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Original article

Analysis of the Antibiofilm Efficacy of Citrus Aurantifolia Juice and Silver Nanoparticles Against Klebsiella Pneumoniae Obtained from Urinary Tract Infections.

Sarmed M. Hussein

Tropical-Biological Research Unit, College of Science, University of Baghdad, Baghdad, Iraq
Email. Sarmed.m@sc.uobaghdad.edu.iq

Abstract
The creation of biofilms by *Klebsiella pneumoniae* is one of the main reasons why people suffer from chronic urinary tract infection (UTI), as it allows the bacteria to live longer and resist antimicrobials. The purpose of this research was to see if lime juice and/or silver nanoparticles (AgNPs) could help prevent biofilms created by *K. pneumoniae* strains isolated from people suffering from UTI. Thirty urine samples were gathered from patients visiting private clinics during the months of November 2024 through January 2025. Microbial isolation and identification were done using traditional microbiology techniques and API 20E. Biofilm formation was determined using the tissue culture plate and Congo red method. The results indicated that *K. pneumoniae* was isolated in 34% of the urine specimens. In the 15 isolates that were analysed for biofilm production, four produced biofilms strongly, nine moderately, and two weakly. Antibiofilm activity of lime juice was observed at all concentrations studied, with 12.5% giving the highest effect. The AgNPs were found to exhibit inhibitory properties, reaching maximum efficacy at 7.8 µg/mL concentration. The use of the combination of lime juice and AgNPs was also more effective against biofilms, especially in isolates 26 and 27. These results confirm that lime juice and AgNPs have promise as effective agents against biofilm formation by *K. pneumoniae*.
Keywords. *Klebsiella Pneumoniae*, Urinary Tract Infection, Biofilm, Lime Juice, Silver Nanoparticles, Antibiofilm Activity.

Introduction

Urinary tract infection (UTI) is a bacterial infection that is prevalent all around the world and has become a significant issue within the healthcare industry [1]. The disease is prevalent worldwide with varying degrees of complications and symptoms [2]. Pathogenesis of UTI begins with the colonization of uropathogenic bacteria that adhere to urothelial cells, thereby initiating infection and progression of the condition. The urine is a nutrient-rich medium where microorganisms can multiply quickly and get into the body via different channels [3]. Due to the ability of bacteria to adhere to the epithelial cells and surfaces of medical devices, biofilm formation occurs on urinary catheters and urinary tracts. Sometimes pollution of the urinary tract occurs due to biofilm formation in catheter treatment sites [4].

K. pneumoniae, *E. coli*, and *Pseudomonas aeruginosa* are some of the main uropathogens that infect people around the world [5]. There are many health organizations across the world that have alerted about the hazards of infection with *K. pneumoniae*. According to the WHO, *K. pneumoniae* causes very serious infections including UTI, pneumonia, and septicemia [6]. The *K. pneumoniae* that led to this infection are categorized as being Gram negative, facultative anaerobic, rod-shaped bacteria that belong to the Enterobacteriaceae family. Some *K. pneumoniae* strains that cause nosocomial infections can become invasive [7]. The high prevalence of resistance to antibiotics in clinically important bacteria indicates there is always a need for new and more effective agents [8]. Therefore, other antibacterial substances like plant extracts are required, such as *Citrus aurantifolia*. *Citrus aurantifolia* has been used for a long time in the food industry in the form of beverages, and its extracts are also used in traditional medicine for promoting immune defense and metabolism, pathogen elimination, and microbial growth inhibition [9].

It has been shown that citrus fruits demonstrate antibacterial effects on both types of Gram bacteria strains [10]. Consumption of citrus fruits has also been linked to low risks of suffering from various chronic diseases [11]. Various researchers have conducted studies on the antimicrobial activities of citrus fruits either alone or as part of the herbal mixture [12], and it has been noted that lime juice has high antimicrobial activity. Other substances can also serve as antimicrobial agents, including nanoparticles. For an extended period, nanoparticles have been identified as possessing a vast antibacterial range against bacteria, fungi, protozoa, and several viruses [13]. Nanoparticles fall under the category of particles that are 1000 times smaller in dimension than microparticles. The high surface area of nanoparticles arises due to their size and causes the nanoparticles to come in contact with bacteria; thus, they have a greater chance of interaction compared to larger particles.

Owing to their diminutive dimensions of fewer than 10 nm, nanoparticles can engage with bacteria, resulting in electrical effects that enhance their reactivity. Currently, nanotherapies are employed as antibiofilm agents by integrating silver nanoparticles into diverse materials [14-16]. Based on all the above-mentioned information, this research was designed to assess bacterial contamination by *K. pneumoniae* in urinary tract infection samples, extract biofilm-producing strains by various techniques, and examine the antibiofilm properties of lime juice and silver nanoparticles on *K. pneumoniae* isolates.

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Materials and Methods

Isolation and Identification

Thirty urine specimens were randomly collected from the clients visiting private clinics located in different regions within the time period ranging from November 2024 to January 2025. The samples were immediately placed in sterilized containers and brought to the laboratory for testing, without any further storage. Samples suspected of a possible urinary tract infection were obtained using the mid-stream clean-catch method and stored in sterile wide-mouth screw-capped containers. One milliliter of the urine sample was incubated at 37°C for 24 hours. with 9 mL of buffered peptone water. The complete volume of the culture was inoculated onto MacConkey agar (Himedia/ India), cystine lactose electrolyte deficient agar (Himedia/ India), and blood agar (Himedia/ India), then incubated at 37 ±1°C for 24 hours. Subsequently, the suspicious colonies were inoculated onto urea agar and triple sugar iron agar (Himedia/ India), punctured to the bottom and streaked along the slope, then incubated for 24 hours at 37°C, followed by the execution of 20 Epi tests.

Biofilm Analysis

Plate Method

Brain heart infusion broth was used to culture all isolates of bacteria enriched with 1% glucose in 96-well plates and incubated at 37°C for 24 hours. In an aerobic environment. Subsequently, the bacterial cells underwent three washes with distilled water. Cells that attached well were preserved in methanol (200 µL) for 20 minutes. Dishes were cleared and air-dried overnight. Remaining cells were stained with crystal violet 0.1% solution for a duration of 15 minutes, followed by rinsing with distilled water. The plates were once more cleansed with distilled water and allowed to air-dry overnight. Crystal violet was affixed to the adhering cells utilising 96% ethanol for all wells. A spectrophotometer was used to assess the plates at 490nm, and this experiment was conducted in triplicate, utilising the absorbance of wells filled with sterile Brain heart broth as controls, with results compared to these controls as illustrated in (Table 1).

Table 1. Categorisation of bacterial attachment with the tissue culture plate technique [17].

OD values	Biofilm
$< OD_{control}$	Not producer
$OD_{control} < OD_{test} \leq 2*OD_{control}$	Weak
$2*OD_{control} < OD_{test} \leq 4*OD_{control}$	Moderate
$4OD_{control} \leq OD_{test}$	Strong
OD= optical density.	

Congo Red Procedures

The procedure followed the methodology outlined by Freeman (1989), who introduced a novel strategy for screening biofilm formation in Staphylococcus, utilising a specially designed solid medium comprising BHI broth (37gms/L), sucrose (50gms/L), agar No. 1 (10gms/L), and Congo Red dye (0.8gms/L). Congo Red dye was originally prepared as a water-based solution and autoclaved at 121°C for 15 minutes without regard to other medium components and added when the medium decreased to 45°C. Culture media plates were subsequently inoculated with bacterial isolates and incubated for 24 hours at 37°C. Favorable outcomes were signified by dried black colonies; moderate biofilm producers were characterised by a grey hue, whilst poor slime producers manifested a pink coloration [18].

Silver NPs suspension

AgNPs were prepared in accordance with [19]; 100 mg of AgNPs were dissolved in 10 mL of sterile distilled water and well stirred. The suspension was ultrasonicated at 40 kHz for 30 minutes, sterilised in an autoclave for 20 minutes at 121°C, and subsequently cooled to room temperature.

Lime Juice Preparation

The extraction of lime juice was conducted using the methodology outlined in [20]. Limes were procured from the marketplace. The fruit was halved utilising a hygienic cutting instrument. The juice was thereafter obtained from each fruit in a hygienic vessel. This operation was performed in a way that safeguarded the juice against contamination during its extraction. This beverage was designated as having a 100% concentration of juice extract. A 50% concentration was attained by mixing 100% juice extract with the requisite amount of sterilised distilled water.

Investigation of the Antibiofilm Properties of Lime Juice

Properties of lime juice as an antibiofilm agent were evaluated using the identical methods employed for assessing biofilm formation as discussed previously, However, rather than employing different concentrations (50, 25, 12.5 and 6.25 %) of biofilm-forming bacteria at a volume of 100 alongside identical volumes of bacterial suspensions, distinct amounts of lime juice were utilised in the wells as triplicates for each concentration, incubated at 37°C for 24 hours, subjected to three washes with distilled water, stained using 0.1% crystal violet, and the optical density was quantified via ELISA reader at 490 nm.

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Evaluation of the Antibiofilm Activity of AgNPs in Relation to Bacterial Isolates

The identical procedure was employed, with the exception of varying doses of AgNPs introduced alongside the suspension into the wells in triplicate for each concentration (500, 250, 125, 62.5, 31.25, 15.6 and 7.8 µg/mL), followed by a 24-hour incubation at 37ºC. All wells were cleansed, marked, and analysed at 490 nm.

Synergistic Evaluation of Antibiofilm Effect of Lime Juice and AgNPs Towards Isolates

The synergistic effects of AgNPs with lime juice in terms of their antimicrobial activities against the biofilms were assessed by performing the Tissue Culture method assay as well. One hundred microliters of the bacterial suspension was added to individual wells, and then AgNPs along with lime extracts (50 µl each) were introduced. Subsequently, incubation occurred at 37±°C for 24 hours, after which the wells were washed three times with distilled water and stained for 15 minutes. Then the stain was removed using ethanol, and the optical density was measured at 490 nm.

Results and Discussion

Identification

The findings of the current study showed the existence of different types of bacterial isolates 44 with different bacterial species in urine samples taken from patients suffering from UTI. It was found that (34%) of the urine samples had *K. pneumoniae* bacteria. Bacterial isolates that have been identified through urine samples in the current study are *Escherichia coli, K. pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis,* and *Staphylococcus aureus*.

Biofilm assay

According to recent study results, all isolated bacteria were found to have the ability to form biofilm but at a variable level of ability, and the percentage of biofilm production by the isolated *Klebsiella* strains is represented as follows: 4 strains were found to produce strong biofilm formation, nine strains exhibited moderate biofilm production, whereas merely two strains demonstrated weak biofilm formation (Figure 1).

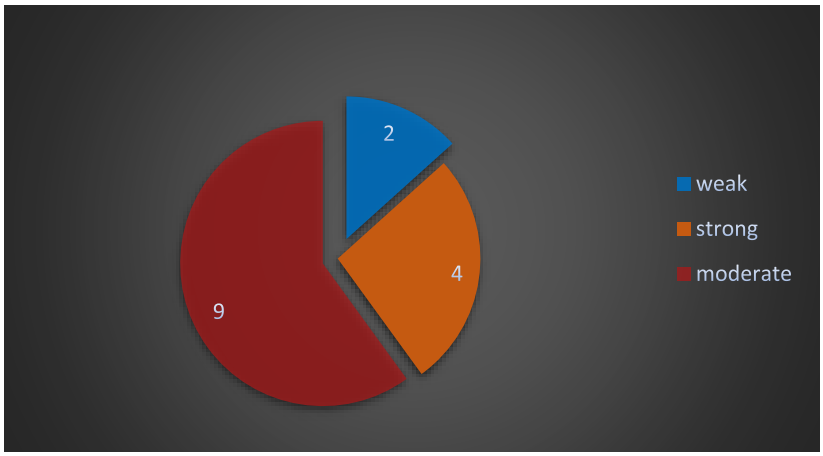


Figure 1. Biofilm produced by K. pneumoniae

Congo Red Agar

The current investigation indicated that the *K. pneumoniae* isolates produce exopolysaccharides, but in variable quantities. A mere 4 isolates (26.66%) yielded positive outcomes by forming black crystalline colonies, while 2 isolates (13.33%) generated dark red colonies, indicating moderate results; conversely, 60% of the isolates exhibited negative results, manifesting as pink colonies. Refer to (Figure 2).

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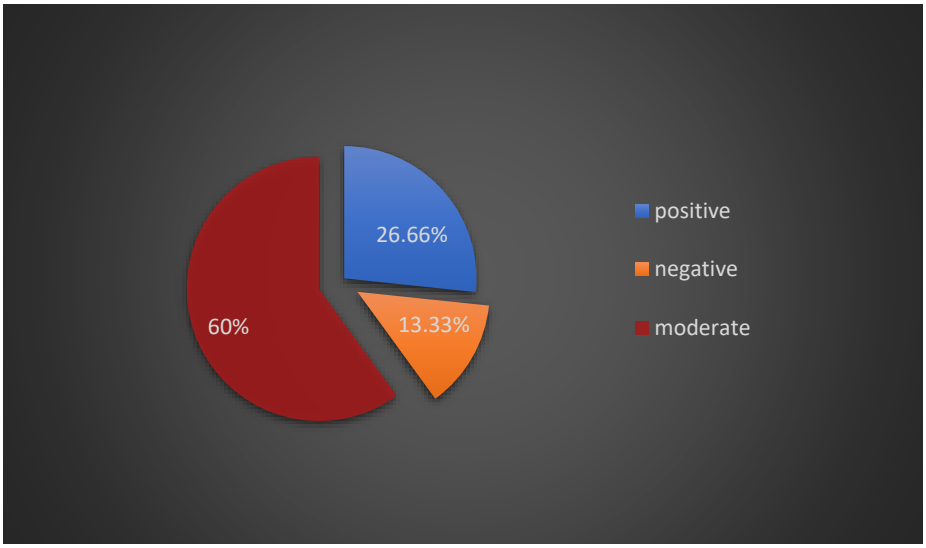


Figure 2. Results of Congo red for K. pneumoniae

Antibiofilm Study of Lime Juice

The findings of the present investigation indicate that all concentrations of lime juice affect biofilm development in *K. pneumoniae*, with the most inhibition reported at a 12.5% concentration, as illustrated in (Figure 3).

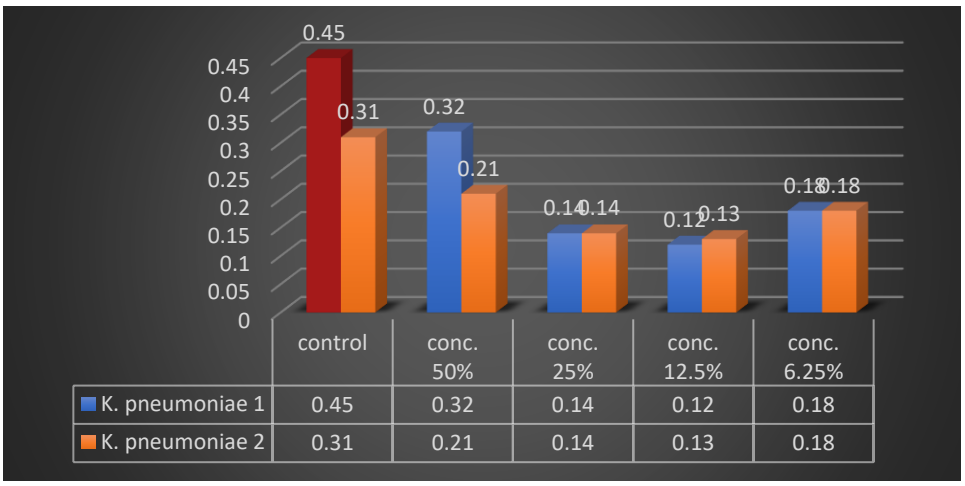


Figure 3. Investigation of the antibiofilm properties of lime juice against K. pneumoniae strains 1 and 2.

The illustration above depicts the impact of varying concentrations of lime juice on biofilm formation, revealing the effective antibiofilm activity of lime juice, as seen by the percentage at each concentration in comparison to the control.

Antibiofilm Efficacy of AgNPs Against Bacterial Strains

In the current study, all synthesised doses of AgNPs demonstrated antibiofilm efficacy against *K. pneumoniae* strains 1 and 2, with the most pronounced activity noted at a concentration of 7.8 µg/mL (Figure 4).

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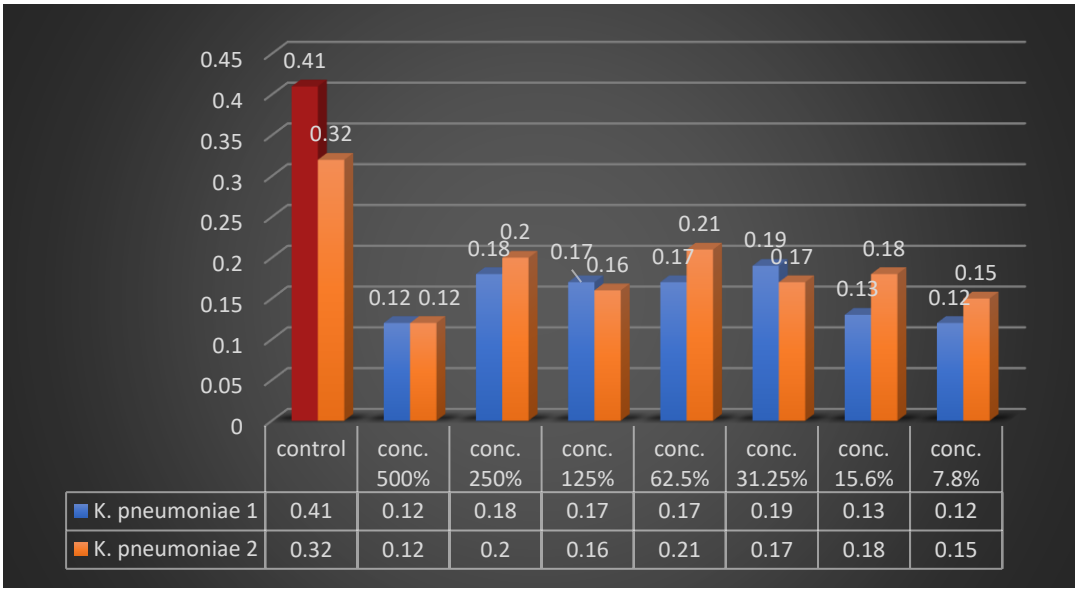


Figure 4. Antibiofilm activity of AgNPs against *K. pneumoniae* 1 and 2

Synergistic Evaluation of Antibiofilm Effect of Lime Juice and Agnps Towards Isolates

In addition, synergism of the effect of AgNPs and lime juice was assessed; it was revealed that the same antibiofilm effect was achieved with their combination, but the most pronounced inhibitory effect on *K. pneumoniae* isolate 1 was noted with the concentration (125 and 12.5 µg/mL) of lime juice and AgNPs, respectively. The antibiofilm activity of *K. pneumoniae* isolate 2 was most significant at a concentration (62.5 and 6.25 µg/mL) of lime juice and AgNPs, respectively (Figure 5).

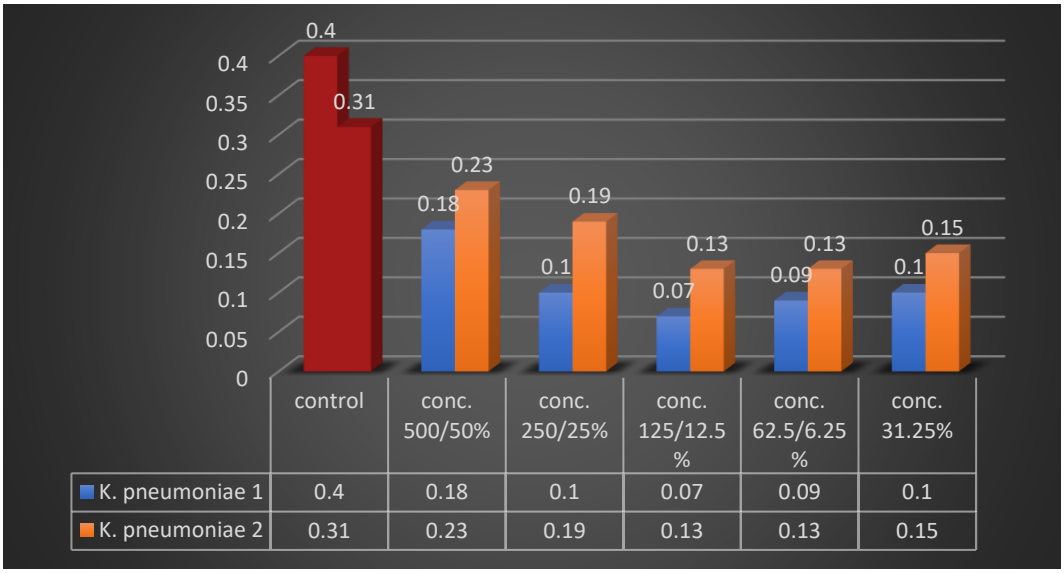


Figure 5. The synergistic effect of AgNPs and Lime juice against K. pneumoniae biofilm 1 and 2.

Discussion

The findings of the current study are consistent with previous studies reporting the presence of *K. pneumoniae* among the common uropathogenic bacteria isolated from urine samples. *K. pneumoniae was* identified as one of the most common uropathogenic bacteria isolated over three years [21]. Similarly, [22] reported that *K. pneumoniae* accounted for 31.7% of urinary tract infection isolates, following Escherichia coli (68.3%). In another Iraqi study, *K. pneumoniae* was isolated from community-acquired and hospital-acquired UTI, with ESBL positivity reported in 21.43% of isolates [23]. The prevalence of this bacterium may be associated with inappropriate antibiotic use, poor hygiene, and catheter-related infections in hospitals [24]. Furthermore, urinary catheter use, inadequate sterilization practices, and increasing antibiotic resistance may enhance bacterial colonization and infection [25].

Biofilm formation is an important virulence factor that contributes to the survival of *K. pneumoniae* on medical devices, including urinary catheters, and is associated with persistent and chronic UTI [26]. Prolonged catheterization increases the risk of biofilm development and infection; patients with catheters retained for three days have a greater than 90% chance of developing an infection, while each additional day increases the likelihood of biofilm development on the catheter surface by 3.55 [27]. *K. pneumoniae* accounts for 6–17% of opportunistic urinary tract infections, many of which

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are associated with biofilm formation within urinary catheters or at their insertion sites [28]. Biofilms also contribute to bacterial survival and increased antibiotic resistance, making catheter-associated UTI an important cause of hospital-acquired infections and increased patient morbidity [29]. The ability of *K. pneumoniae* to form biofilms may vary between strains and is influenced by bacterial virulence factors. Type 1 fimbriae are associated with UTI, whereas type 3 fimbriae contribute to device-associated infections through biofilm formation [30]. Urinary isolates have also demonstrated higher antibiotic resistance and distinct biofilm-forming properties compared with blood and respiratory isolates [31]. Furthermore, biofilm production may be associated with specific sequence types and virulence genes, including wcaG, rmpA, and magA [32]. Regarding biofilm detection, the tissue culture plate method is considered the gold standard compared with the tube method and Congo red method. The Congo red method has limitations in detecting biofilm formation because of its low correlation with other methods and its potential to produce false-positive and false-negative results. [33] reported a sensitivity of 56.9% and a specificity of 50% for the Congo red method, indicating its limited reliability.

Similarly, [34] reported a substantially higher biofilm formation rate using the tissue culture plate method than the Congo red method. These findings support the limitations of the Congo red method as a screening method for biofilm-producing *K. pneumoniae* isolates. Regarding the antibiofilm activity of lime juice, limited research has investigated its direct effect on bacterial biofilm formation, particularly biofilms developing from planktonic bacteria. Previous studies have demonstrated the antibacterial activity of lime extracts against Staphylococcus, Pseudomonas, E. coli, and *K. pneumoniae* [35]. Lime juice has also demonstrated antimicrobial activity against uropathogenic bacteria associated with UTI [36]. Furthermore, ethanolic lime extracts have produced inhibitory zones against *K. pneumoniae*, P. aeruginosa, E. coli, and Enterococcus faecalis [37]. These findings provide supporting evidence for the potential antimicrobial activity of citrus-derived products. Silver nanoparticles (AgNPs) have also demonstrated antibiofilm activity against *K. pneumoniae*. Previous research has reported that AgNPs can reduce extracellular polymeric substance production and increase protein leakage by disrupting bacterial cell membranes [38]. Similarly, silver nanoparticles have inhibited biofilm growth in *K. pneumoniae*, E. coli, and P. aeruginosa after 24 hours. Although the exact mechanisms of AgNP antibacterial activity have not been fully elucidated, their effects may involve reduced quorum-sensing activity, increased antibiotic susceptibility, and oxidative stress leading to DNA damage [39]. The combination of AgNPs with other antimicrobial agents may enhance antibiofilm activity. Such combinations can increase the local concentration of antimicrobial agents near bacterial cells and strengthen their interactions. Previous studies have also reported that combinations of silver nanoparticles with antimicrobial agents can inhibit biofilm formation, reduce biofilm biomass and metabolic activity, and enhance antimicrobial effects [40].

Conclusion

The results obtained from the current study show that there is evident biofilm formation potential in *K. pneumoniae* isolates obtained from UTI samples, emphasizing the role played by this property in the presence of UTI. Out of the 15 *K. pneumoniae* isolates tested for biofilm formation through the Tissue Culture method, 4 isolates were highly capable of forming biofilms, 9 were moderately capable, while 2 were weak biofilm formers. In comparison to the Congo red agar test, there was a much lower number of positive isolates in the latter method, suggesting that the tissue culture method was more informative than the former method. In addition to that, the antibiofilm tests revealed the ability of lime juice and silver nanoparticles (AgNPs) to reduce biofilm biomass of the two selected *K. pneumoniae* isolates (1 and 2) through reduction in optical density when compared to the control. The highest antibiofilm activity of lime juice was seen at a concentration of 12.5%, while the highest reported activity of AgNPs was at a concentration of 7.8 µg/mL. It is evident that the two treatments have the potential to inhibit biofilm formation in vitro. Additionally, it was noted that the combined use of lime juice and AgNPs had a marked effect in decreasing the biofilm biomass in comparison to the controls. In the case of isolate 1, the minimal OD was achieved with a combination of (125, 12.5 µg/mL) lime juice and AgNPs, respectively, with an OD of 0.07 compared to 0.40 in the control. In the case of isolate 2, the minimal OD was obtained at the combination of (62.5, 6.25 µg/mL) lime juice and AgNPs, respectively, with an OD of 0.13 compared to 0.31 in the control. Thus, these results show the efficiency of the proposed treatment in decreasing the biofilm biomass; however, they are not enough to prove the synergy between the two substances. In summary, this study offers proof through experimentation that lime juice and silver nanoparticles, separately and in combination, can prevent biofilm formation by the *K. pneumoniae* tested in urine. The treatment of the isolates with a combination of lime juice and silver nanoparticles showed the lowest optical densities of all the treatments tested. This makes the combination of lime juice and silver nanoparticles a promising approach for future research into the prevention of biofilms. However, since the antibiofilm activity experiment was done using only two selected isolates, further studies using larger numbers of clinical isolates, proper statistical analysis, and proper dosages are recommended.

Conflict of interest. Nil

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