



Study the Effect of Cinnamon and Tea Tree Oils on Biofilm Formation of *Klebsiella Pneumoniae*

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Abstract

Klebsiella pneumoniae is a noteworthy human pathogen. As a virulence factor, these bacteria may create a thick coating of extracellular biofilm. This aids the organism's adhesion to biotic as well as abiotic surfaces, preventing antimicrobial agents from doing their job. Infections caused by bacterial biofilms have become more difficult to treat as a result. Therefore, the present study has been designed to investigate the effects of essential oils, individually or in combination, on the biofilms of *Klebsiella pneumoniae* isolates. In this research, the quantification of biofilm composition for 50 isolates from urine samples indicates the following statistics: [n = 24 (48%)] isolates form a strong biofilm, [n = 12 (24%)] a moderate biofilm, [n = 10 (20%)] a weak biofilm, and [n = 4 (8%)] a non-biofilm. The Minimum Inhibitor Concentration (MIC) and MBC values for essential oils were determined. The results showed that the MIC for tea tree oil was 0.25% and MBC 0.5%. While the MIC for cinnamon oil was 0.125%, and MBC was 0.25%. Afterward, the anti-biofilm effectiveness of essential oils was evaluated. The results showed that both oils had good efficacy against strong biofilm for *Klebsiella pneumoniae* isolates. But in a comparison between them, cinnamon oil showed better results. Due to the efficacy of these two oils, the combined impact of the two oils was discovered in this study. And the results revealed that there was an antagonistic effect. These findings recommend additional essential oils be tested to see how they affect biofilms of *Klebsiella pneumoniae* or other bacteria.

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1. Introduction

K. pneumoniae is getting greater attention as across the world due to a rise in the incidence of severe infections, antibiotic resistance, and increasing challenges in discovering suitable treatments [1, 2]. *K. pneumoniae* is an encapsulated Gram-negative bacterium that may be found in soil, plants, and water, as well as on mammalian mucosal membranes [3, 4]. These bacteria cause a wide range of illnesses in humans, involving respiratory infections, urinary infections, as well as bloodstream infections [5]. Biofilms are microbial colonies that are attached to a surface and embedded in self-produced extracellular polymeric substances (EPS). In general, EPS is made up of polysaccharides, proteins, lipids, and extracellular DNA [6]. The formation and development of

biofilms by bacteria has been considered an essential step in the pathogenesis of many bacterial species [7]. Biofilms can colonize and grow on surfaces of medical implants such as sutures, catheters, and dental implants, and they cause infections that can only be treated by their removal, leading to unaffordable treatment as well mental-illness to patients [8]. Microorganisms are protected by biofilms against antibody opsonization, phagocytosis, and elimination by epithelial cells' cilia [9]. Furthermore, biofilm bacterial inhabitants are considerably more resistant to antibacterial treatments than planktonic cells that are free-living [10, 11]. As a result, using current medication choices to treat an illness after a biofilm has formed is usually fruitless [12]. Several novel ways to treat biofilm infections have developed in recent years. These methods can be used to either prevent or treat biofilm development [13-16]. Plants have had considerable economic significance for millennia, not just as a source of food but also as therapeutic agents [17-19]. One particularly interesting group of compounds is the essential oils obtained from various parts of plants. Although essential oils only represent a small fraction of a plant's composition, they nevertheless confer the characteristics by which aromatic plants are used in the food, cosmetic, and pharmaceutical industries [20]. Essential oils were found to have antibacterial properties against a wide range of harmful bacterial strains in several in vitro experiments [21]. Essential oils are made up of from around 20 to over 100 varying component concentrations. Two or three primary components are frequently found in relatively high quantities 20–70%, whereas the other chemicals are usually only present in trace levels [22]. Tea tree oil (TTO), also known as Melaleuca oil, is a fresh camphor-scented essential oil that ranges in color from light yellow to colorless and transparent. The ability of this essential oil to suppress the growth of numerous microorganisms is one of its distinguishing features. The essential oil of the tea tree has antimicrobial effects due to the presence of terpinen-4-ol and 1,8-cineole [23]. Cinnamon essential oil is recognized for having a high concentration of secondary metabolites such as phenolic compounds, such as eugenol, which can be found in the range of 4 to 10%, and aldehydes, such as cinnamaldehyde, which can be found in the range of 60 to 75 %. Cinnamon gets its taste from the latter component. Cinnamon possesses microbiological and antimicrobial characteristics, making it useful as a food preservative, flavouring agent, and pharmacology [24]. Therefore, the present study has been designed to investigate the effect of essential oils (Cinnamon and Tea tree) individually or in combination on the biofilms of *Klebsiella* isolates.

2. Materials and Methods

2.1. Bacteria isolated and Phenotypic Determination

From 2020/07/1 to 2020/09/25, 50 *K. pneumoniae* isolates were obtained from 133 bacterial isolates from urine samples from various laboratories in Baghdad. For identification, Bacterial isolates were submitted to microscopic, cultural, and biochemical investigations, as well as diagnosis using the vitek 2compact (bioMérieux/ France) equipment.

2.2. Biofilm Formation Assay

Biofilm formation by *K. pneumoniae* was performed with some modifications as follows [25, 26]: After activation of the bacterial isolates on the MacConkey Agar medium. A single colony from each isolate was transferred to polystyrene test tubes containing 3ml of Tryptic Soy Broth (TSB) supplemented with 1% glucose adjusted to the McFarland standard of 0.5 by using a calibrated DensiCHEK Plus Meter (bioMérieux/ France). Each well of a 96-well microtiter plate (3 wells for each isolate) was filled with 150 μ L of sterile broth (TSB with 1 % glucose) and 50 μ L of inoculum broth. After the plate was incubated for 24 h at 37°C, the content of each well was discarded carefully and washed gently with phosphate buffer saline (PBS) to remove free-floating bacteria. Adherent bacteria were fixed with 99% methanol for 10–15 min. The plates were decanted, allowed to dry, and stained for 15 min with 1% crystal violet. Excess stain was rinsed off by washing with tap water. Dissolved the adherent cells with 95% ethanol for 10 min, and subsequently optical density was determined using an absorbance microplate reader (Byonoy/Germany) at a wavelength of 620 nm. The negative control contained (TSB with 1 % glucose) only. The OD cut-off (OD_c) was defined as three standard deviations above the mean OD of the negative control: OD_c = average OD of negative control + (3 \times SD of negative control). The following categories were used to categorize all of the isolates based on their ability to adhere: Non-biofilm producers (OD < OD_c), weak biofilm producers (OD_c < OD \leq 2 \times OD_c), moderate biofilm producers (2OD_c < OD \leq 4 \times OD_c) and strong biofilm producers (4 \times OD_c < OD).

2.3. Determination of the Minimum Inhibitor Concentration and Minimum Bactericidal Concentration

To determine Minimum Inhibitor Concentration (MIC) and Minimum Bactericidal Concentration (MBC) value for each oil broth dilution method was used with some modification as follows [27]: The cell suspension of each isolate was prepared in Muller Hinton broth (MHB) by transferring 1-2 colonies from a 24 h incubated culture, then adjusting it to 0.5 of the McFarland standard. The stock solution for each oil was prepared at a concentration of 8% in dimethyl sulfoxide (DMSO). Two-fold serial dilution was performed in a 96-well microtiter plate from 4% to 0.0625% concentration (for each oil), leaving 100µl volumes in all the wells. 100µl of stock solution was transferred into column 1 and 50µl of sterile MHB into columns 2 to 7. Then, transferred 50µl from each well in column 1 to the wells in column 2 and mixed by pipette 4-6 times. This step was repeated with the other columns to make a serial dilution (The solution drawn from column 7 was discarded). 50µl of each bacterial suspension was added to each well in columns 1 to 7. The positive control included (bacterial suspension with MHB) while the negative control included (essential oils with MHB). After 24h incubation at 37°C, 50µl from each well was transferred to a sterile Muller Hinton agar (MHA) plate and incubated at 37°C for another 24h. The lower concentrations leading to growth or absence of growth were designated as MIC and MBC, respectively. To exclude any errors, every plate was repeated three times (triplicates).

2.4. Impact of Essential Oils on the Preformed Biofilms

The crystal violet staining method was used to detect the anti-biofilm activity of cinnamon oil (Hemani/Pakistan) and tea tree oil (Pranarôm/Belgique) on 24h old pre-formed biofilms [28] as follow: After biofilm formation, the broth medium was Tryptic Soy Broth with 1 % glucose (TSBG) gently aspirated, and plates were washed three times with phosphate-buffered saline solution (PBS) to remove planktonic bacteria. Each EO was added at its MIC concentration and incubated at 37°C for 24 h. After incubation, the content of each well was discarded carefully, then washed with PBS as with the previous step. For fixation of the biofilms, 150 µL of methanol for 10-15 min was added, and the supernatant was removed again. Then, 150 µL of crystal violet (CV) solution at 1% was added to each well and, 15 min later, the excess dye was removed by washing the plates three times with sterile PBS. The bound crystal violet was released by adding 200 µL of 95% ethanol followed by incubation for 10 min at room temperature and optical density was measured at 620nm. The Negative control consists of (MIC value with TSBG) while the positive control consists of (bacterial sample with TSBG).

2.5. Checkerboard Assay

The anti-biofilm activity of the combination of Cinnamon oil (Hemani/Pakistan) and Tea tree oil (Pranarôm/Belgique) against *K. pneumoniae* isolates was evaluated using the Checkerboard method [29] as follow: Seven serial, two-fold dilutions of Cinnamon oil and Tea tree oil were prepared in Muller Hinton broth (MHB) as with the previous steps. Along the x-axis across the checkerboard plate, 50µl of each concentration of the Tea tree oil was added into each well (from Columns 1 to 7). As for the y-axis, 50µl of each concentration of Cinnamon oil was added into each well (from rows A to G). The wells in (Row H and column 12) were considered as control. Each well received 50 µl of bacterial suspension (adjusted to McFarland standard 0.5) and was incubated at 37°C for 24 h. Following incubation, 50 µl from each well was transferred to a sterile Muller Hinton agar (MHA) plate and incubated for another 24 h at 37°C. The fractional inhibitory concentration index (FICI) values were calculated using the following formula while the \sum FICI values are interpreted as follows in Table 1 [30]:

$$\sum \text{FICI} = \text{FIC (A)} + \text{FIC (B)} \quad (1)$$

$$\text{FIC (A)} = \frac{\text{MIC(A) in combination}}{\text{MIC(A) alone}} \quad (2)$$

$$\text{FIC (B)} = \frac{\text{MIC(B) in combination}}{\text{MIC(B) alone}} \quad (3)$$

Table 1: Interpretation of the values of ΣFICI .

Interpretation	Values
Synergetic	≤ 0.5
Partial synergetic	$0.5 - 0.75$
Additive	$0.76 - 1$
Indifferent (non-interactive)	$1 - 4$
Antagonistic	> 4

3. Results and Discussion

K. pneumoniae appeared microscopically as shorter Gram-negative rods after being stained using the Gram staining technique (pink rods colonies). Bacterial samples appeared circular, 2-3mm in size, with a mucoid surface and a pink-red colour on MacConkey Agar medium, and a blue to purple colour on HiCrom™ UTI Agar medium. Oxidase -ve, indole -ve, and citrate utilization +ve were found in *K. pneumoniae* isolates in biochemical assays [31]. All bacterial samples are identified using the vitek 2 compact (bioMérieux/France). The results of *K. pneumoniae* biofilm formation using 96-well microtiter plates showed the following statistics: [n = 24 (48%)] of isolates from strong biofilm, [n = 12 (24%)] moderate, [n = 10 (20%)] weak, and [n = 4 (8%)] do not form biofilm, as shown in Table 2 and Figure 1.

Table 2: Classify biofilm formation ability through the use of a microtiter titer plate [32].

Cut-off value calculation	Mean of OD values results	Biofilm formation abilities
$\text{OD} \leq 0.051$	$\text{OD} \leq 0.051$	None
$\text{ODc} < \text{OD} \leq 2 \times \text{ODc}$	$0.051 < \text{OD} \leq 0.102$	Weak
$2 \times \text{ODc} < \text{OD} \leq 4 \times \text{ODc}$	$0.102 < \text{OD} \leq 0.204$	Moderate
$\text{OD} > 4 \times \text{ODc}$	$\text{OD} > 0.204$	Strong

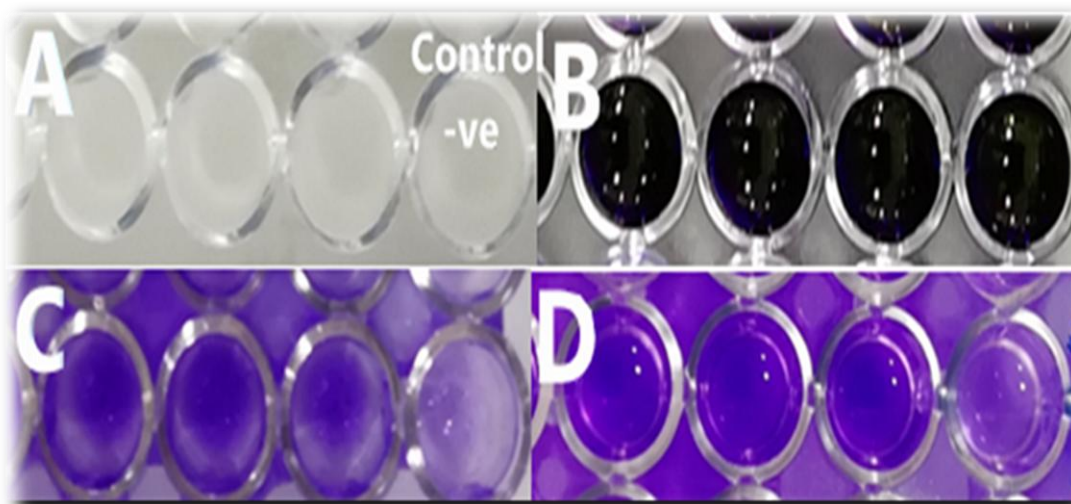


Figure 1: Detection of Biofilm Formation by *K.pneumoniae* (A) After isolates were incubated for 24 h at 37°C, (B) During staining with 1% crystal violet, (C) Biofilm after staining, (D) After dissolving the adherent cells with 95% ethanol. Negative control contained (TSB with 1 % Glucose) Only.

The lower concentrations that cause growth or lack of growth are referred to respectively as MIC and MBC. Two-fold serial dilution was performed on a 96-well microtiter plate from 4% to 0.0625% concentration for each oil. To confirm the result, 50 μ L of each concentration were taken and cultured on the Mueller Hinton Agar and incubated for 24 hours at 37°C. The results showed that the MIC for most of the 24 strong biofilm isolates for Tea tree oil is 0.25% and MBC 0.5% as shown in Figure 2. While the MIC for Cinnamon oil is 0.125% and MBC 0.25% as shown in Figure 3.

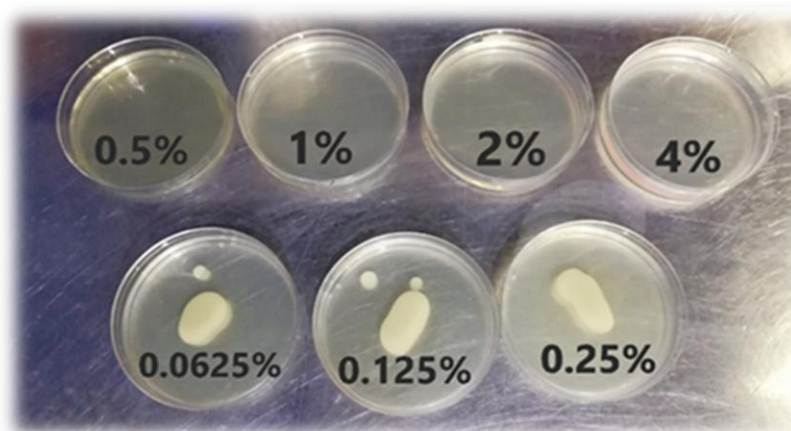


Figure 2: Tea tree oil MIC and MBC concentrations for *K. pneumoniae* isolates were determined through the broth dilution method was performed in a 96-well microtiter plate from 4% to 0.0625% concentration. After 24h of incubation at 37°C, 50 μ L was transferred from each well to a sterile Muller Hinton agar plate and incubated at 37°C for another 24h. That showed the value of MIC was 0.25% while MBC was 0.5%.

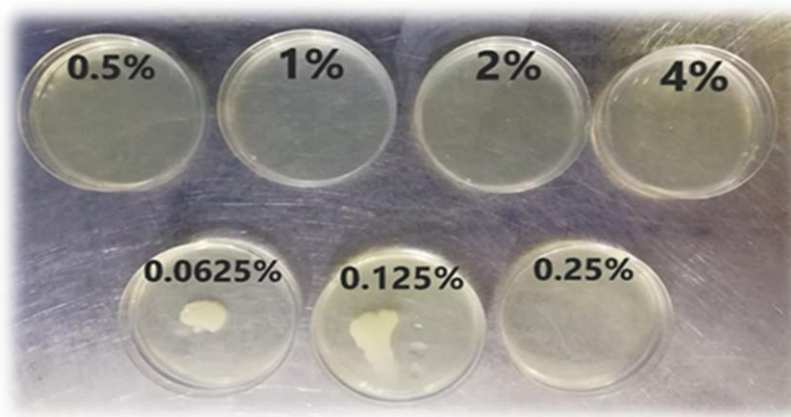


Figure 3: Cinnamon oil MIC and MBC concentrations for *K. pneumoniae* isolates were determined via the broth dilution method was performed on a 96-well microtiter plate from 4% to 0.0625% concentration. After 24h of incubation at 37°C, 50 μ L was transferred from each well to a sterile Muller Hinton agar plate and incubated at 37°C for another 24h. That showed the value of MIC was 0.125% while MBC was 0.25%.

Andrade et al. reported that the MIC of TTO $\leq 0.5\%$ (v/v) for *K. pneumoniae* and other different bacteria [34]. Carson et al. reported that the MIC of Tea tree oil against *Escherichia coli* and *Staphylococcus aureus* was 0.25% and 0.05% respectively [35]. Lang et al. reported that *cinnamomum cassia* essential oil has bactericidal activity at a concentration of 0.125% against *pseudomonas aeruginosa* [36]. Prabuseenivasan et al. reported that Cinnamon oil can inhibit both gram-positive and gram-negative bacteria. It also shows promising inhibitory activity even at low concentrations, so it could be a good source of antibacterial agents [37]. The essential oils (Cinnamon oil and Tea tree oil) were evaluated for their anti-biofilm activity on 24h old pre-formed biofilms and

this was detected by the crystal violet staining method. The results showed that both oils had good efficacy against strong biofilm for *K. pneumoniae* isolates, as seen in the Figures 4 and 5. Iseppi et al. Tea tree oil was tested as an anti-biofilm agent against *K. pneumoniae* and other gram-negative bacteria and found to have good anti-biofilm capability [38]. Condò et al. reported that Cinnamon oil was tested on 18, 24, 48, and 72-hour mature biofilms. It exhibited the best results, showing significant activity against *K. pneumoniae* and other bacterial pathogens [39]. EOs are efficient in combating nosocomial infections and have been utilized as a cleaning solvent for sanitizing medical equipment and surfaces [40]. In a comparison between the two oils, Cinnamon oil showed better results. The antimicrobial activity of Cinnamon oil has been related to its cinnamaldehyde content [41]. Terpinen-4-ol is considered to be the principal active component of Tea Tree oil [42]. It has been reported that EOs containing aldehydes or phenols, such as cinnamaldehyde, citral, carvacrol, eugenol or thymol as major components, showed the highest antibacterial activity, followed by EOs containing terpene alcohols. Other EOs containing ketones or esters, such as β -myrcene, α -thujone, or geranyl acetate, had much weaker activity. While terpene hydrocarbon-containing volatile oils are usually inactive [43]. The Essential oil (EO) action on biofilm inhibition and dispersal can be related to reactivity, hydrophobicity, and the diffusion rate of the EO in the matrix, as well as the biofilm composition and structure [44]. The main constituents of EO can act in several ways to disturb the biofilm's development, such as blockage of the quorum-sense system, or through interference with bacterial motility [45]. Anti-biofilm agents can have different therapeutic applications depending on their effects on the biofilm: compounds that interfere with biofilm formation could be exploited in the prophylaxis of implant surgery or for the coatings of medical devices, whereas agents able to disperse biofilm structure could be administered in combination with conventional antibiotics for the treatment of biofilm-associated infections [46]. The Checkerboard technique evaluates antibacterial combinations' activity in 2-fold serial dilutions at clinically acceptable doses. Anti-bacterial from several classes are typically included in the test combinations. The fractional inhibitory concentration index (FICI) is used to examine the data generated by the checkerboard experiment [47]. The results showed that the MIC of Tea Tree oil was 0.25%, and in combination with Cinnamon oil was 0.0625%, while the MIC of Cinnamon oil was 0.125%, and in combination with Tea Tree oil was 0.5%. The FIC and FIC Index values were calculated and interpreted as seen in Table 3 According to the equation and values mentioned earlier.

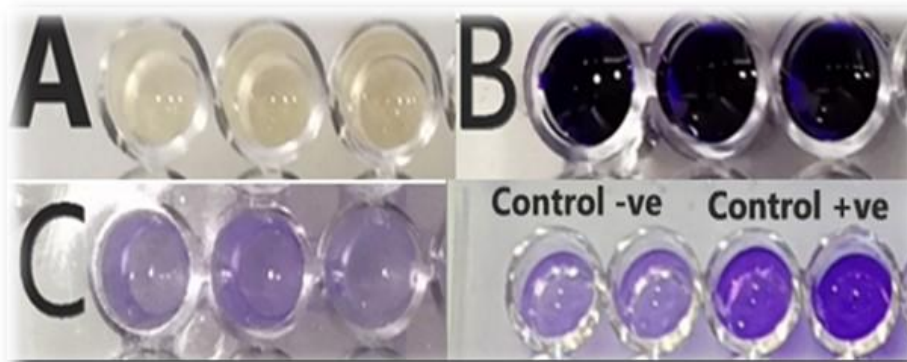


Figure 4: Anti-biofilm Efficacy of Tea Tree Oil against *K. pneumoniae* Isolates by Tissue Culture Plate Method (A) After 24 hours of incubation at 37°C, (B) During staining with 1% crystal violet, and (C) After dissolving adherent cells in 95% ethanol. The Negative Control Consists of (MIC value with TSBG) While the positive control consists of (Bacterial sample with TSBG).

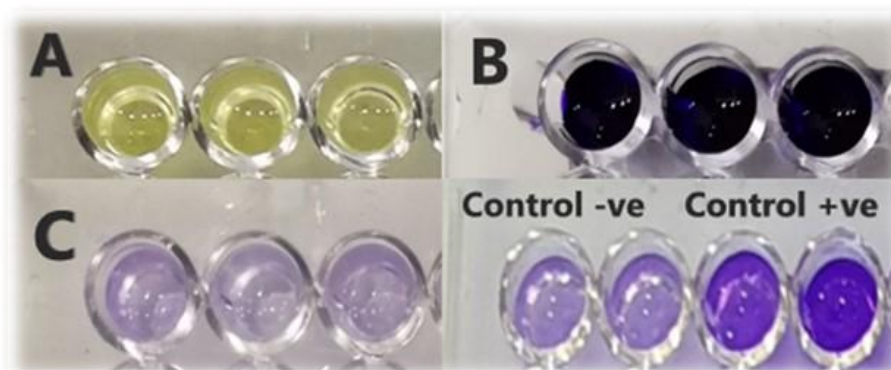


Figure 5: Anti-biofilm Efficacy of Cinnamon Oil Against *K. pneumoniae* Isolates by Tissue Culture Plate Method. (A) After 24 hours of incubation at 37°C, (B) During staining with 1% crystal violet, and (C) After dissolving adherent cells in 95% ethanol. The Negative control consists of (MIC value with TSBG) while the positive control consists of (bacterial sample with TSBG).

Table 3: FIC and FICI values for the combination of Tea tree and Cinnamon oils.

Combination	MIC alone	MIC combined	FIC	FICI	Remark
Tea tree	0.25	0.0625	0.25	4.25	Antagonism
Cinnamon oils	0.125	0.5	4		

FIC: fractional inhibitory concentration. FICI: fractional inhibitory concentration index.

The results show that the combination of Tea tree and Cinnamon oils has antagonism effects. The interaction between Essential Oil (EO) compounds can produce four possible types of effects: synergistic, additive, indifferent, and antagonistic effects. Synergism is observed when the effect of the combined substances is greater than the sum of the individual effects. An additive effect is observed when the combined effect is equal to the sum of the individual effects. While the absence of interaction is defined as indifference. Antagonism is observed when the effect of one or both compounds is less when they are applied together than when individually applied [48]. In many cases, the activity results from the complex interaction between the different classes of compounds such as phenols, aldehydes, ketones, alcohols, esters, ethers, or hydrocarbons found in EOs [49]. Several studies have found that a number of these compounds exhibited significant antimicrobial properties when tested separately [50, 51]. Different terpenoid components of EOs can interact to either reduce or increase antimicrobial efficacy [52]. Most studies attributed additive and synergism effects to phenolic and alcohol compounds. Generally, compounds with similar structures exhibit additive rather than synergistic effects. An antagonistic effect has been attributed to the interaction between non-oxygenated and oxygenated monoterpene hydrocarbons [53]. There are a limited number of studies on the effects of the test medium's physical and chemical parameters on the interaction between essential oil components and their antimicrobial activities. Physical (temperature) and chemical (sodium chloride) parameters were also found to modulate the antimicrobial responses of the mixtures. Sodium chloride was found to have antagonistic effects when combined with carvacrol and p-cymene against *Bacillus cereus*. It was also observed that carvacrol and p-cymene worked synergistically, but this effect was reduced when sodium chloride was added [54]. It has been reported that the combination of Cinnamon and Clove EOs showed better antimicrobial activity in the vapour phase than in the liquid phase [53]. Also, Combined effects can vary based on the target bacterial species, highlighting the importance of the evaluation of antibacterial complexes for each target bacterial species, as shown by research on the complex formulated from Lauric arginate (LAE) and EO, which showed synergistic effects against *Listeria monocytogenes* but antagonistic effects against *Escherichia coli* O157: H7 and *Salmonella enterica* [55]. López et al. reported some generally accepted mechanisms for the synergistic action of antimicrobial combinations: the sequential inhibition of a common biochemical pathway, inhibition of protective enzymes, combinations of cell wall active agents, or the action of cell wall active agents to enhance the uptake of other antimicrobials [56]. Likewise, some mechanisms produce antagonism of antimicrobial combinations. Although these are less well known, generally they include the combinations of bactericidal and bacteriostatic agents, the use of compounds that act on the same target of the microorganism, or chemical (direct or indirect) interactions

among compounds such as the reduction of the active aqueous terpene solubility by non-aqueous monoterpene hydrocarbons [57].

4. Conclusions

In this study, the ability of fifty *K. pneumoniae* isolates collected from urine samples of people with urinary tract infections to produce biofilms was revealed. The outcomes revealed that nearly half of the isolates formed strong biofilms, while the other half produced moderate to weak biofilms, and a small percentage of them were non-productive. Tea Tree and Cinnamon oils both exhibit good effects against the strong biofilm of *K. pneumoniae*. But in comparison between them, Cinnamon oil showed better results. The mixture of the two oils, on the other hand, produces an antagonistic impact. The results in this study recommended seeking the effect of more essential oils in single or combination form might be of great interest for future medication or disinfectant development.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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