

Synergistic Effects of Silver Nanoparticles Combined with Amoxicillin or *Cymbopogon schoenanthus* Essential Oil on the Formation of Bacterial Biofilm

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Abstract

Objective: Multidrug-resistant bacteria create an urgent need for new materials and treatment combinations. This study aims to identify effective antibiofilm agents derived from nanomaterials, either alone or in combination with antibiotics or essential plant oils, and to evaluate their potential in preventing biofilm formation.

Methods: Four bacterial pathogens were selected for their resistance to more than two classes of antibiotics: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Biofilm formation was assessed using the Crystal violet staining method, with the highest biofilm producer selected for further analysis. The biofilm formation and bacterial Exopolysaccharide (EPS) content were measured and compared after treatment with Ag nanoparticles, whether used alone or combined with Amoxicillin or the essential oil extracted from *Cymbopogon schoenanthus*.

Results: All treatments effectively inhibited both bacterial growth and biofilm formation. The most significant activity was observed with the combination of AgNPs and plant oil, followed by the combination of AgNPs and Amoxicillin, and then nanoparticles alone. All treatments demonstrated significant inhibitory effects compared to the control group (which did not receive any inhibitory substances). The addition of Amoxicillin or plant oil also reduced the minimal inhibitory concentrations (MIC) for *K. pneumoniae*, which were determined to be 6.0 µg/ml for nanoparticles alone, 4.5 µg/ml for nanoparticles combined with the antibiotic, and 3 µg/ml for nanoparticles combined with plant oil. Furthermore, the toxicity of nanoparticles against *Artemia salina*, a test organism, was assessed. Treatment with AgNPs also decreased EPS production by *K. pneumoniae* by 39.6%, with further reductions of 54% and 80% observed in the presence of the antibiotic or plant oil, respectively. The effectiveness of the plant oil is attributed to the numerous secondary metabolites it contains.

Conclusion: This research highlights the promising potential of using AgNPs with either Amoxicillin or *C. schoenanthus* oil to fight against antibiotic-resistant bacterial infections.

Keywords: Exopolysaccharide, silver nanoparticles, amoxicillin, *Klebsiella*, *Cymbopogon*

Introduction

Bacterial biofilms are characterized by their adhesion to surfaces due to the presence of protective Extracellular Polymeric Substances (EPS) which are a complex mixture of predominantly sugars in addition to low quantity of proteins and phospholipids.¹ These biofilm forming bacteria are resistant to antibiotics and adhere to various surfaces, including glass, plastic, tissues, medical devices, and implants, causing chronic infections of soft tissue and many health problems.^{2,3} Biofilm can consist of single or multiple bacterial species of various pathogenic Gram-positive and Gram-negative bacteria, including *P. aeruginosa*, *E. coli*, and *K. pneumoniae*, which is known for its biofilm production and high antibiotic resistance.⁴ After a biofilm matures, antibiotics can kill the released planktonic cells; however, the bacteria within the biofilm can survive, leading to recurrent infections. Therefore, biofilm infections often exhibit recurrent symptoms even after multiple rounds of antibiotic therapy.² Also, bacterial aggregation contributes to antibiotic tolerance, while resistance arises from various internal and external factors, including mutations.⁵ Cells in bacterial biofilm can be 10 to 1,000 times more resistant to antimicrobial agents than free-living bacteria. For instance, *Propionibacterium acnes* cells grown in biofilms have slower metabolic rates and are more resistant to antimicrobial agents compared to free cells, which leads to treatment failures and increased morbidity, mortality, and healthcare costs.⁶⁻⁸

The thick layers of EPS around bacterial biofilms may play a crucial role for several reasons, such as preventing antimicrobial agents and chemicals from entering bacterial cells and reducing their adsorption or interaction with the biofilm, which makes biofilm removal difficult, resulting in many persistent illnesses.⁹ In contrast, planktonic bacteria outside the biofilms are highly active and more susceptible to antibiotics.¹⁰ The delayed growth rate of some biofilm cells is not due to nutritional deficiency but rather a general stress response triggered by biofilm formation, which protects the cells from various external pressures, including heat, pH fluctuations, and antimicrobial agents.¹¹

Preventing the initial bacterial adhesion is crucial for controlling biofilm development.³ There is growing interest in developing new methods for treating and preventing biofilms, particularly to combat their increasing antibiotic resistance. Novel biofilm inhibitors, especially nanomaterials, control biofilms on medical devices, and AgNPs have garnered attention due to their broad antibacterial properties and the mechanism of action involving generating reactive oxygen species, disrupting bacterial membrane integrity, and interfering with cellular processes such as respiration and replication.¹² Importantly, AgNPs can also overcome some of the mechanisms of resistance expressed by bacteria, making them an attractive candidate for use in combination with antibiotics. Amoxicillin, a broad-spectrum β -lactam antibiotic, is widely used as an effective agent against some bacterial infections. It works by

inhibiting the synthesis of bacterial cell walls, leading to cell lysis and death. However, the emergence of resistant bacterial strains has compromised the efficacy of Amoxicillin, raising the need for adjunct therapies that can potentially restore its effectiveness.

The combined effects of AgNPs and Amoxicillin have not been well demonstrated, and the type of interaction, synergistic or antagonistic, is not well studied. For example, a study by Zhang et al.^{13,14} found that combining AgNPs with Amoxicillin enhanced antimicrobial activity against methicillin-resistant *S. aureus* compared to either agent alone. This combination therapy decreased MIC of antibiotic and increased bacterial cell permeability, allowing for better antibiotic uptake.¹⁵ Moreover, another investigation indicated that AgNPs can reverse the resistance of bacteria to Amoxicillin by impairing the function of efflux pumps and beta-lactamases, enzymes commonly associated with antibiotic resistance.¹⁶ They added that the synergy observed may be attributed to several mechanisms.

The perennial grass *Cymbopogon schoenanthus*, commonly known as camel grass, possesses aromatic properties and various medicinal uses, serving as a potential antimicrobial and antibiofilm agent against some bacterial pathogens. Although *Cymbopogon* species are widely distributed in Saudi Arabia, there has been limited characterization of the full range of bioactive constituents in their essential oils, which is necessary to explain their inhibitory activities. Additionally, geographical origin may affect the oil content.^{17,18} AgNPs, amoxicillin, and plant essential oils are well-known as inhibitory substances that impact bacterial growth and biofilm formation; however, the assessment of their combined effects and potential mechanisms of action is not well documented. Current research focuses on novel strategies to biocontrol biofilm formation and EPS production, including the use of nanomaterials alone or in combination with antibiotics or plant oils.

Material and Methods

Bacterial Strains

The test organisms, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700613, *Pseudomonas aeruginosa* ATCC-27863, and *Staphylococcus aureus* ATCC 25923 were obtained from King Abdulaziz University Hospital (KAUH) in Saudi Arabia and maintained on nutrient agar plates at 4°C until used¹⁹ (Wong et al., 2023). These bacteria were identified using the Vitek 2 Identification System (VITEK® 2: Healthcare, BIOMERIEUX), while the Antibiotic resistance pattern was detected using disc diffusion assay. Class III biological safety cabinets are utilized for experiments involving antimicrobial agents.

Source of Silver Nanoparticles and Antibiotics

AgNPs (500 ppm) are in a solution containing 99.9% non-ionic crystalline AgNPs with a size of 6–12 nm. It was obtained from Saudi Arabia (<https://saudi-arabia-ksa.desertcart.com/products>). Analytical grade Amoxicillin powder was obtained from Mike's Falconry Supplies (20185 SE Seibert Lane, Sandy). The two previous materials were prepared in appropriate concentrations, varying between 1.0 to 100 µg/ml in sterile distilled water.

Plant Essential Oil

Healthy samples of *Cymbopogon schoenanthus* were collected from Taif city, Kingdom of Saudi Arabia, during the winter of 2024 in the morning when the oils were most concentrated. All plant materials were identified at the Biology Department, Faculty of Science, KAU, Jeddah. A voucher specimen was deposited in the Faculty of Science herbarium. The essential oil of *C. schoenanthus* was extracted through steam distillation of fresh leaves. The steam carried the vaporized essential oils, which were then condensed and carefully separated from the hydrosol using a pipette and stored in a dark glass bottle for chemical analysis using NMR analysis at the Central Lab, Faculty of Pharmacy, Cairo University, Egypt.

Measuring Biofilm Formation

The tested bacteria were cultivated in Tryptone Soy Broth medium, and 200 µl of overnight bacterial culture (0.5 McFarland standard) was inoculated to a well of microtiter plate containing 800 µl of Luria-Bertani broth. Plates were incubated at 37°C for 4, 8, 12, 16, 20, and 24 hrs to allow biofilm formation. After incubation, the medium was removed, and the wells were rinsed with phosphate-buffered saline to eliminate non-adherent cells. Biofilm was measured using the crystal violet method after being stained with 1 ml of 0.1% crystal violet solution for 15 minutes. Excess dye was washed off with distilled water, and the bound crystal violet was solubilized in one ml of ethanol (95%), and the absorbance of the solution was measured at 640 nm using a microplate reader (model DNM-9602, Wincom, Hunan) as described by Jastaniah et al.²⁰

Inhibition of Biofilm Viability with Ag Nanoparticles, Antibiotics, and Plant Oil

The experiment focused on targeting antibiotic-resistant bacteria before biofilm formation. The ability of Ag nanoparticles, Amoxicillin, and plant oil of *C. schoenanthus* to prevent biofilm formation was assessed using a crystal violet staining method. Bacterial inoculum was prepared from a 24-hour Tryptone Soy Broth culture, and the suspensions were adjusted to 0.5 McFarland turbidity standards (1.5×10^8 CFU/mL) in a 96-well microplate. Bacteria were treated with Ag nanomaterial, plant oil, and Antibiotic (5 µg/ml), and their mixture with AgNPs (1:1 v/v), and the plates were incubated at 37°C for different incubation periods, 0 to 24 hrs. The quantity of biofilm was measured. After incubation, plates were stained, and the absorbance was measured to quantify biofilm viability using the crystal violet staining method described before.²⁰ The percentage of biofilm inhibition was detected compared to the control (cells without inhibitory effects).

Determination of Minimum Inhibitory Concentration (MIC)

To determine the MIC of AgNPs alone or with antibiotics or plant oil, a serial dilution method was performed in 96-well microtiter plates. Different concentrations of each tested agent (1–10 µg/ml) were prepared and tested against the bacterial pathogens using the microdilution method following the guidelines of the Clinical and Laboratory Standards Institute. The lowest concentration that inhibited visible growth was recorded as the MIC.²¹

Cytotoxicity Test

The cytotoxicity of AgNPs alone or with antibiotics or plant oil was assessed by *Artemia salina* larvae (Brine shrimp) as test organism and determining their LD₅₀ values, which indicate the lethal concentration to 50% of the population. The nanoparticles alone (1–10 µg/ml) or with either Amoxicillin or *C. schoenanthus* oil (1:1 v/v) were prepared at varying concentrations, and their effects on *Artemia* viability were measured after a 24-hrs incubation period. Using Brine Shrimp Hatchability Assay, the concentration resulting in 50% inhibition was calculated.²²

Evaluation of the Antibacterial Activity of Nanoparticles Alone and with Antibiotic or Plant Oil

The antibacterial efficacy of the AgNPs was assessed using the agar well diffusion method against four bacterial pathogens. Briefly, the tested bacterial strain was spread on agar plates, and wells were created (5 mm) with sterile cork polar to add the nanoparticles, tested antibiotic, or plant essential oil (100 µl) of the stock solution containing (5 µg/ml). The antibacterial activity of the mixture of 1:1 v/v nanoparticles and either plant oil or antibiotic was also detected. Plates were incubated at 37°C for 24 hrs, and inhibition zones were measured (mm) to determine the tested material's antibacterial activity.²³

Exopolysaccharides Content of *K. pneumoniae* biofilm

The effect of AgNPs with either antibiotic or plant oil on the Exopolysaccharide content of *K. pneumoniae* biofilm was examined. The sample preparation involved adding 2.5 ml of double-concentrated tryptic soy broth (2X) in a test tube, 0.5 ml of the tested bacteria, and 2 ml of either antibiotic, nanoparticles, or plant oil (3 µg/ml, less than the MIC). The effect of a 1:1 mixture of the nanoparticles and antibiotic or nanoparticles and plant oil was tested. All samples were incubated for 24 hrs. After incubation, bacterial cells were removed by centrifugation, and EPS in the supernatant was extracted using two volumes of chilled absolute ethanol and incubated for another 24 hrs at 4°C to precipitate.²⁴ The resulting EPS pellet was dried at room temperature, and 0.05 mg of the EPS pellet was dissolved in 0.5 ml of deionized water. The total extracellular carbohydrate content was quantified using the phenol-sulfuric acid method by measuring absorbance at 495 nm using a visible light spectrophotometer.^{25,26} The EPS from untreated bacteria served as a control. The EPS inhibition percentage was estimated using the following formula:

$$\text{Inhibition percentage} = (1 - \text{concentration of EPS of control (mg/l)} / \text{concentration of EPS with Inhibitor (mg/l)}) \times 100$$

Statistical Analysis

Statistical analysis was conducted using Statistical Software, *t*-test and a one-way analysis of variance (ANOVA) to determine the significance between treatment and control, and within the different treatments. Data were reported as mean ± standard deviation (SD) of three independent experiments, and a *P*-value of <0.05 was considered statistically significant.

Results

The antibiotic resistance patterns of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* showed that all the tested bacteria were resistant to at least two types of antibiotics, except for *K. pneumoniae*, which was more resistant overall. It was resistant to Nalidixic Acid, Nitrofurantoin, Cephalothin, and Norfloxacin (Table 1). All bacterial isolates were screened for biofilm formation using the Crystal violet stain, and biofilm formation increased over time, reaching a maximum at about 16 hours. *K. pneumoniae* forms a strong biofilm, followed by *E. coli* and *P. aeruginosa*, and finally *S. aureus* (Figure 1). *K. pneumoniae* was chosen for more detailed studies.

Cymbopogon schoenanthus (Table 2) was collected and identified, and the plant oil was extracted to detect its effect on bacterial growth and biofilm formation. The metabolite profiles of *Cymbopogon* oil were characterized via NMR and the NMR spectra was analyzed for characteristic peaks corresponding to specific functional groups in the compounds which belonging to various classes. The results from NMR analysis was compared with literature values and standard references to confirm and identity the presence of defined metabolites like terpenes, alcohols geraniol, and aldehydes (Figure 2).

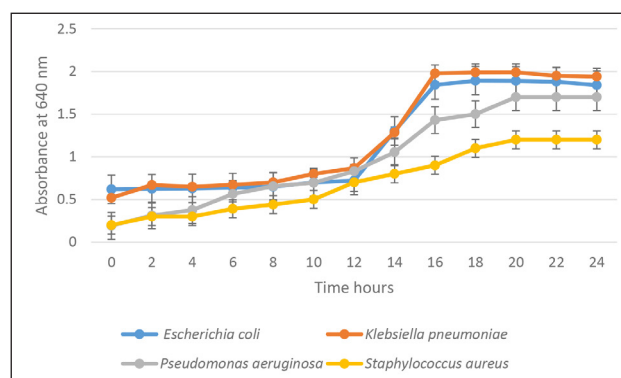


Fig. 1 The biofilm formation by the four selected bacterial isolates, grown under normal growth conditions.

Table 1. The antibiotic-resistant patterns of the studied bacterial pathogens, detected by the diameter of inhibition zone (mm) ± SD

Pathogenic bacteria	The tested antibiotics					
	Nalidixic Acid (30 µg)*	Nitrofurantoin (30 µg)	Cephalothin (30 µg)	Ampicillin (25 µg)	Cotrimoxazole (25 µg)	Norfloxacin (10 µg)
<i>Escherichia coli</i>	25.5 ± 0.4	25.5 ± 0.7	ND	ND	20.9 ± 3.0	25.0 ± 0.3
<i>Klebsiella pneumoniae</i>	ND	ND	ND	17.9 ± 0.9	15.8 ± 0.6	ND
<i>Pseudomonas aeruginosa</i>	ND	ND	23 ± 0.5	25.7 ± 3.0	25.7 ± 0.8	17.2 ± 0.3
<i>Staphylococcus aureus</i>	19.6 ± 0.3	25.3 ± 0.7	ND	ND	15.6 ± 0.8	20.0 ± 0.8

*µg/disc. ND: not detected.

The AgNPs (Ag nano), antibiotic, and plant essential oil exhibited strong inhibitory activity against all tested bacteria; as the bacterial index ranged from 20.25 to 20.57 mm; however, the differences were not significant at $P \leq 0.05$ (Table 3).

Moreover, all the tested bacteria were inhibited by AgNPs with inhibition zone diameters (IZD) ranged from 17–23 mm and the maximum activities were against *P. aeruginosa* and *E. coli* as the IZD were 24 and 23 mm, respectively. While the lowest activity was recorded against both *K. pneumoniae* and *S. aureus*, presence of antibiotic or plant oil enhanced the inhibitory activity and significantly increased the IZD to 23 and 27 mm for *K. pneumoniae* and 27 and 29 mm for *S. aureus* (Table 3). The highest significant inhibitory effects were noted for AgNPs in combination with either the antibiotic or plant oil on the growth of the bacterial pathogens compared to the control (AgNPs alone), with the highest inhibitory activity recorded against *E. coli* for the mixture of AgNPs and plant oil (IZD, 33.0 mm).

Table 2. The used plant for oil extraction, common name, and family

	Scientific name	Common name	Plant family	Extracted part
	<i>Cymbopogon schoenanthus</i> L.	Camel grass	Poaceae	Leaf

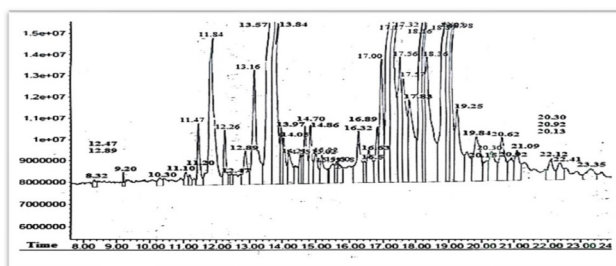


Fig. 2 The chemical pattern of *C. schoenanthus* oil detected by NMR analysis.

The MIC of the Ag nanoparticles, antibiotic, or plant oil was calculated, revealing that the lowest MIC (3.0 µg/ml) was for the mixture of AgNPs and plant oil against *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*. The recorded MIC values for the four tested bacterial pathogens ranged from 4.5 to 8 µg/ml for the Ag nanoparticles, from 4 to 4.5 µg/ml for the combination of AgNPs and antibiotic, and from 3 to 4 µg/ml for the mixture of AgNPs and plant oil (Table 4). MIC of the mixture of AgNP sand antibiotic or AgNPs and plant oil was lower compared to AgNPs alone (control), which means a synergetic activity.

The calculated LD₅₀ was 10 µg/ml for the Ag nanoparticles, while it decreased to below 10 µg/ml when combined with the antibiotic or plant oil (Table 5).

Figure 3 shows the effect of AgNPs alone or with either antibiotic or plant oil on biofilm formation by *K. pneumoniae* after different incubation periods. The maximum biofilm inhibition was recorded after 18 hrs of incubation, while *K. pneumoniae* (control treatment) showed maximum biofilm formation (**Figure 3**). It was clear that *K. pneumoniae* formed a strong biofilm, which was inhibited by 51.66, 63.50, and 62.62% by AgNPs alone, AgNPs + antibiotic, and AgNPs + plant oil, respectively (**Table 6**).

The effect of AgNPs and antibiotic and AgNPs and plant oil on EPS formation by *K. pneumoniae* was summarized in Table 7. Maximum EPS inhibition (80%) was recorded by a mixture of AgNPs and plant oil, followed by treatment by

Table 4. The MIC of the tested materials for some pathogenic bacteria using the Broth Microdilution method

Pathogenic isolates	MIC of the nano alone or combined with antibiotics or plant oil (µg/ml)		
	AgNPs (control)	AgNPs and Amoxicillin	AgNPs and Plant oil
<i>Escherichia coli</i>	5.5 ± 0.5	4.0 ± 0.5*	4.0 ± 0.5*
<i>Klebsiella pneumoniae</i>	6.0 ± 0.5	4.5 ± 0.5*	3.0 ± 0.5*
<i>Pseudomonas aeruginosa</i>	4.5 ± 0.5	4.0 ± 0.5	3.0 ± 0.5*
<i>Staphylococcus aureus</i>	8.0 ± 0.5	4.5 ± 0.5*	3.0 ± 0.5*

*Significant result compared to control.

Table 3. The antimicrobial activities (diameter of inhibition zone, mm) of Ag nano alone or combined with either Antibiotic or *C. schoenanthus* oil on four bacterial pathogens

Pathogenic isolates	Tested materials					One way anova test
	AgNPs (Control)	Antibiotic (Amoxicillin)	Plant oil [#]	AgNPs and Antibiotic	AgNPs and Plant oil	
<i>Escherichia coli</i>	23 ± 1.71	20 ± 2.11	21 ± 2.90	23 ± 2.24	33 ± 2.58*	**
<i>Klebsiella pneumoniae</i>	17 ± 0.61	20 ± 1.34*	21 ± 2.21*	23 ± 2.20*	27 ± 2.81*	**
<i>Pseudomonas aeruginosa</i>	24 ± 0.71	21 ± 1.34*	21 ± 0.65*	25 ± 0.43	25 ± 0.51	**
<i>Staphylococcus aureus</i>	18 ± 0.73	20 ± 1.13	20 ± 0.85	29 ± 2.33*	27 ± 0.94*	**
Bacterial Index**	20.50	20.25	20.75	25.0*	28.0*	
One way anova test	<i>P</i> = 0.043**	<i>P</i> = 0.392	<i>P</i> = 0.793	<i>P</i> = 0.0432**	<i>P</i> = 0.0503**	

*Significant result using t-test compared to control, **The inhibitory effect on different bacteria (last row) or the effect of different treatment on bacteria (last column) was significant using one way anova test at $P \leq 0.05$, #The calculated mean value of inhibition zone diameters for the four tested bacterial pathogens, ##: *C. schoenanthus* oil

AgNPs and antibiotic (54% inhibition), and finally AgNPs alone (39.64%). The quantity of EPS in the biofilm decreased in the presence of nanomaterial, and the decrease was apparent in the presence of antibiotics or plant oil. The inhibition stays almost constant up to 12 hrs of bacterial incubation, then starts a sharp reduction till 15 hrs, followed by a slight increase, and reaches its maximum content after 20 hrs of growth (Figure 4). A summary of the antimicrobial, MIC, and antibiofilm activities of AgNPs alone or associated with antibiotic or *C. schoenanthus* oil and their effects on *K. pneumoniae* are summarized in Figure 5.

Table 5. Toxicity (% of cell mortality) of different concentrations of Ag nano, Ag nano and antibiotic and Ag nano and plant oil using brine shrimp lethally test

Tested materials	Concentration of tested material (µg/ml)				
	2.5	5.0	7.5	10	LD ₅₀ (µg/ml)
AgNPs (control)	0.0*	10 ± 0.1	10.1 ± 3.1	50.0 ± 5.1	10.0
AgNPs and Antibiotic	0.0	0.0	10.4 ± 2.1	40.4 ± 3.2	>10
AgNPs and Plant oil	0.0	0.0	10.4 ± 2.8	30.1 ± 2.4	>10
Negative control (Dist. water)	0.0	0.0	0.0	0.0	ND

*No cell mortality. ND: not detected.

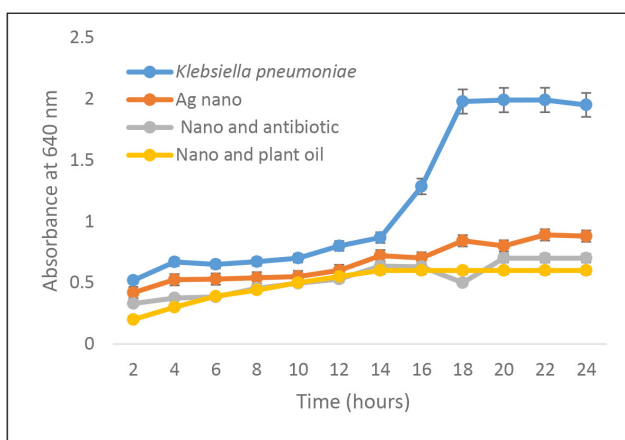


Fig. 3 Effect of Ag nano alone or with either antibiotic or plant oil on biofilm formation by *Klebsiella pneumoniae* in relation to control (bacteria alone) after different incubation periods.

Table 6. Effect of nano alone or with either antibiotic or plant oil on biofilm formation after by *Klebsiella pneumoniae* 24 hrs

Treatment	Biofilm (A _{690nm})	Biofilm inhibition (%)	Degree of biofilm
Control 1 (not treated)	1.99	0.0	Strong
AgNPs (Control 2)	0.984 ± 0.5*	51.66	Weak
AgNPs and Antibiotic	0.753 ± 0.5**	63.50	Weak
AgNPs and Plant oil	0.743 ± 0.5**	62.67	Weak

*Significant results compared to control 1 at $P \leq 0.05$, *Significant results compared to control 2 at $P \leq 0.05$.

Table 7. Impact of AgNPs alone or with either antibiotic or plant oil on Exopolysaccharide (EPS) production by *K. pneumoniae* after 24 hrs of incubation

Treatment	EPS (OD ₄₉₀ nm)	Production of EPS (%)	Inhibition (%)
No treatment (Control 1)	1.551 ± 0.25	100	00.00
AgNPs (Control 2)	0.951 ± 0.22	61.36	39.64*
AgNPs and Antibiotic	0.719 ± 0.23	46.00	54.00**
AgNPs and Plant oil	0.289 ± 0.17	20.00	80.00**

*Significant result compared to control 1 and *Significant result compared to control 2 at $P \leq 0.05$.

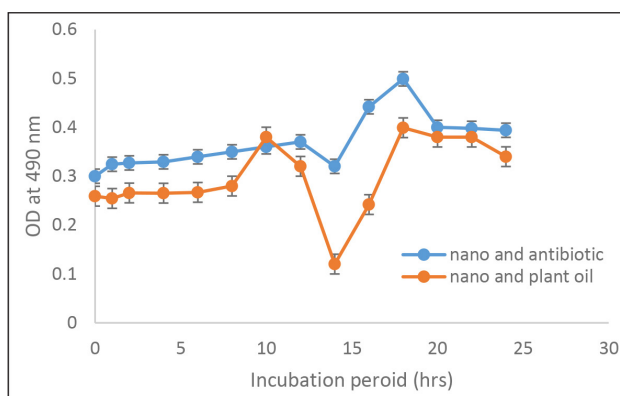


Fig. 4 Effect of AgNPs with either antibiotic or plant oil on Exopolysaccharide production by *Klebsiella pneumoniae* after different incubation periods.

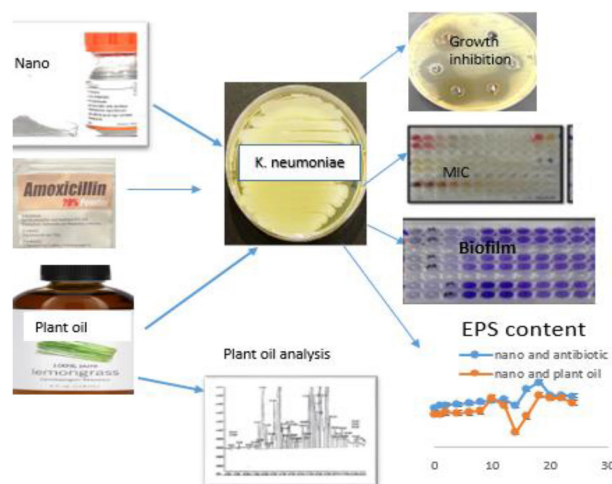


Fig. 5 Summary of the effects of AgNPs particles alone or associated with antibiotic or *C. schoenanthus* oil on growth, MIC value, biofilm formation, and EPS production by *Klebsiella pneumoniae*.

Discussion

These four bacterial pathogens, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* are difficult to treat because of their increased resistance to antibiotics and strong biofilm formation, which is a critical virulence factor for several bacterial pathogens.²⁷ The resistance of bacteria within biofilms reaches 1,000 times greater than that of free cells.^{2,28–30}

Biofilm formation is an important feature in the four studied bacterial pathogens, and maximum activity was recorded for *K. pneumoniae*. Preventing growth and biofilm formation with effective antimicrobial agents is essential; however, current methods are limited due to the presence of exopolysaccharide, which makes cells within biofilm show high resistance to antimicrobial agents.^{31–33} Thus, there is an urgent need for new treatments like Nanoparticles and plant essential oil beyond antibiotics to control bacterial biofilms.

AgNPs are effective agents to inhibit both resistant bacterial growth and biofilm formation which is in accordance with other studies.^{33,34} Until now, limited number of metallic nanoparticles have exhibited bactericidal properties.³⁵ For instance, AgNPs show broad-spectrum antimicrobial activity against various pathogens, including *K. pneumoniae*, and the mechanisms of action involve disrupting bacterial cell walls, generating reactive oxygen, and interfering with the formation of biofilm and cell membrane.^{12,36} For instance, AgNPs can damage bacterial DNA, proteins, plasma membranes, EPS, and biofilm architecture, leading to oxidative stress and cell death³⁷ (Khan et al., 2019). Furthermore, AgNPs can disrupt the signaling molecules involved in quorum sensing, impairing the ability of bacteria within biofilm to coordinate.^{33,38} Salunke et al. (2014)³⁹ found that AgNPs inhibited the growth of *Acinetobacter baumannii*, *E. coli*, and *S. aureus* at low concentrations, achieving disruption rates of 88%, 67%, and 78% for the biofilms of these bacteria, respectively. Similarly, gold nanoparticles have demonstrated effective antibiofilm activities.⁴⁰ Its effects depend on properties such as size and surface charge, which are critical in determining its interaction with bacterial cells. Similarly, Nazir et al.¹ studied the roles of AgNPs in the reduction of EPS production and weakening the structural integrity of biofilms, which makes bacteria more susceptible to antibiotics and enhances the effectiveness of these agents in penetrating deeper into biofilm.⁵ The possibility of formulating new antimicrobial agents that combine the strengths of AgNPs and traditional antibiotics or plant oil is possible now and this highlight the need for careful evaluation of dose- response relationships to ensure patient safety.⁶

The results of this study indicate that AgNPs can significantly inhibited the growth of *K. pneumoniae*, *E. coli*, *P. aeruginosa*, and *S. aureus* and enhance the effectiveness of Amoxicillin, which acts against both Gram-positive and Gram-negative bacteria by inhibiting the biosynthesis and repair of the bacterial cell wall in addition to preventing the formation of biofilms.³³ This is particularly relevant for pathogens like *K. pneumoniae*, which is known for the ability to form biofilms, resistance to multiple antibiotics, and association with various infections, including pneumonia, urinary tract infections, and sepsis, particularly in healthcare settings; therefore, innovative strategies are crucial for combating this pathogen.⁴¹ Combining AgNPs with conventional antibiotics may enhance the inhibitory activities of nanomaterials due to enhance antibiotic uptake by cells, disrupt bacterial growth and biofilm formation which reduce the MIC of amoxicillin against some pathogens.^{3,42,43} This synergism may also result from the disruption of biofilm structures, allowing for better penetration of antimicrobial agents.⁴⁴ Zhang et al.^{13,14} reported that AgNPs combined with antibiotics like ciprofloxacin induced a more significant reduction in both growth and biofilm formation of *P. aeruginosa* compared to either agent alone. Koo et al. (2017)⁴⁵ demonstrated that certain polymeric

nanoparticles can disrupt signaling in *Streptococcus mutans*, leading to decreased biofilm formation and increased susceptibility to antibiotics. Very limited studies were found about the action of both AgNPs and antibiotics.

Due to the rise of antibiotic resistance in bacteria, it is necessary to explore alternative strategies to combat bacterial infections. Among these innovative approaches, the combination of AgNPs and plant oils has emerged as a promising avenue for inhibiting bacterial growth and disrupting biofilm formation. Certain plant oils can inhibit bacterial growth, block bacterial biofilm, and disrupt membrane integrity, quorum sensing, and bacterial communication.^{9,18} The combined effects of nanoparticles and plant oil on bacterial physiology may lower the minimum inhibitory concentrations and the side effects associated with high concentrations of nanoparticles. Plant oils are typically less toxic compared to antibiotics and effectively dissolve biofilms of *P. aeruginosa* and *Staphylococcus* by affecting membrane permeability and EPS within biofilms.^{2,46,47} Mathpal et al. (2015)⁴⁸ reported excellent antibacterial activity of *C. schoenanthus* essential oil against *S. aureus* and *E. coli*, and it has potent antifungal activity against *Candida* spp.⁴⁹ The oil from *C. schoenanthus* is enriched in neral, geranial, aconitic acid, isoorientin, caffeic acid, linalool, myrcene, citramalate, and citral, in addition to having a high phenolic content.^{17,50} Moreover, essential oils with nanoparticles are promising strategies that minimize the side effects of nanomaterials, enhance antibacterial effects, and may improve the ability to combat biofilms. Similarly, the essential oil from *Ocimum tenuiflorum* prevents *S. aureus* adhesion while TiO_2 inhibits growth and biofilm formation by *S. aureus*, *S. epidermidis*, and *E. coli*.⁵¹ Additionally, there were very few studies on the combined effects of plant oil and Ag nanoparticles; hence, more research was required to remedy this information leak.

Similarly, plant oils are generally regarded as safe and have been used traditionally for medicinal purposes, making them suitable candidates for use in combination therapies. They are known for their antimicrobial properties due to the presence of bioactive compounds such as phenols, terpenoids, and fatty acids demonstrated effectiveness against a wide range of microbial pathogens. These compounds can disrupt microbial membranes and inhibit enzymatic pathways critical for bacterial survival. When AgNPs are combined with plant oils, the resulting synergy can lead to enhanced antibacterial effects and allows for the use of lower concentrations of both NPs and plant oils and reducing their side effects.³⁷ Traditional antibiotics often fail to penetrate biofilms effectively, leading to persistent infections. The incorporation of NPs and plant oils can increase antibiotic penetration and disrupt biofilms. Additionally, essential oils can prevent biofilm formation by inhibiting quorum sensing communication, important for biofilm development.

The toxicity was measured using *A. salina* as a test organism which is a well-established practice in toxicological testing. It provides a reliable method for comparing results across different studies due to its sensitivity to a wide range of toxic compounds. Furthermore, *A. salina* is easy cultured and maintained in laboratory settings, making it a popular choice for assessing toxicity. Furthermore, the size and surface chemistry of nanoparticles significantly impact their toxicity, and smaller particles are more toxic. The results of this study showed no significant toxicity at lower

levels of AgNPs, with moderate toxicity observed at higher concentrations. Generally, compared to traditional antibacterial agents or plant oils, NPs exhibit high environmental toxicity.⁵² As a result, when NPs are combined with antibiotics or essential oils, the overall toxicity is lower than with nanoparticles alone.

Conclusion

The synergistic effects of AgNPs to enhance the effectiveness of Amoxicillin or plant oil could significantly improve treatment outcomes and provide a viable approach for biocontrol of resistant bacterial strains in clinical settings. Further research

is needed to elucidate the mechanisms of action and to evaluate the long-term biocompatibility of such combinations.

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Conflict of Interest

The author has revealed no conflicts of interest throughout this study. ■

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