a negative aspect and the antibiotic kanamycin a positive aspect. The size of the zone that was inhibited was calculated after the plates were incubated at 37°C for 18 hours [16].

Antibacterial activity and MIC of Cinnamon extract

We started with a 10 mg/ml solution of the extract of cinnamon and performed several concentration doublings. These dilutions were conducted in microtiter plates using the Mueller-Hinton broth. The concentrations achieved were from 1 to 1024 µg/ml. 20 different microorganisms were incorporated into each well. This sample was compared to the McFarland's typical No. 0.5 (1.5 × 108 CFU/ml). Negative controls well containing Muller-Hinton broth were without bacteria. The microtiter plates were maintained at 37°C for 18-20 hours. After two hours, the color of the wells was assessed after adding 20 µl of resazurin to each well. Small quantities of resazurin that color the blueish broth pink were located in the diluted broth. These are known as the MIC levels. [17].

Biofilm formation assay for *K. pneumonia*

In this research, the method of evaluating biofilm formation by *K. pneumoniae* was altered; the previous method was used as a guide. Of the 50 clinical isolates, 34 were evaluated for their capacity to form biofilms using a modified method of microtiter plates. In the early stages, the isolates were cultured in brain heart infusion (BHI) broth containing 2% sucrose and incubated at 37°C for 24 hours. Each microtiter plate was coated with 180 ml of BHI broth and 20 ml of a bacterial suspension that was standardized to have 0.5 McFarland. After the seals were made, the microtiter plates were incubated at 37°C for 24 hours. After incubation, the wells were rinsed three times with water to remove cells that didn't attach to the surface. To dissolve the cells that are adhered to the plate, 200 µl of 99% methanol was added to each well and allowed to sit for 15 minutes. The cells were then left to dry for 30 minutes at room temperature. The biofilm was then covered with 200 ml of 1% crystal violet for a period of 15 minutes. The extra color was subsequently removed. The optical density (OD) at 630nm was determined using a microplate reader, the biofilm's color was then dissolved with 96% ethanol. After the staining process, the wells were rinsed with sterile water that was purified of any microorganisms or parasites. The OD values were employed to categorize the biofilm's development into different classes according to the listed factors in **table (1).** This method was employed to determine the quantitative capacity of *K. pneumoniae* to form biofilms.

Antibiofilm activity of Cinnamon extract against K. pneumoniae

Mahdi's team (2019) documented that the same method was employed in the development of biofilms in experiments with cells [21]. After making sterile brain heart infusion broth (BHIB) that was supplemented with 2% sucrose, 180 µl of the treated broth was added to each well. Later, 20 µl of the *K. pneumoniae* suspension were added that were equal to 0.5 MacFarland's standard. Only 180 ml (BHIB) and 20 µl of bacterial suspension that was not treated with cinnamon were added to the control wells, as the positive control had to be supplemented with 180 ml (BHIB) and 20 µl of cinnamon's extract as a negative control. After incubation, the medium was removed from the wells and washed three times with sterile PBS to remove all loose *K. pneumoniae* cells, the medium was then dried for 15 minutes at room temperature. Next, 200 µl of crystal violet (0.1%) was added to each well and left to stand for 20 minutes. After three washes with PBS (pH 7.2) that removed any unbound colorant, the stained wells were dried for 15 minutes at room temperature. After adding 200 µl of 95% ethanol to each well, the optical density of the cells was read at 630nm using an ELIZA reader. Ultimately, the cells were permitted to sit for 15 minutes at a room temperature. Next, 200 µl of crystal violet (0.1%) was added to each well and left to stand for 20 minutes. After three washes with PBS (pH 7.2) that removed any unbound colorant, the stained wells were dried for 15 minutes at room temperature. After adding 200 µl of 95% ethanol to

each well, the optical density was read at 630nm using an ELIZA reader.

Statistical analysis

Statistical analysis is significant in the analysis of quantitative data because it can describe the data and assess the relationship between different data sets. The data in this report are expressed as counts or percentages. The significance of the differences was determined with SPSS (version 26). For variables that are normally distributed, paired and an independent test of significance was conducted. For variables that are not distributed normally, the Wilcoxon test, Mann-Whitney U test, and chi-square test were employed. The importance of the data was paramount to the p value being smaller than 0.05.

Ethical approval

The investigation was approved by the Ethics Committee of the Technical University of Munich. Each participant was explained the study and asked to sign a document that would confirm their participation. Additionally, the patient was informed that his information would be protected.

Table 1. Evaluating the formation of biofilms using the microtiter plate method.

Optical density	Adherence
OD ≤ ODc	Non–adherent
2ODc > OD > ODc	Weak
4 ODc > OD > 2ODc	Moderate
OD >4 ODc	Strong
Cut off value (ODc) = average OD of negative control + (3 *Standard Deviation).	

Results

Identification of bacterial isolates

Of the 100 bacterial isolates, 50 were initially considered to be *Klebsiella pneumoniae*. The isolates were identified by their physical and chemical properties, and the diagnosis was confirmed with the Vitek 2 Compact System.

Antibiotic sensitivity test *of K. pneumonia* isolates

Figure 1 displays the outcomes of the disc dilution test for isolating antibiotic sensitivity to ten different antibiotics, common antibiotics that are used to treat *K. pneumoniae* were chosen for this investigation. Our research showed that a large percentage of bacterial isolates (98%) were amoxicillin resistant. While imipenem lists the lowest resistance rate as 6%.

Biofilm formation of isolates

The capacity of 34 *K. pneumoniae* strains from patients to form biofilms on polystyrene surfaces was studied in this research. Based on their optical density (OD) values (0.0721-0.144, 0.144-0.288, and ≥ 0.288), the results demonstrated that 6 isolates (18.75%) formed weak biofilms, 11 (34.375%) formed moderate biofilms, and 15 (46.875%) formed strong biofilms **(Table 2).**

Antibacterial activity Cinnamon extract against *K. pneumoniae* isolates

Different concentrations of cinnamon extract (128, 1024 μg/ml) were employed to determine the area of the inhibitory effect of the substance using the disk diffusion method. As the concentration of cinnamon extract increased, the zone of inhibition also increased. The results demonstrated that the zone of inhibition was greatest at a concentration of 1024 μg/ml, while the greatest degree of inhibition was achieved at a concentration of 124 μg/ml. The antibacterial effect of cinnamon extract was assessed against 15 *Klebsiella pneumoniae* that formed obvious biofilms, as demonstrated in **table (3) and figure (3).**

Antibiofilm activity Cinnamon extract against *K. pneumoniae* isolates

The outcomes in the table demonstrate the impact of cinnamon extract on decreasing the formation of biofilm by *K. pneumoniae*. The Optical Absorbance Values (OD) of all of the isolates before being treated with the extract were high, ranging from 0.296 to 0.013, and from 0.604 to 0.056, which indicates that they have a strong capacity to form biofilms. After the addition of the cinnamon extract, a significant decrease in the optical density values was observed, the range of decrease was from 0.087 to 0.142. This significant decrease in abundance demonstrates the effectiveness of cinnamon's extract in preventing biofilm formation. It also demonstrates the capacity of this extract as an antibiotic to be used alongside treatment for *K. pneumoniae* infections **(Table 4).**

Table 2. Evaluating the formation of biofilms using the microtiter plate method.

intensity	**OD630 Limits number of isolates**	**Number of isolates**	**percentage) %**
Non-biofilm producer	< 0.0721	0	0
Weak	0.0721-0.144	6	18.75%
Moderate	0.144- 0.288	11	34.375%
Strong	≥ 0.288	15	46.875%

Table 3. Results of Cinnamon's antibacterial effects.

Isolates	**The diameter of the cinnamon inhibitory zone at 128 µg/ml mean ±SD**	**The diameter of the cinnamon inhibitory zone at 1024 µg/ml mean ±SD**
Kle1	12.7±1.5	18.2±1.2
Kle 2	13.2±1.2	15.5±0.5
Kle 3	7.3±1.5	10.8±1.3
Kle 4	11±1	17.7±0.6
Kle 5	11.1±0.7	19.5±1.3
Kle 6	13±1	17.2±0.8
Kle 7	15.3±0.5	20±1.5
Kle 8	15.8±0.8	22.8±0.3
Kle 9	15.2±1	21.3±2
Kle 10	14.7±0.3	23±1
Kle 11	12.2±1	15.5±0.9
Kle 12	16±1	18±0.6
Kle 13	16.8±1	20.1±1.6
Kle 14	13.8±1.3	16.3±0.8
Kle 15	14±1	19.3±0.6

Table 4. Result of antibiofilm activity of Cinnamon extract.

Bacteria number	**O.D before Cinnamon extract**	**O.D after Cinnamon extract**
K1	0. 296 ±0.013	0.123±0.024
K2	0.302 ±0.008	0.105±0.037
K3	0.403 ±0.016	0.119±0.068
K4	0.372 ±0.011	0.128±0.050
K5	0.399 ±0.018	0.142±0.025
K6	0.477 ±0.050	0.115±0.044
K7	0.361 ±0.54	0.096±0.005
K8	0.502 ±0.030	0.099±0.010
K9	0.475 ±0.062	0.125±0.030
K10	0.314 ±0.037	0.108±0.019
K11	0.301 ±0.011	0.090±0.010
K12	0.604 ±0.056	0.114±0.028
K13	0.460 ±0.031	0.090±0.030
K14	0.442 ±0.050	0.110±0.033
K15	0.350 ±0.049	0.087±0.012

Figure 1. Percentage of isolated *K. pneumonia* strains' antibiotic resistance.

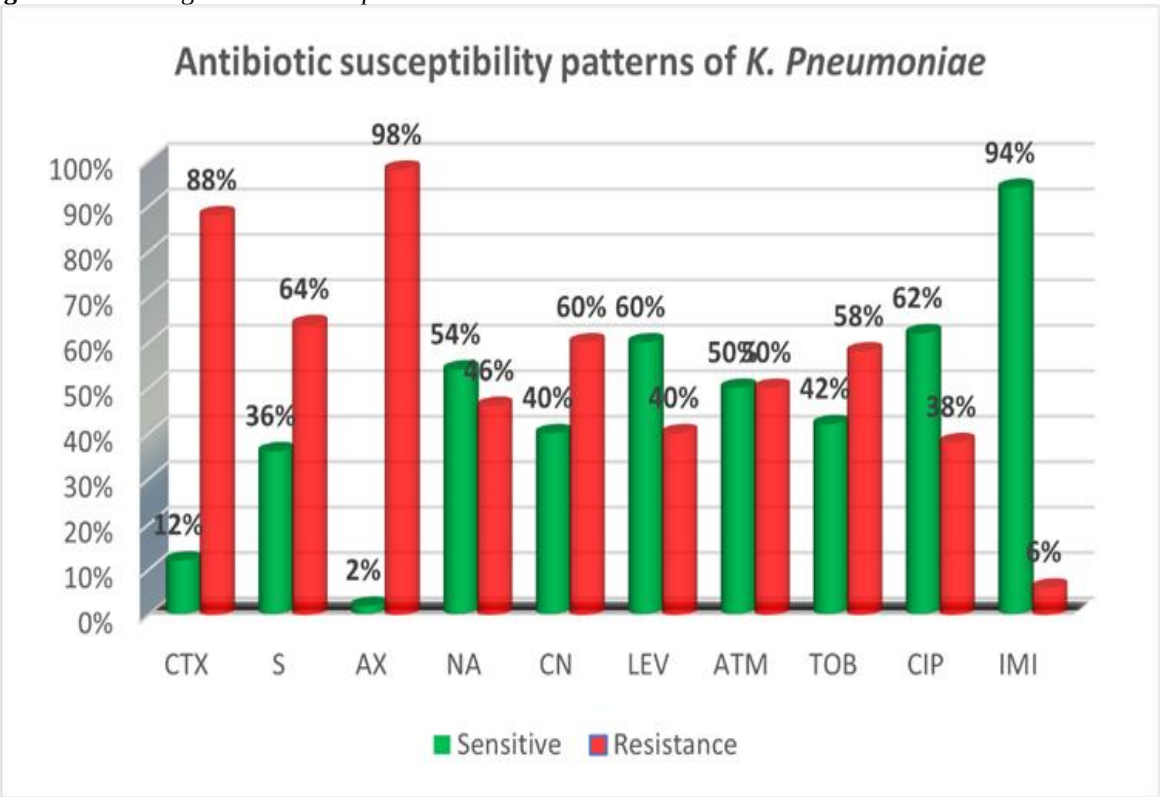


Figure 2. Biofilm formation of *K. pneumoniae* isolates.

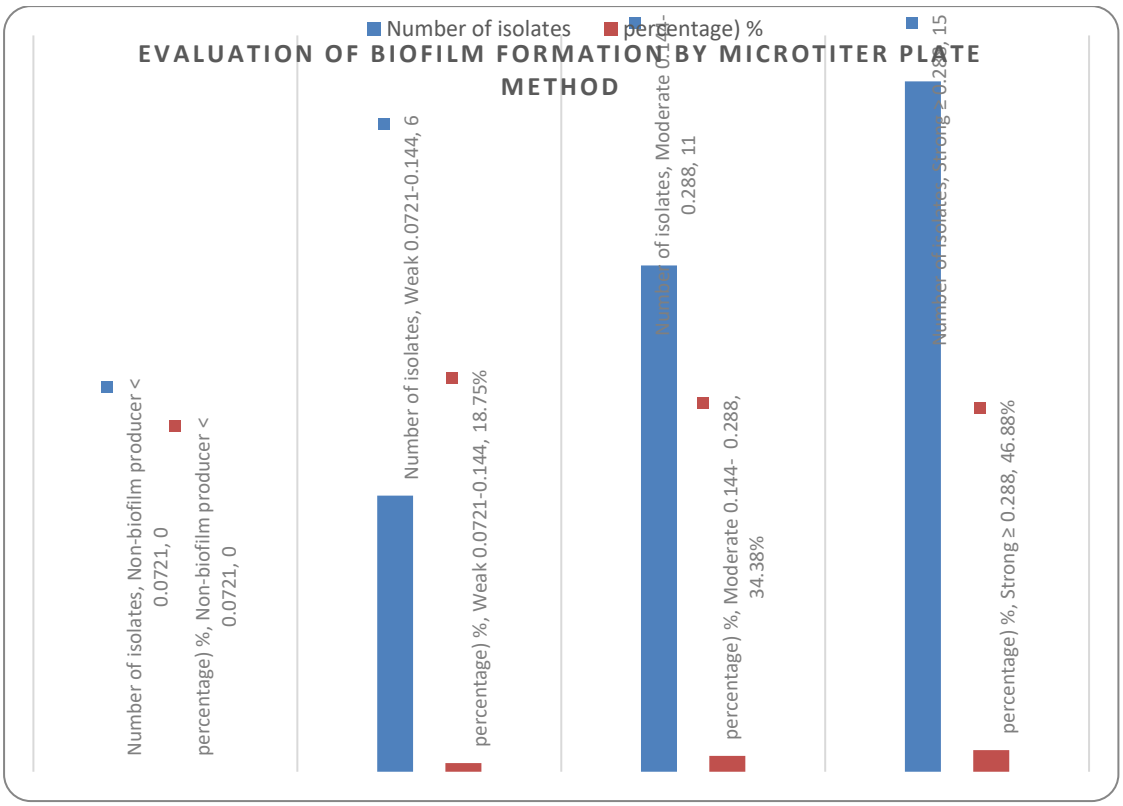


Figure 3. Zone diameter of the extract and gentamicin against *Klebsiella pneumoniae.*

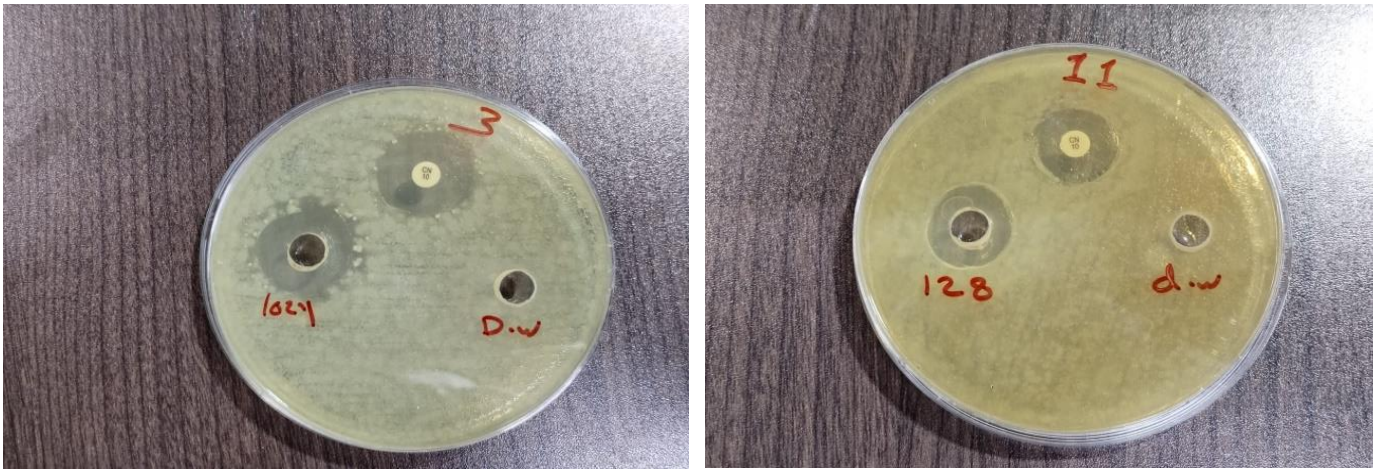


Figure 4. Antibacterial activity of Cinnamon extract against *Klebsiella pneumoniae.*

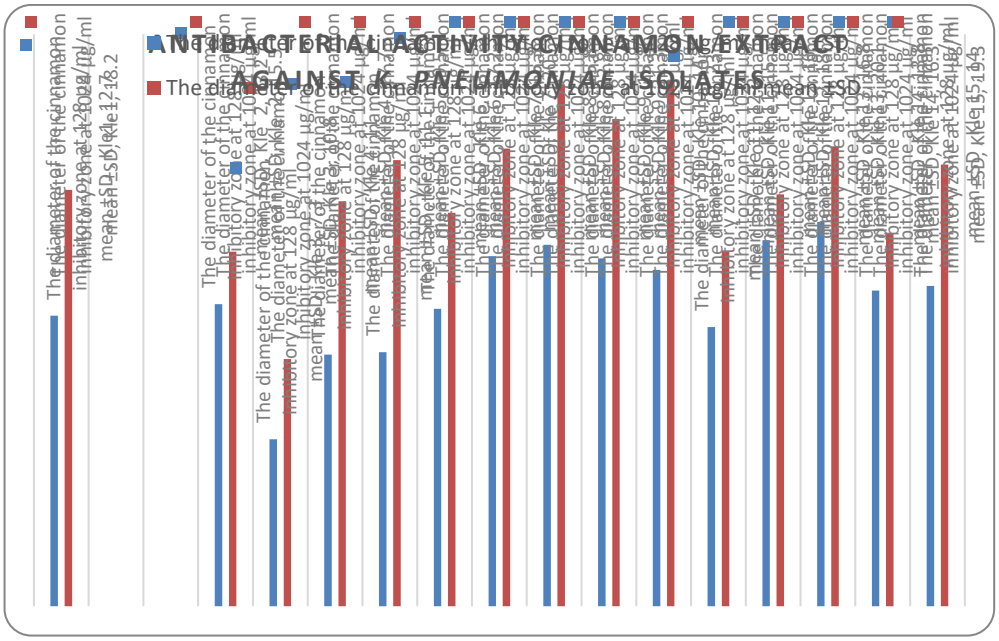
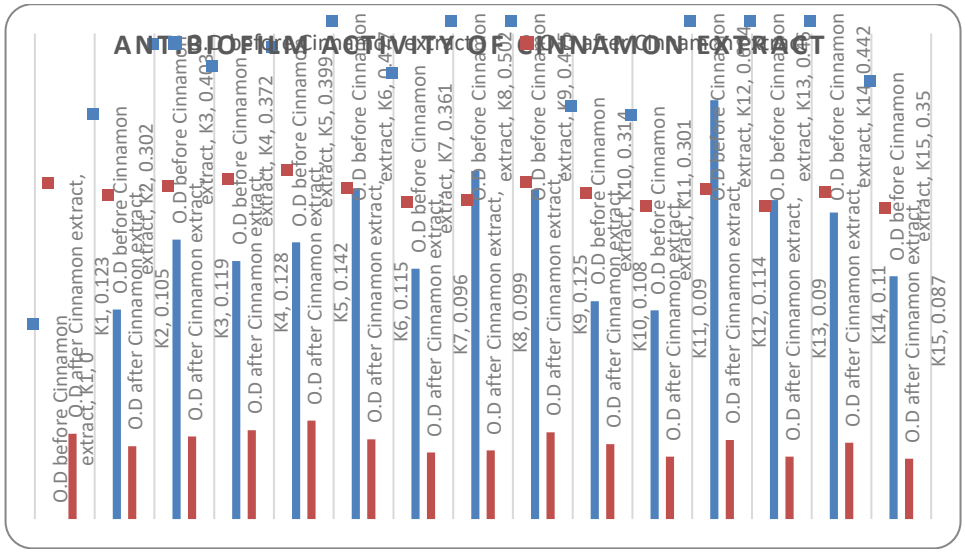


Figure 5. Activity Cinnamon extract against *K. pneumoniae* isolates.



Discussion

Klebsiella pneumoniae has evolved several mechanisms to defend against the effects of antibiotics, including the production of various carbapenemases and extended spectrum β-lactamases (ESBLs) [18*]. Klebsiella pneumoniae* has been considered a dangerous infectious agent in recent years because of the increasing number of serious infections, including blood poisoning and pneumonia, as well as the decrease in effective treatments. The rise of antibiotic-resistant bacteria is attributed to the acquisition of additional genetic properties by *K. pneumoniae* strains. The outcomes of this investigation are derived from the findings of **Rafeeq et al.** [18]. This investigation documented the susceptibility of different isolates to specific antibiotics, and it also documented the prevalence of highly pathogenic and MDR (or resistant to multiple drugs) isolates. Both are crucial to developing a treatment regimen for infections caused by this bacterium [20]. Another investigation from [21] reported that amoxicillin was ineffective against all Gram-negative bacteria in the urine. The capacity of the pathogen to form biofilms on both biological and non-biological surfaces, such as catheters and other objects, is crucial to the development of antibiotic resistance [22]. These findings are in agreement with previous studies that have demonstrated that the thickness of biofilms differs between bacterial strains and is associated with the different production of quorum sensing chemicals, this is a key mechanism in the development of biofilms **Shadkam et al.** [22]. Our results indicate that 34 of the 50 isolates were capable of forming biofilms. This was illustrated by the expression of type 3 fibers. *Klebsiella pneumoniae* isolates were demonstrated to be extremely potent producers of biofilm. In their investigation, they documented that 25% of the isolates developed full biofilms, 19% developed moderate amounts of biofilm, and 25% developed weak biofilms [24]. Previous investigations have demonstrated that the antimicrobial properties of cinnamon are attributed to the combination of cinnamon oil and pure cinnamaldehyde [25]. The primary chemical components of cinnamon oil are eugenol (75.520%) and eugenol acetate (4.403%). Total number of chemical components: 10. Previous investigations have documented that cinnamon oil contains two significant chemical components: eugenol and its acetate counterpart. These chemical compounds with inflammation disrupt the bacterial cell membranes, this results in cell death [26]. A local study from **El-Sherbiny** in 2024 [26] studied the effects of cinnamon on bacteria that are Gram positive or negative. The outcomes of this investigation were similar to the present study: cinnamon extract had a significant impact. The cinnamon extract exhibited a encouraging inhibitory role even at low concentrations. It has been observed to inhibit both bacteria that are Gram-positive and those that are Gram-negative. Cinnamon extract is a powerful source of compounds with antimicrobial properties [28]. Biofilm infections are increasing in frequency; this has a significant impact on the efficacy of treatment. New methods of eliminating or preventing biofilm infections are essential, along with the evaluation of the effectiveness and potential of currently available antibiotics. The currently available data also suggest that toxic, expensive, and difficult to access drugs should be used to treat infectious diseases. Therefore, herbal and phytotherapeutic agents should be widely studied for their potential in treating microbial diseases, especially biofilm control [29]. The current investigation examined the capacity of cinnamon leaves to eliminate the percentage of harmful bacterial biofilms [30]. This influence could be attributed to the presence of eugenol in the leaves of cinnamon. Eugenol's antimicrobial effects are attributed to the detection of wall damage and deformation, shrinkage, and leakage of internal compartments. Eugenol can cause alterations in cellular structure by interacting with the cell wall and the matrix surrounding the cell [31]. The inhibitory effect of cinnamon extract on the formation of biofilms by *K. pneumoniae* was studied at a concentration of 100 mg/m³. After the treatment of bacterial isolates with the MIC of cinnamon extract, the results demonstrated that the bacteria were dependent on the extract and that biofilm formation was inhibited. As demonstrated in **table (4),** the OD values of the biofilms of 15 *K. pneumoniae* isolates were significantly greater before treatment ($p < 0.001$). Interestingly, this suppressed the formation of biofilms by the cinnamon extract [31].

Conclusion

Klebsiella pneumoniae is a significant invasive bacterium that frequently causes nosocomial outbreaks and exhibits varying levels of virulence and antibiotic sensitivity. The medical

community faces difficulty in determining the bacterial variables crucial during infection in light of the rise of certain difficult-to-treat diseases brought on by *K. pneumoniae*. This study has shown that cinnamon extract has great potential for bactericidal effects. We think that the results of this study, along with earlier research, confirm cinnamon's antibacterial capabilities.

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Declaration of competing interest

None.

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