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Research Article

Antimicrobial, Antibiofilm and Antiurease Activities of Microbially Synthesized Silver Nanoparticles against *Proteus mirabilis*

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Abstract: Nanoparticles (NPs) are tiny materials ranging in size from 1 to 100 nm and have unique magnetic, electrical, and optical characteristics differing from bulk materials. They have a broad spectrum of applications in different industries. Several physical and chemical techniques have been applied to produce metal NPs. Alternatively, green synthesis offers an environmentally friendly and simple means for NP preparation. In the present study, silver NPs were produced by the *Pseudomonas aeruginosa* OG1 strain. Characterization of NPs was performed by TEM, SEM, and XRD. These NPs were used against pathogenic *Proteus mirabilis*, which shows high-level urease activity and forms clear biofilms. Silver NPs obtained in the present study were applied to inhibit the growth, urease production, and biofilm formation of *P. mirabilis*. Growth inhibition zones of 9 mm and 11 mm and, 60 % and 85% antibiofilm effects were obtained by 100 µg mL^{-1} and 200 µg mL^{-1} NPs, respectively. The urease activity of *P. mirabilis* was completely inhibited in both concentrations. These results show that AgNPs can be used as effective antimicrobial, antibiofilm, and antiurease agents in the fight against pathogens.

Mikrobiyal Olarak Sentezlenen Gümüş Nanopartiküllerin *Proteus mirabilis*'e Karşı Antimikrobiyal, Antibiyofilm ve Antiüreaz Aktiviteleri

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Öz: Nanopartiküller (NP), 1 ila 100 nm arasında değişen küçük malzemelerdir ve bulk malzemelerden farklı benzersiz manyetik, elektriksel, optik özelliklere sahiptir. Farklı endüstrilerde geniş bir uygulama alanlarına sahiptirler. Metal NP'leri üretmek için çeşitli fiziksel ve kimyasal teknikler uygulanmıştır. Alternatif olarak, yeşil sentez, NP hazırlama için çevre dostu ve basit bir yol sunar. Bu çalışmada, gümüş NP'ler *Pseudomonas aeruginosa* OG1 suşu tarafından üretilmiştir. NP'lerin karakterizasyonu TEM, SEM ve XRD ile yapılmıştır. Bu NP'ler, yüksek düzeyde üreaz aktivitesi gösteren ve berrak biyofilmler oluşturan patojenik *Proteus mirabilis*'e karşı kullanılmıştır. Bu çalışmada elde edilen gümüş NP'ler *P. mirabilis*'in büyümesini, üreaz üretimini ve biyofilm oluşumunu engellemek için uygulanmıştır. Sırasıyla 100 µg mL^{-1} ve 200 µg mL^{-1} konsantrasyonlardaki NP'lerle 9 mm ve 11 mm'lik büyüme inhibisyon bölgeleri ve %60 ve %85 antibiyofilm etkileri elde edildi. *P. mirabilis*'in üreaz aktivitesi her iki konsantrasyonda da tamamen inhibe edilmiştir. Bu sonuçlar, AgNP'lerin patojenlerle mücadelede etkili antimikrobiyal, antibiyofilm ve antiüreaz ajanları olarak kullanılabileceğini göstermektedir.

1. Introduction

Nanotechnology has advanced rapidly in recent years as a result of the numerous applications of nanoparticles (NPs) in disciplines such as optics, electronics, energy, food, agriculture, biology, engineering, environment, textile, cosmetics, medicine, pharmacy, and biomedicine (Kapoor et al., 2021; Ozdal & Gurkok, 2022a). NPs are very small materials that range in size from 1 to 100 nanometers. Most NPs are made up of just a few hundred atoms. NPs differ greatly from bulk materials in that when size lowers, the surface area-to-volume ratio grows significantly, resulting in NPs with numerous new and unique properties (Alavi & Varma, 2021).

NPs can arise naturally or be man-made through physical, chemical, and biological means. The first two processes have drawbacks such as the use of hazardous and poisonous chemicals, low efficiency, and excessive energy consumption at high temperatures (Ahmad et al., 2019; Ozdal & Gurkok, 2022b). Given the aforementioned disadvantages, research has concentrated on the synthesis of NPs employing low-cost, biocompatible, non-toxic, and environmentally friendly biological components (Koul et al., 2021). Another advantage of this green synthesis approach is that it is usually performed at ambient temperature or with mild heating. As a result, the use of NPs generated using physicochemical methods in biomedical applications has been limited, owing to concerns about toxicity and bioincompatibility (Shah et al., 2015).

Today, microorganisms are commonly used in the biosynthesis of NPs because it is a simple, cost-effective, and ecologically benign technique. This method is also promising for large-scale NP generation (Khandel & Shahi, 2018). Microorganisms are important nano-factories because their various reductase enzymes may decrease, accumulate, and detoxify heavy metals (Singh et al., 2016). Enzymes, proteins, polysaccharides, biosurfactants, pigments, and other biological compounds are present in microorganisms and play a vital role in the production of NPs (Ozdal & Gurkok, 2022a).

Because of their biocompatibility, they have numerous potential applications in the sector of medicine. In this context, NPs are used to combat potentially harmful pathogens. *Proteus mirabilis* is a Gram-negative bacterium that causes urinary tract infections mostly through biofilm development (Wasfi et al., 2020). Biofilm generation and antibiotic resistance are rapidly becoming major global issues in the public health system (Ozdal & Gurkok, 2022b). As a result, in recent years, the effects of various NPs such as Ag, ZnO (Khan et al., 2021), ZnO, CuO (Mohamed et al., 2021), and Se (Salem et al., 2022) on biofilm formation and microbial growth have been studied. Bacterial ureases are recognized as key virulence factors in human health such as kidney stone formation, catheter occlusion, stomach inflammation, peptic ulcers, and dental plaque creation (Loharch & Berlicki, 2022). Urease is also necessary for the biofilm consortium to remain stable (Morou-Bermudez et al., 2015). NPs with the ability to inhibit urease include Ag, Au (Ali et al., 2021), and ZnO (Sajjad et al., 2021; Ashraf et al., 2023). Considering this information, the biofilm and urease of *P. mirabilis* may be promising therapeutic targets for bacterial control. As a result, the antibacterial, antibiofilm, and antiurease activities of biosynthesized AgNPs against *P. mirabilis* were studied in this study.

2. Material and Methods

2.1. Microorganisms

Pseudomonas aeruginosa OG1 (NCBI KC453990) isolated in a previous study (Ozdal et al., 2016) was used for the microbial synthesis of AgNPs. *Proteus mirabilis* (ATCC 12453) was used to investigate the biological activities of the synthesized AgNPs.

2.2. Biosynthesis of silver nanoparticles

For the synthesis of AgNPs, the *P. aeruginosa* OG1 strain was incubated in Nutrient Broth (NB) for 24 hours at 30 °C and 150 rpm. The cell suspension (500 µL, OD_{600} 1) was inoculated into 100 mL of Luria Bertani Broth (LB) containing 1 mM $AgNO_3$. NP biosynthesis was carried out at 30 °C, 150 rpm for 48 hours. To purify AgNPs at the end of the period, the cell suspensions were sonicated in an ultrasonic bath (Apple/S30) at 100 W for 10 minutes and washed using hexane, ethanol, and ddH_2O,

respectively. Each step was centrifuged at 10 000 g for 10 minutes. Control experiments without $AgNO_3$ were performed simultaneously.

2.3. Characterization of silver nanoparticles

A UV-visible spectrophotometer (Shimadzu, Japan), TEM (HT-7700), X-ray diffractometer (XRD Bruker D2, K, =1.54 A, scanning angle 70°), and SEM (Zeiss Sigma 300) were used to characterize AgNPs (Çakıcı et al., 2019).

2.4. Investigation of the antimicrobial effect of silver nanoparticles

The antibacterial activity of AgNPs against *P. mirabilis* bacteria was tested using the agar disc diffusion method (Bauer et al., 1966). Bacterial culture was cultivated in NB for 24 hours at 37 °C and 160 rpm. Bacterial dilutions of 0.5 McFarland standard (1.5×10^8 cfu mL^{-1}) were prepared, and aliquots of 100 µL were dispersed on Nutrient Agar (NA) plates. On these plates, paper discs containing 100 µg and 200 µg AgNPs were inserted. Negative control disc containing 25 µL sterile ddH_2O and positive control disc containing 25 µg gentamicin were placed on these plates. After 24 hours of incubation at 37 °C, antimicrobial activity was assessed by measuring inhibition zones around AgNPs-impregnated discs.

2.5. Investigation of antibiofilm effect of silver nanoparticles

A biofilm inhibition assay with AgNPs was tested against *P. mirabilis* in 96-well culture plates. The bacteria were incubated in 10 mL Tryptic Soy Broth (TSB) with 1% glucose for 24 hours at 37 °C. After 24-hour incubation, 177.5 µL of the growth medium, 12.5 µL of AgNPs (final concentrations of 100 µg mL^{-1} and 200 µg mL^{-1}), and 10 µL of test bacteria dilutions were mixed into each well of the plates. In the positive controls, 10 µL of bacterial dilutions were mixed with 190 µL of the growth medium, while the negative control contained growth medium only. In antibiotic control, 12.5 µL of gentamicin (final concentration of 25 µg) was added instead of AgNPs. After incubation at 37 °C for 24 hours, unattached cells were removed by washing three times with distilled water and adherent cells were stained with 200 µL of 0.4% crystal violet for 30 minutes. Later, the excess crystal violet was removed and the wells were washed with distilled water three times. The residual stained biofilm was suspended in 200 µL of 70% ethanol at room temperature for 30 minutes. The OD of the wells was measured using a microplate reader at 570 nm (Thermo Scientific Inc., Multiskan GO, Finland) (Bai et al., 2019). Biofilm inhibition in the wells containing AgNPs (test group) was compared with the control group and calculated with the formula 1:

$$\text{Biofilm inhibition (\%)} = [(\text{Control } OD_{570}\text{nm} - \text{Test } OD_{570}\text{nm}) / \text{Control } OD_{570}\text{nm}] \times 100 \quad (1)$$

2.6. Investigation of the antiurease effect of silver nanoparticles

Urease activity was determined using the Nesslerization method with spectrophotometric analysis at 425 nm (Doriya & Kumar, 2016). Urease assay was performed in test tubes containing 3.8 mL of NB and 200 µL of urea solution (0.6 M urea prepared in Tris Buffer, pH 7). AgNPs (final concentrations of 100 µg and 200 µg) and 30 µL of test bacterial suspension of *P. mirabilis* were added. Positive control tubes were prepared in the same way without the addition of test NPs. Negative controls contained only NB and urea in the same proportions. In antibiotic control, gentamicin (final concentration of 25 µg) was added instead of AgNPs. All tubes were incubated at 37 °C, 150 rpm for 24 hours. The tubes were then centrifuged at 6 000 rpm. The supernatant (100 µL) was mixed with 900 µL of urea solution and left at 30 °C for 15 minutes. The urease reaction was stopped with 100 µL of 10% Trichloroacetic acid (TCA) and centrifuged at 6 000 rpm. To analyze the released ammonia, 200 µL of the supernatant was mixed with 200 µL of Nessler's Reagent and 1.6 mL of ddH_2O and incubated for 5 minutes at room temperature. The absorbances of the mixtures were measured at 425 nm. Urease inhibition rates were calculated by the formula 2:

$$\text{Urease inhibition (\%)} = [(\text{Control } OD_{425}\text{nm} - \text{Test } OD_{425}\text{nm}) / \text{Control } OD_{425}\text{nm}] \times 100 \quad (2)$$

3. Results and Discussion

3.1. Characterization of silver nanoparticles

The reduction of the ions in $AgNO_3$ in the *P. aeruginosa* OG1 culture to AgNPs was observed with the change of the color of the culture medium from yellow to brown, as vibrations occurred on the plasma surface. As is known, the formation of silver nanoparticles is understood by the color of the medium turning dark brown or black (Sharifi-Rad et al., 2021; Ozdal & Gurkok, 2022b).

Microorganisms have been used frequently in the biosynthesis of NPs in recent years and *Pseudomonas* species is one of the most preferred microorganisms in this regard and have been used in biosynthesis of various particles such as AgNPs (John et al., 2020; Yang et al., 2020; Pernas-Pleite et al., 2022), AuNPs (Husseiny et al., 2007) and ZnO-NPs (Abdo et al., 2021).

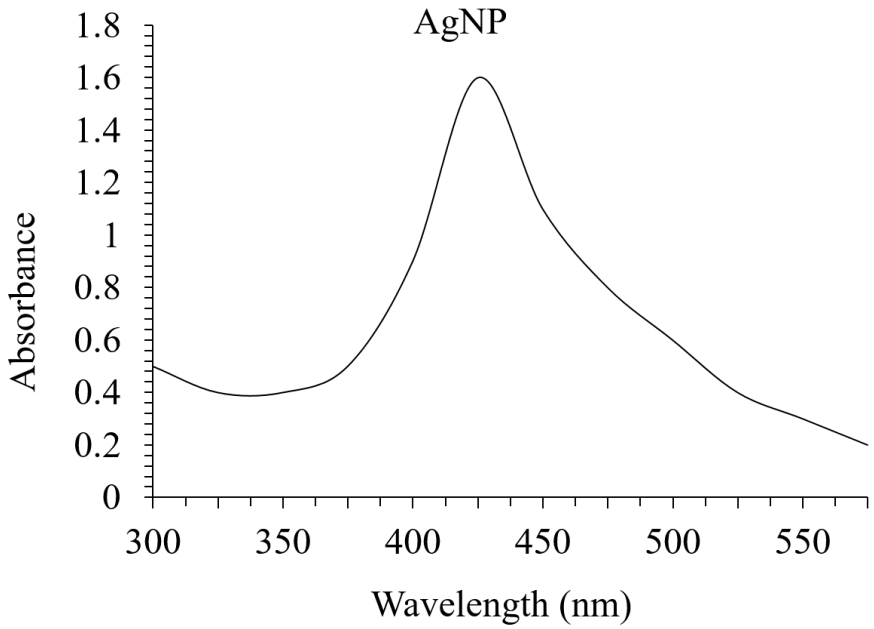

Figure 1. UV-Vis absorbance peak of microbially synthesized AgNPs.

The UV-visible spectrum of the synthesized AgNPs was determined by a spectrophotometer (Figure 1). It was observed that AgNPs peaked at 415 nm in the spectrum taken by UV-visible spectrophotometer in the wavelength range of 350-600 nm. Similar to the results of this study, many studies have indicated the formation and presence of spherical and/or near-spherical AgNPs, with the SPR peak between 410 and 460 nm (Chandrakar et al., 2021; Sharifi-Rad et al., 2021; Hu et al., 2022).

The images of AgNPs taken with TEM (60.0 K X magnification) at 100 nm (Figure 2.a) and with SEM (50.00 K X magnification) at 200 nm (Figure 2.b) scale are shown in Figure 2. AgNPs were found to have a size less than 30 nm. The energy-dispersive X-ray spectroscopy (EDX) analysis was used to evaluate the composition of synthesized AgNPs, as shown in Figure 2c. The EDX spectrum showed mostly Ag, carbon (C) and oxygen (O) components forming the structure of the AgNPs sample.

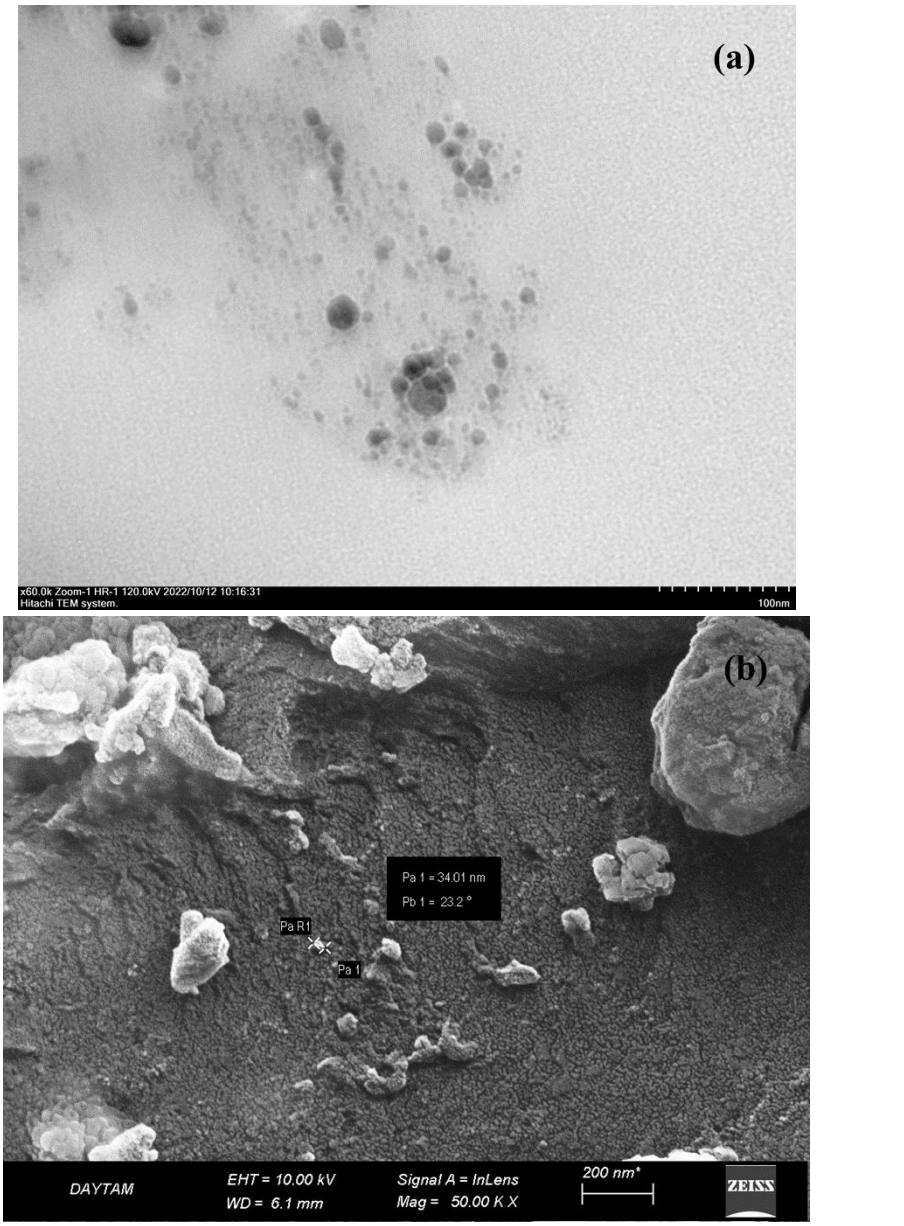

Figure 2. TEM (a), SEM (b) images, and corresponding EDX spectrum (c) of AgNPs.

Figure 3 depicts the crystal structure of produced AgNPs as determined by XRD analysis. The distinctive spherical crystal structure of silver at 2θ was shown by four intense peaks at 38.2, 46.1, 64.1, and 77.3. These peaks indicate that a spherical nanosilver was synthesized (Elbahnasawy et al., 2021; Abdulkareem et al., 2022).

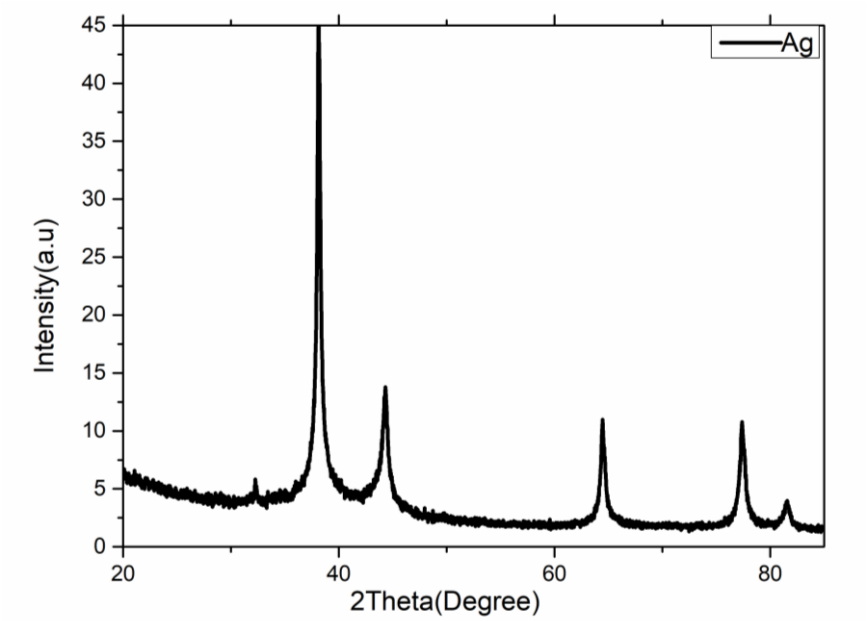

Figure 3. XRD pattern of AgNPs.

3.2. Antibacterial, antibiofilm and antiurease effects of silver nanoparticles

Nanoparticles are known to have a wide range of biological activities such as antimicrobial, antibiofilm, and antiurease effects which vary considerably depending on the type, shape, and size, the synthesis method, and the concentration of the nanoparticles, the exposure time and the nature of pathogenic microorganisms.

The antibacterial, antibiofilm, and antiurease characteristics of AgNPs are summarized in Table 1. After 24-hour incubation on NA with paper discs soaked with 100 µg $disc^{-1}$ and 200 µg $disc^{-1}$ AgNP, *Proteus mirabilis* showed growth inhibition zones of 9 and 11 mm, respectively, while the positive control gentamicin disks resulted in 15 mm growth inhibition zone and negative control disk containing ddH_2O showed no zone. As mentioned before, the biological effects of nanoparticles such as the observed antimicrobial effect vary depending on many parameters. Obtaining close antimicrobial results with the positive control using a pure antibiotic with nanoparticles indicates a promising result.

Antimicrobial effects of AgNPs against a range of microorganisms have been demonstrated (Lashin et al., 2021; Chandrasekharan et al., 2022). Ag-NP synthesized by *Streptomyces roseolus* was used in the range of 0.39–25 mg mL^{-1} (which is quite higher than the current study) against Gram-positive *L. monocytogenes*, *Staphylococcus aureus*, *B. subtilis*, *B. cereus*, Gram-negative bacteria *E. coli* O157:H7, *K. pneumonia*, *A. hydrophilia*, and yeast *C. albicans*. They observed higher antibacterial effect against *E. coli* O157:H7 (23.66 mm inhibition zone) and lower antimicrobial activity against *C. albicans* (18.66 ± 0.3 mm inhibition zone) and *B. subtilis* (19.66 ± 0.3 mm inhibition zone) (Elnady et al., 2022). The antibacterial activity of AgNPs synthesized by tea leaves extract was evaluated against Gram-negative foodborne pathogens, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13773, *S. typhimurium* ATCC 14028, and *S. enteritidis* ATCC 13076, and 15 mm, 10 mm, 20 mm, and 20 mm clear zones were obtained (Loo et al., 2018). Antimicrobial effect of AgNPs synthesized by photo-irradiation was evaluated against *S. warneri* and 14 mm zone of inhibition was obtained (Dong, et al., 2017). In another study, antibacterial activity of AgNPs synthesized by *P. aeruginosa* against multidrug-resistant isolates from intensive care unit has been reported (Abeer Mohammed et al., 2022). The antimicrobial activity of these AgNPs was used against *A. baumannii* and 18 mm inhibition zone was obtained using 1 and 4 mM AgNPs. The same nanoparticle was also used against *K. pneumoniae*

and 19.17 mm inhibition zone was observed at a concentration of 1 mM of AgNPs and 22 mm using 4 mM of AgNPs (Abeer Mohammed et al., 2022). The antimicrobial effects of AgNPs synthesized by *Lactobacillus acidophilus* (Hussein & Alsharifi, 2021) and leaves extracts of *Ficus benghalensis* (Maniraj et al., 2019) against *P. mirabilis* have been studied previously, but, to the best of our knowledge, antimicrobial effects of AgNPs synthesized by *P. aeruginosa* against *P. mirabilis* was investigated for the first time in this study.

In the presence of 100 and 200 mg mL-1 AgNPs, biofilm production was reduced by 60% and 85%, respectively. (When gentamicin (25 µg) was added to the cultures, biofilm formation was not observed because bacteria did not grow). Mohanta et al. (2020) reported that photosynthesized AgNPs have considerable anti-biofilm efficacy against human pathogens *E. coli*, *P. aeruginosa*, and *S. aureus*. According to the researchers, AgNPs' anti-biofilm effect was linked to the reduction of bacterial exopolysaccharide (EPS) production, which is required for biofilm formation (Seo et al., 2021). Bharathi et al. (2018) found dose-dependent biofilm inhibitory action of AgNPs against *S. aureus* and *E. coli* at concentrations ranging from 25 to 100 µg mL^{-1}. At a concentration of 100 µg mL^{-1}, the biosynthesized AgNPs inhibited *S. aureus* and *E. coli* biofilm formation by 90%.

Antibiofilm properties of AgNPs synthesized by *Pseudomonas alloputida* have been reported against *Bacillus subtilis*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, *S. aureus*, and *S. epidermidis* pathogens (Pernas-Pleite et al., 2022). This result showed that infections due to biofilms can be prevented and treated with AgNPs. Commercial AgNPs have also been evaluated for their antibiofilm activities and resulted in 50% biofilm inhibition at concentrations about 50 ppm for *E. coli* and slightly higher for *S. aureus* (Lange et al., 2021).

Urease is a virulence factor found in a variety of pathogens that is necessary by the host organism. Urease synthesis in *P. mirabilis* was completely inhibited during incubation with AgNPs at both dosages. (When gentamicin (25 µg) was added to the cultures, urease synthesis was not observed because bacteria did not grow). NPs may interact with the enzyme's active site or allosteric site, creating structural modifications. Because of its non-competitive method of NP binding, urease loses its enzymatic properties (Ponnuvel et al., 2015). Urease inhibition has previously been demonstrated by NPs such as Ag, Au (Ullah et al., 2022), SiO_2, TiO_2, and ZnO (Khan et al., 2017).

Although the antiurease activities of NPs are relatively less studied, it has been reported that AgNP and AuNP synthesized with *Crataegus oxyacantha* extract have antiurease activity and resulted in 99,25 % biofilm inhibition in *Canavalia ensiformis* urease (Ali et al., 2021). ZnONPs synthesized by *Boerhavia diffusa linn* have also been reported to have 81.3% urease inhibition rate (Ashraf et al., 2023). AgNPs from *Fagonia cretica* extracts stabilized by short chain heterocyclic thiol namely Ethyl 6-methyl-4-phenyl-2-thioxo1,2,3,4-dihydropyrim-idine-5-carboxylate (DHPM) was evaluated in a study performed by Khan et al. (2022) and they observed urease inhibition of 40.3%, which is lower than that obtained in this study. However, as far as is known, there is no study on the antiurease activity of AgNPs synthesized with *P. aeruginosa* against *P. mirabilis*.

Antibacterial, antibiofilm, and antiurease activities of AgNPs against *P. mirabilis* were demonstrated for the first time in the current study. Because NPs act on bacteria via several targets/mechanisms, it is extremely difficult for bacteria to develop resistance to them (Lee et al., 2019; Ozdal & Gurkok 2022a). Therefore, NPs are promising agents, as an alternative to antibiotics, against pathogens that develop antibiotic resistance.

Table 1. Antibacterial, antibiofilm, and antiurease activities of AgNPs against *P. mirabilis*

	Gentamicin	**AgNP concentrations**	
AgNPs Biologic Activities	**25 µg**	**100 µg**	**200 µg**
Antibacterial Activity (inhibition zone)	15 mm	9±0,5 mm	11±0,5 mm
Antibiofilm activity	100%	60±3%	85±4%
Antiurease activity	100%	100%	100%

4. Conclusion

In this study, it was determined that biologically synthesized AgNPs have antibacterial, antibiofilm, and antiurease effects against the pathogen *P. mirabilis*. The use of AgNPs can help develop new approaches to prevent biofilm-associated infections and biofilm formation. Due to their diverse activities, AgNPs have the potential to be used in the antimicrobial treatment of pathogenic microorganisms, especially antibiotic-resistant bacteria. Based on this study, green synthesized AgNPs show great promise in the environmental, food, and pharmaceutical industries.

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