

# Evaluation of the anti-biofilm potential of biosynthesized silver nanoparticles against *Escherichia coli* and *Klebsiella pneumoniae* isolated from urinary tract infection patients in Zahedan, 2023

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## Abstract

Urinary tract infections (UTIs) are among the most prevalent infections worldwide and a significant contributor to morbidity and mortality, with bacterial biofilm formation playing a critical role in their persistence and resistance to treatment. This study evaluated the biofilm-forming ability of *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) isolates obtained from UTI patients and investigated the anti-biofilm activity of biosynthesized silver nanoparticles (AgNPs) against biofilm-producing strains. Bacterial identification and confirmation were performed using selective culture media and polymerase chain reaction (PCR) assays. Biofilm production and the anti-biofilm efficacy of AgNPs were assessed through a quantitative microtiter plate assay, while antimicrobial susceptibility testing was conducted using standard antibiotic disc diffusion methods. The results revealed a high prevalence of biofilm-positive strains, with *E. coli* exhibiting a higher proportion of strong biofilm producers compared to *K. pneumoniae*. Antibiotic resistance analysis demonstrated significant resistance in both uropathogens, particularly to beta-lactam antibiotics. The synthesized AgNPs exhibited potent anti-biofilm activity, effectively inhibiting biofilm formation in both bacterial species. Interestingly, despite the higher biofilm-forming capacity of *E. coli*, AgNPs displayed greater inhibitory effects on *E. coli* biofilms compared to those of *K. pneumoniae*. These findings underscore the clinical relevance of biofilm formation in UTI pathogenesis and highlight biosynthesized AgNPs as a promising therapeutic agent for managing biofilm-associated infections. This study emphasizes the need for innovative approaches to combat biofilm-related antimicrobial resistance and improve UTI treatment outcomes.

**Keywords:** Urinary tract infection, Uropathogenic *Escherichia coli*, *Klebsiella pneumoniae*, Anti-biofilm activity, Silver nanoparticles

## 1. Introduction

Urinary tract infections (UTIs) are among the most prevalent hospital-acquired infections, affecting approximately 150 million people globally each year [1]. These infections, caused primarily by bacterial pathogens, can affect various parts of the urinary system, including the urethra, bladder, ureters, and kidneys [1]. UTIs are particularly common in women, with 50% experiencing at least one infection in their lifetime and 25% suffering from recurrent episodes. Recurrent UTIs, which often occur within weeks or months of the initial infection, present significant challenges in clinical management [2]. They are associated with increased morbidity and mortality, exacerbated by the rise of antibiotic resistance, particularly among Gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* [3]. The primary causative agent of UTIs is uropathogenic *Escherichia coli* (UPEC), responsible for 70–80% of cases [1]. UPEC strains are extraintestinal pathogens capable of colonizing sites such as the bloodstream, central nervous system, and urinary tract, with biofilm formation being a key virulence mechanism [4].

Biofilms enhance bacterial resistance to antibiotics and the host immune response, contributing to persistent infections and complications, particularly in cases involving medical devices such as catheters [5]. Similarly, *K. pneumoniae* is a significant uropathogen, presenting treatment challenges due to its multiple virulence factors, including biofilm formation, polysaccharide capsule production and secretion of hydrolytic enzymes [6]. Both UPEC and *K. pneumoniae* rely on their ability to adhere to host tissues, facilitating colonization, biofilm development, and invasion of the bladder epithelium, which triggers inflammatory responses [7]. The emergence of extended-spectrum beta-lactamase (ESBL)-producing strains of UPEC and *K. pneumoniae* has further complicated UTI treatment. These strains exhibit high resistance to beta-lactam antibiotics, underscoring the need for novel therapeutic approaches [8]. Understanding biofilm formation in these pathogens is critical for developing effective treatments and designing vaccines to prevent and control UTIs caused by these bacteria. Antibiotics remain the primary treatment for UTIs, but the growing prevalence of multidrug-resistant uropathogens has significantly reduced the efficacy of standard therapies. This not only complicates treatment but also contributes to the spread of antibiotic-resistant bacteria in healthcare settings [9]. Over the past decade, uropathogens have developed various resistance mechanisms, resulting in decreased susceptibility to first-line antibiotics. Given the central role of biofilms in mediating antibiotic resistance, there is increasing interest in identifying therapeutic strategies that can inhibit biofilm formation at different stages [10]. Recent research has shifted toward alternative therapeutic strategies due to the limitations of conventional antibiotics and the side effects of chemical antimicrobial agents. Advances in nanotechnology over the last decade have highlighted the antimicrobial potential of metal nanoparticles. Studies have demonstrated that inorganic nanoparticles with sizes below 100 nm exhibit significant bactericidal activity [11]. The nanoscale size of these particles increases their surface activity, enhancing interactions with bacterial cells. Electrostatic attraction between negatively charged bacterial membranes and positively charged nanoparticles facilitates their adherence to bacterial surfaces, leading to membrane disruption, leakage of intracellular contents, and cell death [12]. Nanoparticles composed of metals such as silver, gold, chromium, zinc, titanium, copper, magnesium, and their oxides have shown strong antimicrobial properties. Coating surfaces with nanoparticles can prevent biofilm formation by inhibiting bacterial adhesion, exerting direct antibacterial effects, or both. Additionally, combining nanoparticles with conventional antibiotics or other antimicrobial agents offers a promising strategy to combat antibiotic resistance [13].

This study aims to address the urgent need for novel therapeutic approaches to combat biofilm-associated UTIs caused by multidrug-resistant pathogens. Specifically, it investigates biofilm production and antibiotic susceptibility in *E. coli* and *K. pneumoniae* strains isolated from patients with UTIs in Zahedan. Furthermore, the study evaluates the anti-biofilm activity of biosynthesized silver nanoparticles against strains with high biofilm production and antibiotic resistance. The findings of this research are expected to provide valuable insights into the potential use of silver nanoparticles as an alternative or adjunctive therapy for managing biofilm-associated UTIs.

## 2. Experimental

### 2.1. Materials and methods

#### 2.1.1. Isolation and identification of uropathogenic bacterial isolates

Between September 2023 and March 2024, 110 *Escherichia coli* (*E. coli*) isolates and 35 *Klebsiella pneumoniae* (*K. pneumoniae*) isolates were collected from patients diagnosed with urinary tract infections (UTIs) at Imam Ali, Social Security, Khatam, and Ali Asghar Children's Hospitals in Zahedan. These hospitals were selected for their diverse patient populations, enabling the collection of samples from patients of varying clinical characteristics, including both sexes, different age groups, and both inpatients and outpatients. Initial identification of the isolates was performed using selective differential media, including eosin methylene blue agar and MacConkey agar (Merck, Germany) [14]. Final confirmation of *E. coli* and *K. pneumoniae* was conducted using polymerase chain reaction (PCR) with primers specific to the *tufA* and *16S rRNA* genes [15]. Genomic DNA extraction for PCR analysis was carried out using the boiling method, following the protocol established by Kai-Larsen et al. [16]. PCR assays were conducted using an Eppendorf thermal cycler (Hamburg, Germany). All primers and reagents were sourced from SinaGen (Tehran, Iran). The PCR products were analyzed using agarose gel electrophoresis (1–2%) (Invitrogen, Norway) in 0.5% TBE buffer and stained with ethidium bromide (0.5 mg/mL) (Sigma, Germany). Visualization of the gels was performed using a gel documentation system (Biometra, Germany).

## 2.2. Biofilm production analysis

Biofilm production was quantitatively assessed for all confirmed bacterial isolates using the microtiter plate method, as described by O'Toole et al. [17]. The optical density (OD) of each well was measured at 570 nm using an ELISA reader (Stat Fax 2100). Based on the OD values, the isolates were categorized into strong, moderate, weak and negative biofilm producer groups.

## 2.3. Antibiotic resistance assay

The antibiotic susceptibility of biofilm-positive strains was evaluated against 11 commonly used antibiotics for UTI treatment using the standard disk diffusion method on Mueller-Hinton agar (Merck, Germany), following Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. The antibiotics tested included ampicillin (AM, 10 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CRO, 30 µg), ceftiofur (FOX, 30 µg), imipenem (IMP, 10 µg), gentamicin (GN, 10 µg), amikacin (AK, 30 µg), ciprofloxacin (CIP, 5 µg), levofloxacin (LEV, 5 µg), and sulfamethoxazole/trimethoprim (STX, µg), all sourced from Cypress Diagnostics (Belgium).

## 2.4. Biosynthesis of silver nanoparticles

Silver nanoparticles were biosynthesized using an aqueous extract of *Mesembryanthemum cordifolium*. The plant extract was prepared through maceration, where fresh leaves of the plant were washed thoroughly with distilled water, air-dried, and ground into a fine powder. The powdered material was then mixed with distilled water in a percolation funnel and allowed to extract bioactive compounds for 24–48 hours under controlled conditions [19]. The resulting extract was filtered through Whatman No. 1 filter paper to remove solid residues, yielding a clear aqueous extract rich in phytochemicals. To synthesize silver nanoparticles, a solution of silver nitrate ( $\text{AgNO}_3$ ) was prepared and mixed with the plant extract in varying ratios. The reaction mixture was stirred continuously at room temperature, and the formation of silver nanoparticles was visually confirmed by a color change from pale yellow to dark brown, indicating the reduction of silver ions ( $\text{Ag}^+$ ) to metallic silver ( $\text{Ag}^0$ ) [20]. The reduction process was facilitated by phytochemicals such as flavonoids, phenolic compounds, and terpenoids present in the plant extract, which acted as natural reducing and stabilizing agents. The reaction conditions, including pH, temperature, and the concentration of silver nitrate and plant extract, were optimized to control the size and morphology of the nanoparticles.

## 2.5. Characterization

The nanoparticles were further characterized using a UV-Vis spectrophotometer (Jenway 6715, UK) at wavelengths of 400–700 nm. The size, shape, and purity of the nanoparticles were determined using X-ray diffraction (XRD; D8-Advance, Bruker, Germany) and transmission electron microscopy (TEM; MIRA3, Tescan, Czech Republic) [21].

## 2.6. Antimicrobial activity assay

To evaluate the antimicrobial activity of the synthesized silver nanoparticles, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. A preliminary assessment of antimicrobial potency was conducted using the disk diffusion method by measuring the zones of inhibition [22]. The MIC and MBC were subsequently determined using the broth microdilution method in 96-well microtiter plates (Orange) [23].

## 2.7. Determination of anti-biofilm activity of synthesized nanoparticles

The anti-biofilm activity of the synthesized silver nanoparticles was assessed at a concentration equivalent to  $\frac{1}{2}$  MIC using a quantitative microtiter plate assay combined with crystal violet staining [17]. The efficacy of the nanoparticles in inhibiting biofilm formation in *E. coli* and *K. pneumoniae* strains was quantified by measuring the mean optical density (OD) of the treated wells ( $\text{OD}_t$ ), negative control wells ( $\text{OD}_{nc}$ ), positive control wells ( $\text{OD}_c$ ), and blank control wells ( $\text{OD}_s$ ). The percentage inhibition of biofilm formation was calculated using the formula:

$$\text{Inhibition of biofilm formation (\%)} = \frac{[(\text{OD}_c - \text{OD}_s) - (\text{OD}_t - \text{OD}_{nc})]}{[\text{OD}_c - \text{OD}_s]} \times 100$$

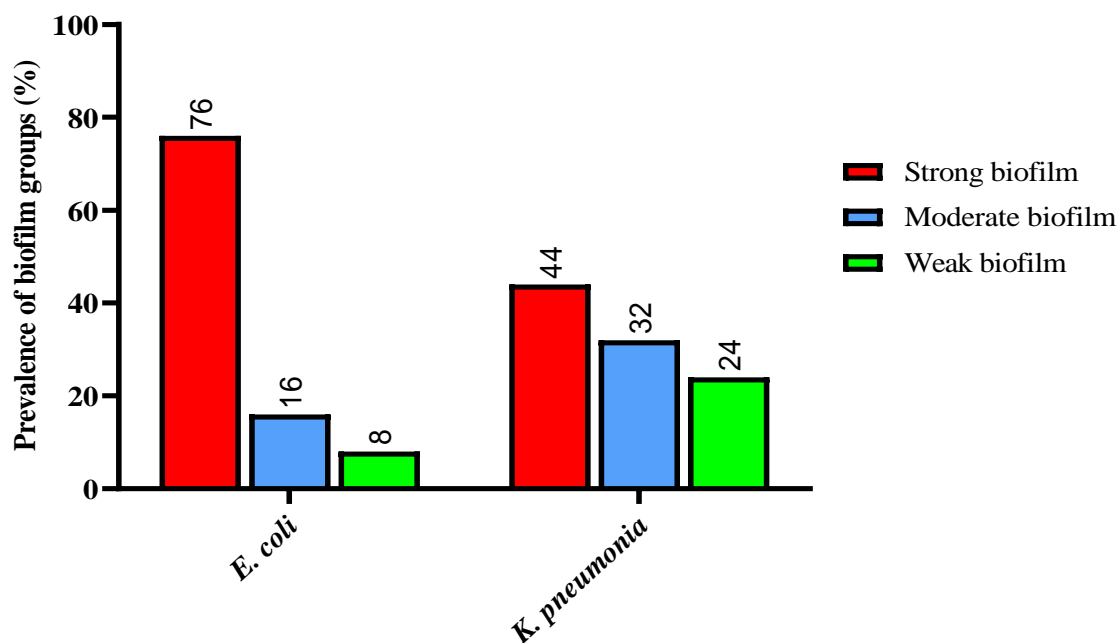
### 3. Results and discussion

#### 3.1. Identification of uropathogenic *Escherichia coli* and *klebsiella pneumoniae* isolates

A total of 110 *E. coli* and 35 *K. pneumoniae* isolates were initially cultured from patients with urinary tract infections (UTIs) in hospitals across Zahedan using MacConkey agar and eosin methylene blue (EMB) agar. Of these, 83 *E. coli* isolates (75%) and 31 *K. pneumoniae* isolates (89%) were preliminarily identified. PCR analysis using primers specific to each bacterial genus confirmed these results, verifying that all isolates belonged to the respective bacterial species.

#### 3.2. Determination of biofilm-forming ability

The biofilm-forming ability of the 114 confirmed strains was quantitatively assessed using the microtiter plate method and optical density (OD) measurement at 570 nm after 24 hours of incubation. Among the isolates, 51 *E. coli* strains (61%) and 25 *K. pneumoniae* strains (81%) demonstrated biofilm-forming capacity, while the remaining strains were classified as biofilm-negative. Based on the quantity of biofilm production, the strains were categorized into strong, moderate, and weak biofilm producers. Overall, *E. coli* strains exhibited a higher prevalence of biofilm production and a greater proportion of strong biofilm producers compared to *K. pneumoniae* strains (Fig. 1).

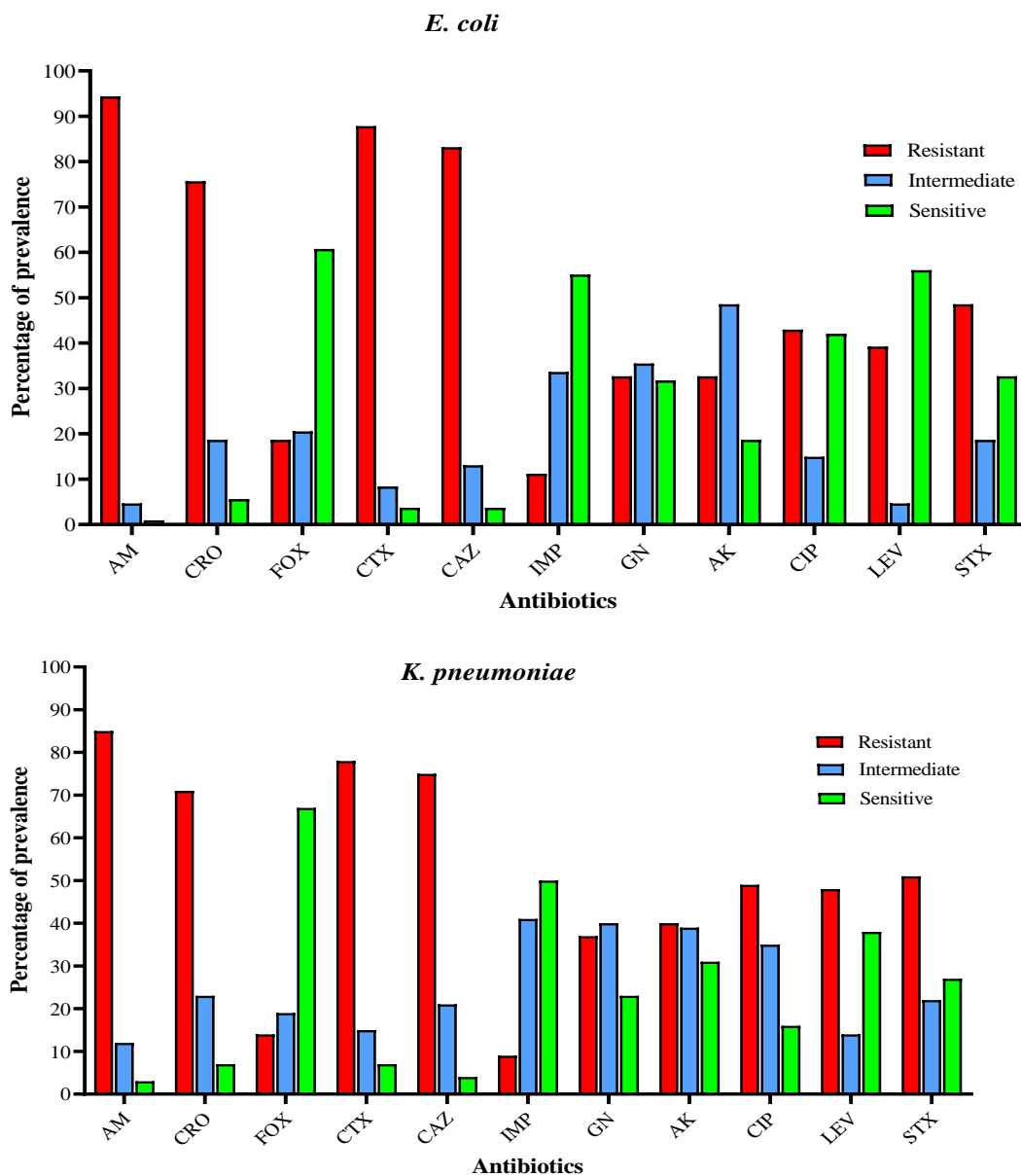


**Fig. 1.** Prevalence of strong, moderate, and weak biofilm groups among uropathogenic *E. coli* and *K. pneumoniae* strains.

One of the key characteristics of UTIs is their tendency to recur, particularly in women and children [1]. Recurrence is often attributed to the ability of uropathogens to form biofilms and intracellular bacterial communities. The adhesion of bacteria to uroepithelial cells initiates biofilm formation, providing protection against the hydrodynamic forces of urine and triggering host signaling pathways [24]. Biofilms are composed of a dense extracellular matrix that shields bacteria from antimicrobial agents and host defenses, leading to persistent and chronic infections that are difficult to treat [25]. Notably, biofilm-producing bacteria exhibit antibiotic resistance levels 500 to 5000 times higher than their planktonic counterparts [26]. These findings highlight the importance of investigating biofilm formation and developing rapid diagnostic methods for biofilm-producing bacteria to guide appropriate treatment strategies, prevent infection recurrence, and avoid severe complications such as pyelonephritis and prostatitis.

### 3.3. Antibiotic resistance determination

Antimicrobial susceptibility testing of biofilm-positive strains revealed high levels of resistance to several antibiotics commonly used to treat UTIs. The highest resistance was observed against ampicillin, cefotaxime, ceftazidime, and ceftriaxone. Notably, *E. coli* strains exhibited higher resistance rates to most antibiotics compared to *K. pneumoniae* strains (Fig. 2).

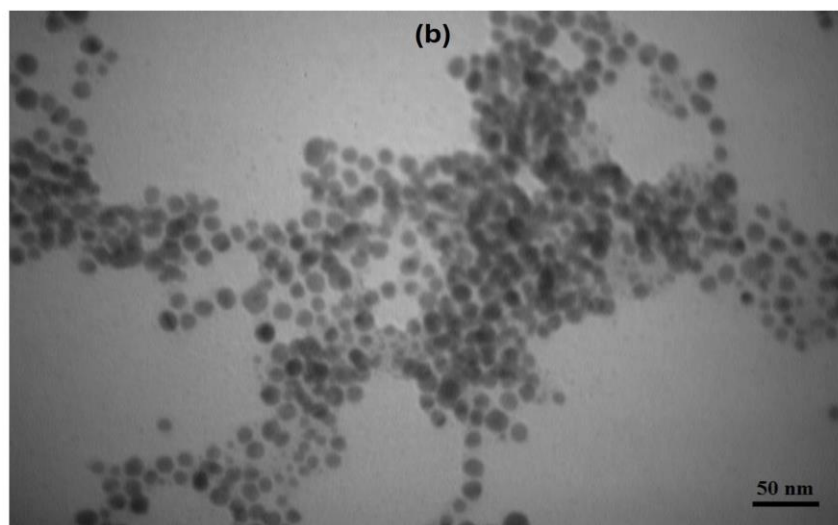
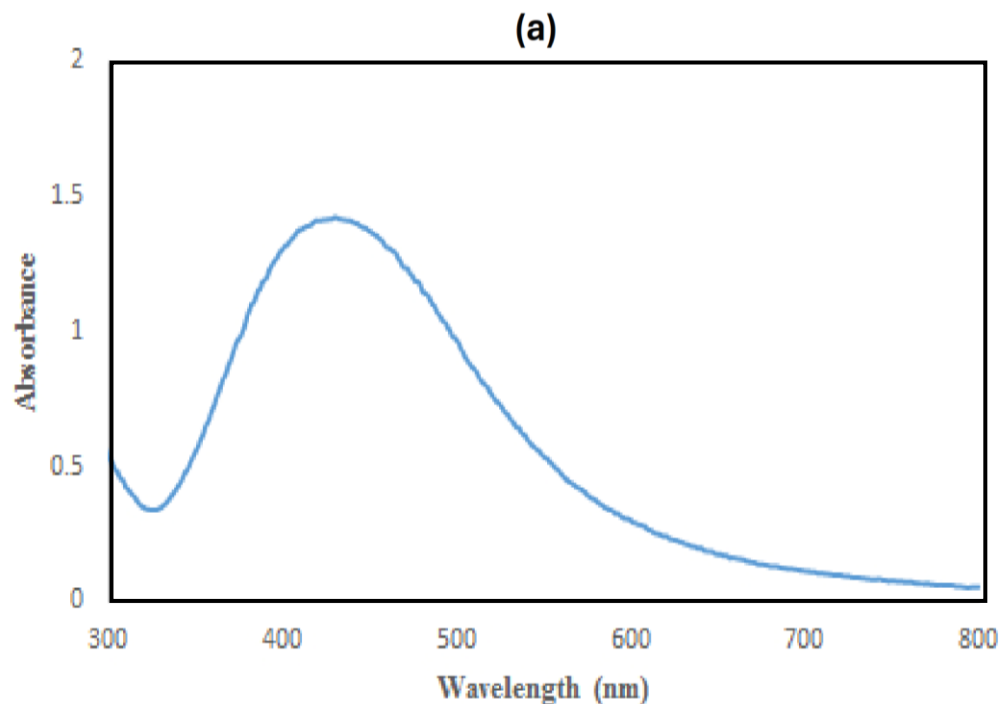


**Fig. 2.** Prevalence of resistant, intermediate, and susceptible groups in *E. coli* and *K. pneumoniae* strains against the tested antibiotics.

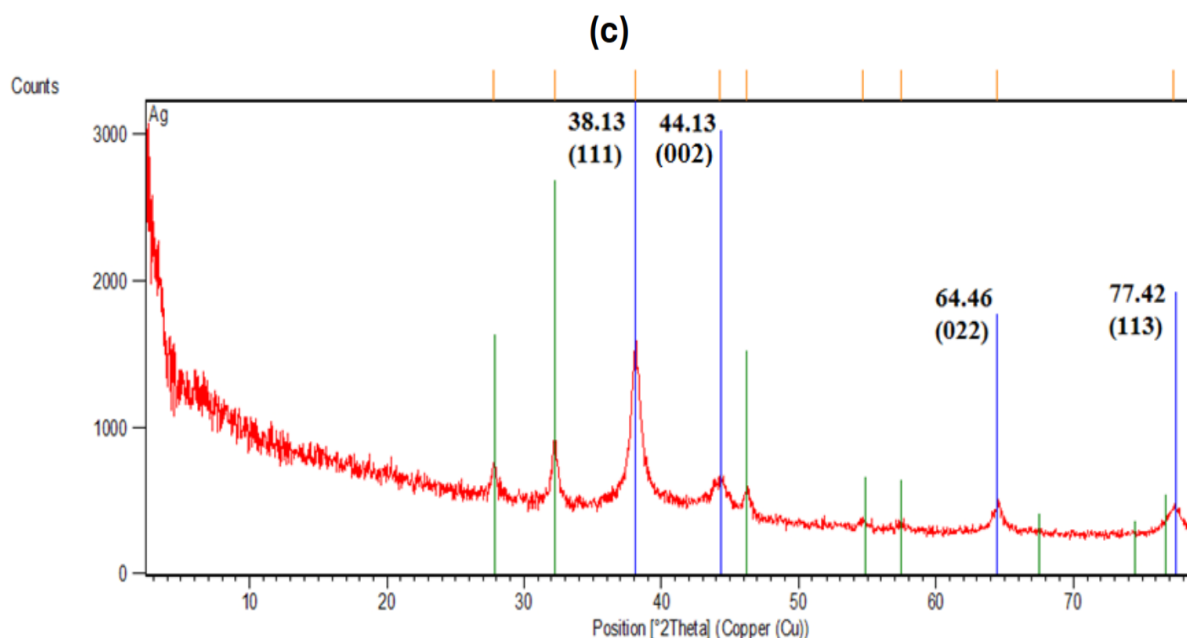
Given the increasing prevalence of antibiotic resistance and the variable susceptibility of uropathogens in different regions, studying the resistance patterns of biofilm-producing bacteria is essential. In this study, strains with strong biofilm-forming capacity were highly prevalent, suggesting that these bacteria have adapted to form robust biofilms as a defense mechanism against the immune system and antibiotics. This finding emphasizes the need for alternative therapeutic strategies to overcome biofilm-associated resistance.

### 3.4. Confirmation of silver nanoparticle synthesis

The successful synthesis of silver nanoparticles was confirmed through UV-Vis spectrophotometry, transmission electron microscopy (TEM), and X-ray diffraction (XRD) analysis (Fig. 3). UV-Vis spectrophotometry revealed a characteristic absorption peak of surface plasmon resonance associated with nanoparticles, confirming their formation in the presence of the plant extract. TEM analysis indicated that the nanoparticles had a diameter of 40–60 nm, while XRD analysis revealed sharp peaks, confirming the crystalline structure and successful synthesis of the nanoparticles.



↓ continuing



**Fig. 3.** UV-Vis spectrophotometry, transmission electron microscopy, and X-ray diffraction analysis of synthesized silver nanoparticles.

Silver nanoparticles have emerged as promising antimicrobial agents due to their unique properties, including a large surface area, strong antibacterial activity, and the ability to penetrate biofilm structures. AgNPs exert their antimicrobial effects by disrupting bacterial cell walls and interfering with essential cellular functions such as protein synthesis and nucleic acid replication. The results of this study showed that biosynthesized AgNPs effectively inhibited biofilm formation, even at low concentrations. This efficacy can be attributed to the biocompatible and non-toxic nature of the biosynthesis method, which enhances the safety of AgNPs for medical applications [20].

### 3.5. Antimicrobial activity of synthesized silver nanoparticles

The antimicrobial activity of the synthesized silver nanoparticles was evaluated using the disc diffusion test and broth microdilution method (Table 1 and Table 2). The results demonstrated a concentration-dependent antibacterial effect of the nanoparticles against both *E. coli* and *K. pneumoniae*. Notably, *E. coli* strains were more sensitive to the synthesized nanoparticles compared to *K. pneumoniae* strains.

**Table 1.** Zone of inhibition diameter using the disc diffusion method in selected strain of *E. coli* and *K. pneumoniae* in the presence of different concentrations of silver nanoparticles.

Silver nanoparticles	Diameter of the growth zone (mm)								
	Synthesized nanoparticle concentration ( $\mu\text{g/mL}$ )								
	0.5	1	2	4	10	20	50	100	200
<i>E. coli</i>	0	7	8	12	13	15	20	21	24
<i>K. pneumoniae</i>	0	0	7	8	10	14	16	19	19

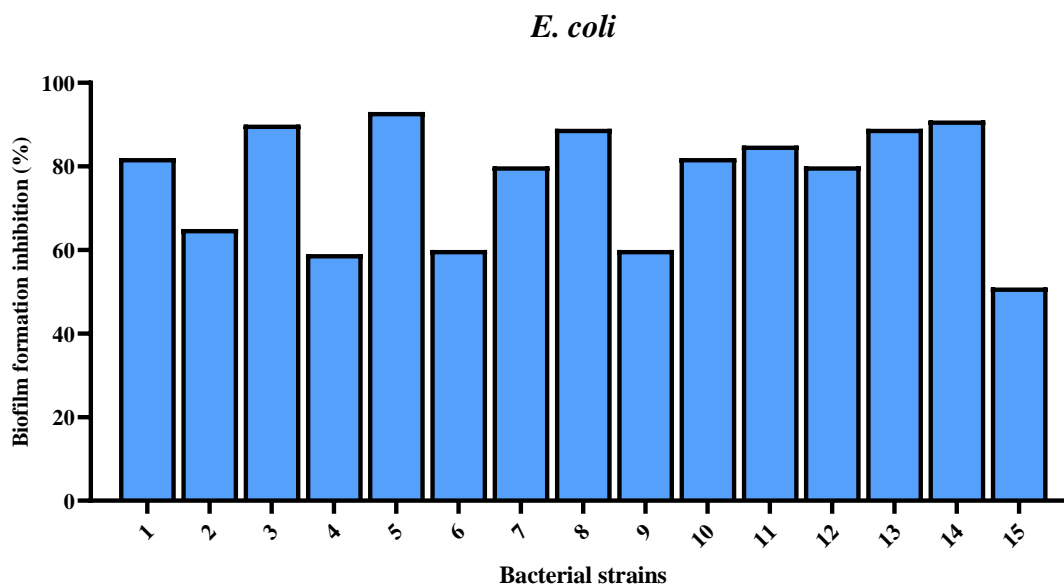
**Table 2.** Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) selected strain of *E. coli* and *K. pneumoniae* in the presence of silver nanoparticles.

Silver nanoparticles ( $\mu\text{g/mL}$ )	<i>E. coli</i>		<i>K. pneumoniae</i>	
	MIC	MBC	MIC	MBC
	6.25	1.562	12.5	25

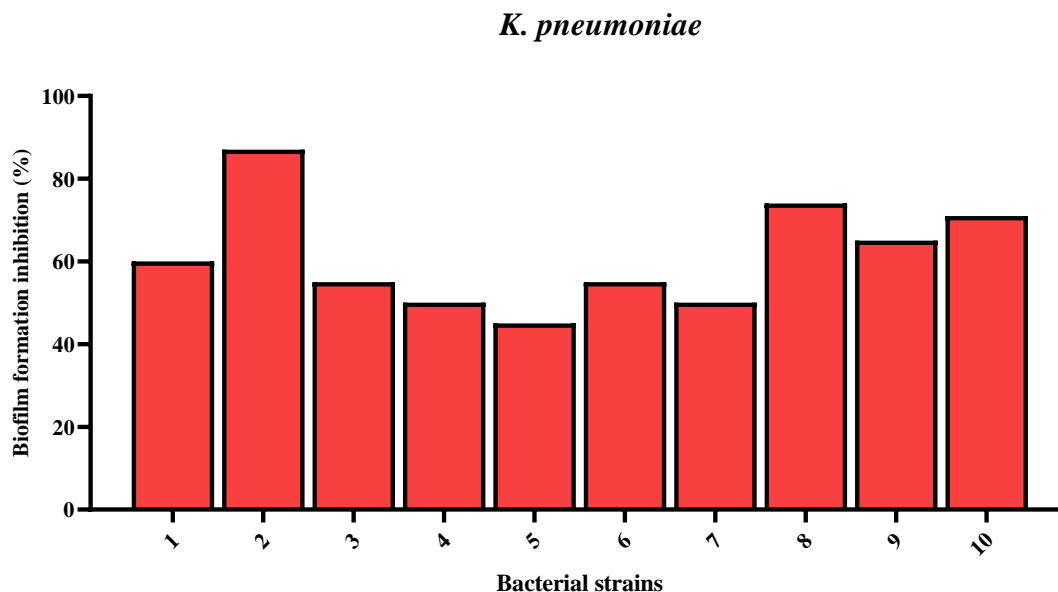
Advances in nanotechnology over the past decade have highlighted the potential of metal nanoparticles to combat biofilm-associated infections. Previous studies have demonstrated the ability of silver nanoparticles to penetrate biofilms and disrupt their structure. For example, Becker et al. [27] reported that AgNPs effectively inhibited biofilms formed by gram-negative bacteria such as *E. coli* and *K. pneumoniae*. Similarly, Li et al. [28] showed that biosynthesized AgNPs reduced biofilm formation by more than 80% in antibiotic-resistant strains. Patel et al. [29] further demonstrated that AgNPs exert anti-biofilm effects through mechanisms such as reactive oxygen species (ROS) production and bacterial cell wall disruption, which decrease biofilm density and prevent its spread. Rezaei et al. [30] also reported that biosynthesized AgNPs inhibited the growth and biofilm formation of antibiotic-resistant *E. coli* and *K. pneumoniae*, suggesting their potential as a complementary approach in UTI treatment.

### 3.6. Anti-biofilm activity assay

The anti-biofilm activity of silver nanoparticles was assessed at a concentration equivalent to  $\frac{1}{2}$  MIC in 15 selected *E. coli* strains and 10 selected *K. pneumoniae* strains. The results indicated that the nanoparticles effectively inhibited biofilm formation in both bacterial species, with *E. coli* strains showing a greater reduction in biofilm formation compared to *K. pneumoniae* strains (Fig. 4).



↓ continuing



**Fig. 4.** Percentage of biofilm formation inhibition of selected *E. coli* and *K. pneumoniae* strains in the presence of  $\frac{1}{2}$  MIC concentration of silver nanoparticles.

Biofilm inhibition by nanoparticles can occur through the prevention of bacterial adhesion to surfaces, their intrinsic antibacterial properties, or both [31]. Other nanoparticles, such as magnesium fluoride, have also shown efficacy in preventing biofilm formation by pathogens like *E. coli* and *Staphylococcus aureus*. For instance, catheters coated with magnesium fluoride nanoparticles demonstrated resistance to biofilm-associated infections [32]. Furthermore, synergistic effects have been observed when combining nanoparticles with antibiotics or plant extracts. Krychowiak et al. [33] reported that the combination of AgNPs with *Drosera binata* plant extract exhibited enhanced anti-biofilm activity against *S. aureus*. In this study, *K. pneumoniae* strains exhibited greater resistance to biofilm inhibition by AgNPs compared to *E. coli*. This difference may be attributed to the genetic and structural characteristics of the two bacterial species. Nevertheless, biosynthesized AgNPs were effective in reducing biofilm formation in both cases, highlighting their broad-spectrum potential [34].

The rapid spread of drug-resistant uropathogens has outpaced the development of new antibiotics, necessitating the exploration of alternative therapeutic strategies. Natural antimicrobial substances, such as medicinal plant extracts and metal nanoparticles, have gained attention as potential replacements for conventional antibiotics. Biosynthesized AgNPs offer several advantages, including the use of environmentally friendly resources and reduced risks to human health and the environment compared to chemically synthesized nanoparticles [35]. This sustainable approach to nanoparticle production may enhance the safety and efficacy of antimicrobial treatments.

### 3.7. Limitations and future directions

The study was conducted in vitro and requires in vivo validation because human systems operate under different conditions. Future research should investigate the synergistic ability of AgNPs in combination with common antibiotics to obtain antimicrobials that are more effective. Present study from our group have demonstrated potential targets for AgNPs, which regulate biofilm-production; however, further investigations into the specific molecular pathways through which AgNPs act may shed more light on their therapeutic application in the design of targeted treatments.

#### 4. Conclusion

Biofilms are a major contributor to hospital-acquired infections, forming on medical surfaces such as catheters and surgical instruments. The use of silver nanoparticles offers a promising strategy to prevent biofilm formation and reduce the risks associated with these infections. This study demonstrated the *in vitro* antimicrobial and anti-biofilm activity of biosynthesized AgNPs against uropathogens, providing insights into their potential use in preventing and managing UTIs. However, further research is needed to optimize their clinical applications, including determining safe dosages, understanding their mechanisms of action, and evaluating their effects on other bacterial strains. The findings of this study may contribute to the development of effective antimicrobial agents with anti-biofilm properties, offering new solutions for the treatment of UTIs and other antibiotic-resistant infections.

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#### Conflict of Interest

The authors declare no conflict of interest.

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