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Effectiveness of Silver Nanoparticles (AgNPs) Synthesized Using Waste Lime Peel Extract (Citrus aurantifolia Swingle) Against Bacteria Causing Nosocomial Infections

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ABSTRACT

Introduction: *Nosocomial infections remain a challenge because they increase morbidity, mortality, hospital stay, and healthcare costs. Rising bacterial resistance to conventional antibiotics has encouraged the search for alternative antibacterial agents. Green-synthesised silver nanoparticles have attracted attention because of their antimicrobial activity, while lime peel waste provides phytochemicals that act as reducing and stabilising agents during nanoparticle synthesis.*
Methods: *This laboratory-based experimental study used a post-test-only control-group design. Silver nanoparticles were biosynthesised using waste lime peel extract and characterised by colour change, UV-Vis spectrophotometry, Fourier Transform Infrared spectroscopy, and Scanning Electron Microscopy. Antibacterial activity against Pseudomonas aeruginosa was evaluated in vitro using disc diffusion at concentrations of 25, 50, 75, 100, and 125 µg/mL, with controls. All groups were tested in triplicate. Data were analysed using Shapiro-Wilk, Levene's test, and One-Way ANOVA.*
Results: *Biosynthesis produced a colour change, indicating nanoparticle formation. Characterisation demonstrated a maximum absorption peak around 380 nm, functional groups associated with phytochemical reduction and capping, and spherical particles ranging from 13 to 53 nm, with an average size of 31 nm. All tested concentrations inhibited Pseudomonas aeruginosa and were categorised as strong. The largest inhibition zone among nanoparticle groups was observed at 125 µg/mL (18.20 ± 0.81 mm), although levofloxacin showed greater inhibition. Statistical analysis showed significant differences among treatments ($p < 0.05$).*
Conclusions: *Biosynthesised AgNPs derived from lime peel extract showed antibacterial activity against opportunistic Gram-negative bacteria. However, further studies are needed to determine MIC/MBC values, evaluate cytotoxicity, and assess activity against a wider bacterial spectrum.*

Introduction

Nosocomial infections, also known as healthcare-associated infections (HAIs), are a serious global health challenge (Essam *et al*., 2024). These infections are acquired by patients while receiving healthcare for other conditions (Khan *et al*., 2017). Common nosocomial infections include pneumonia, urinary tract infections, bloodstream infections, and surgical site infections (Sartelli, 2022). The prevalence of nosocomial infections increases morbidity and mortality, prolongs hospital stays, and imposes a major economic burden on healthcare systems worldwide (Stewart *et al*., 2021).

The global prevalence of nosocomial infections is estimated to be approximately 14%. In Southeast Asia, the prevalence is 21.6%, with Indonesia exhibiting the highest rate at 30.4%. In East Java, hospital-based data indicate that the point prevalence of HAIs was 11.2% in 2021 and

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decreased to 7.7% in 2023 (Artalia & Rosa, 2025; Raoofi et al., 2023; Wah Goh et al., 2023). The main drivers of morbidity and mortality associated with nosocomial infections are the causative agents, especially bacteria. Common bacterial pathogens include Staphylococcus aureus (including Methicillin-Resistant Staphylococcus aureus [MRSA]), Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Enterococcus species (Tafin *et al*., 2024). A critical issue in the management of these infections is the alarming rise in antibiotic resistance. Bacteria have developed various mechanisms to evade the effects of antibiotics, rendering many conventional antimicrobial drugs ineffective (Donaliazarti, 2022; Nurjanah *et al*., 2020). The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria poses a serious threat to public health, necessitating the development of new and effective antimicrobial agents and strategies (Salam *et al*., 2023).

In response to the growing challenge of antibiotic resistance, there has been an intensive global effort to explore alternative antimicrobial therapies. These efforts include revisiting traditional medicinal practices and utilizing modern scientific advances such as nanotechnology (Fadlilah dan Gozali, 2022). Nanotechnology offers great potential in the development of antibacterial agents, one of which is silver nanoparticles (AgNPs). AgNPs have received significant attention because of their strong broad-spectrum antimicrobial activity against bacteria, viruses, and fungi (Bruna *et al*., 2021; Urnukhsaikhan *et al*., 2021). The antibacterial effects of AgNPs are multifaceted and include damage to bacterial cell walls and membranes, disruption of essential enzymatic pathways, interaction with DNA and proteins, and the generation of reactive oxygen species. This multi-target approach makes it more difficult for bacteria to develop resistance to AgNPs compared with single-target antibiotics (Yin *et al*., 2020).

Conventionally, AgNPs are synthesized using chemical methods. However, these methods often involve harsh chemicals, high energy consumption, and toxic by-products, raising concerns about environmental impact and potential toxicity in biomedical applications (Rathi *et al*., 2024). Consequently, there has been growing interest in green synthesis or biosynthesis methods for nanoparticle production. Biosynthesis uses biological entities such as bacteria, fungi, algae, or plant extracts as reducing and stabilizing agents for metal ions. Plant extracts are especially favored because they are abundant, affordable, easy to handle, and contain various phytochemicals (such as flavonoids, polyphenols, and terpenoids) that can mediate the reduction of silver ions (Ag+) to elemental silver (Ag0) and stabilize the resulting nanoparticles (Nalawati *et al*., 2021).

Waste lime peel (Citrus aurantifolia Swingle) is an abundant agricultural and food-service waste product that has not yet been fully utilized. Lime peel is known to contain various phytochemical compounds with potential as reducing and stabilizing agents in nanoparticle synthesis. The use of waste lime peel for the biosynthesis of AgNPs not only adds value to this waste material, but also aligns with the principles of green synthesis and environmental sustainability (Purwajanti *et al*., 2023). Although the biosynthesis of silver nanoparticles (AgNPs) using various plant extracts has been widely studied, research specifically utilizing lime peel waste extract for AgNP synthesis and evaluating its antibacterial activity against bacteria that cause nosocomial infections remains very limited. This research gap is becoming increasingly important in light of the serious threat posed by nosocomial bacteria, particularly *Pseudomonas aeruginosa*, which is known for its high level of antibiotic resistance. To the best of our knowledge, only a limited number of studies have specifically investigated the potential of biosynthesised AgNPs derived from lime peel waste to combat clinically relevant nosocomial bacteria. Therefore, this study focuses on evaluating the antibacterial activity of biosynthesised AgNPs using lime peel waste extract against *Pseudomonas aeruginosa*, with the aim of providing strong scientific evidence regarding the potential of agricultural waste as a source of new antibacterial agents to help address infection-related challenges in hospital settings.

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The research question in this study is: How effective are silver nanoparticles (AgNPs) biosynthesized using waste lime peel extract against bacteria that cause nosocomial infections? The urgency of this study lies in addressing antibiotic resistance, as nosocomial infections caused by antibiotic-resistant bacteria pose a serious threat to health. The use of waste lime peel as a raw material for AgNP synthesis contributes to the reduction of organic waste, provides economic value, and supports the concept of a circular economy. In terms of potential clinical application, if the resulting AgNPs prove effective against nosocomial bacteria, this study may serve as a basis for developing topical antibacterial formulations or medical device coating materials to prevent infections in hospital settings. The objective of this study is to analyze the antibacterial effectiveness of biosynthesised silver nanoparticles (AgNPs) produced using lime peel waste extract against bacteria that cause nosocomial infections.

Methods

This study employed a laboratory experimental study with post-test only control group design and a green synthesis approach.

1. Time and Place of the Study

The research was conducted from August to December 2025. The study was carried out at the Microbiology and Chemistry Laboratories of STIKES Banyuwangi. Scanning Electron Microscope (SEM), UV-Vis (Thermo Scientific Genesys 10s), and Fourier Transform Infrared Spectroscopy (FT-IR) analyses were conducted at LPPT UGM.

2. Equipment and Materials

The equipment used in this study included SEM, a Genesys 10S FT-IR UV-Visible spectrophotometer, a magnetic stirrer, a mortar and pestle, and glassware. The materials required in this study were waste lime peel (Citrus aurantifolia Swingle) obtained from fruit juice or beverage vendors and restaurants in Banyuwangi Regency, East Java, demineralized water, AgNO3 (Merck), sodium citrate (Merck), HCl (Merck), Mayer reagent, Dragendorff reagent, tricarboxylic acid/TCA (Merck), acetonitrile (Merck), distilled water, Whatman No. 1 filter paper, McFarland solution (Merck), 1% levofloxacin, Nutrient Agar (NA) medium (Merck), and *Pseudomonas aeruginosa* ATCC 27853 bacteria obtained from the Microbiology Laboratory of IPB, Bogor.

3. Research Procedure

a. Synthesis of Silver Nanoparticles from Lime Peel (Citrus aurantifolia Swingle)

Waste lime peel (Citrus aurantifolia Swingle) was obtained from fruit juice or beverage vendors and restaurants in Banyuwangi Regency, East Java. After washing, the peels were dried in an oven. Once dry, the peels were blended and sieved to 50 mesh. To prepare the mixture, 5 g of peel powder was mixed with 50 mL of sterile deionized water at a 1:10 ratio. The resulting mixture was sonicated in a water-bath sonicator (Sibata) at 60°C for 4 hours. The mixture was filtered through Whatman No. 1 filter paper and stored at 4°C. To isolate plant particles from the extract, centrifugation was performed at 4,000 rpm for 10 minutes. The resulting supernatant was collected as lime peel extract, while the pellet residue was discarded. The obtained lime peel extract was used for the preparation of silver nanoparticles, in which 10 mL of extract was mixed with 90 mL of 1 mM AgNO3 solution. The solution was kept in a dark environment and incubated for 24 hours at 60°C. The color of the AgNO3 solution gradually changed from colorless to pale yellow and finally to dark reddish-brown, indicating the formation of Citrus-AgNPs. To separate the silver nanoparticles from the mixture, centrifugation was performed at 12,000 rpm for 30 minutes, followed by washing the pellet twice with deionized water at the same centrifugation speed and duration. The pellet was collected for subsequent freeze-drying to obtain stable silver nanoparticles.

b. Characterization of Lime Peel Silver Nanoparticles (Citrus aurantifolia Swingle)

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To confirm the surface plasmon resonance of the synthesized lime peel silver nanoparticles, the synthesis mixture was analyzed using a UV-Vis spectrophotometer (Thermo Scientific Genesys 10s). The maximum absorption of silver nanoparticles is in the wavelength range of 400–430 nm. To investigate the involvement of phytoconstituents in the synthesis of lime peel silver nanoparticles (Citrus aurantifolia Swingle), Fourier transform infrared spectroscopy (FTIR) analysis was carried out over the range of 500–4,000 cm−1. This test was performed at LPPT UGM. The size and shape of the nanoparticles were determined using SEM (Scanning Electron Microscope) at 3,000× magnification, 200 resolution, and an accelerating voltage of 11 kV. SEM analysis was used to observe the morphological characteristics, shape, and size of the nanoparticle samples.

c. Antibacterial Activity of Citrus-AgNPs

Preparation of the bacteria causing nosocomial infection: Pseudomonas aeruginosa used in the antibacterial study was isolated and identified. The antibacterial activity of Citrus-AgNPs was determined based on colony formation using an in vitro Petri dish assay. The bactericidal activity of Citrus-AgNPs was evaluated using the disk diffusion method. Nutrient Agar (NA) medium was poured into sterilized Petri dishes and allowed to stand overnight. Then, 100 mL of the prepared bacterial suspension was added to the NA medium, and the bacteria were spread across the surface of the Petri dish until dry. The turbidity of the suspension was adjusted using a spectrophotometer at 530 nm to obtain a final concentration corresponding to the 0.5 McFarland standard (~1.5 × 10^8 CFU/mL). Six discs were used, each containing lime peel silver nanoparticles at concentrations ranging from 25 to 125 μg/mL in increments of 25 (25, 50, 75, 100, 125). In addition, a control disc containing chlorhexidine at a concentration of 125 μg/mL was included. The samples were then incubated at 37°C for 24 hours. After the incubation period, the diameters of the inhibition zones were measured. Measurements were taken in the horizontal and vertical directions and then averaged. This study received ethical approval with approval number 317/02/KEPK-STIKESBWI/VIII/2025. Measurements were taken in both horizontal and vertical directions and then averaged. Each group was tested in triplicate.

Results

1. Characterization of Silver Nanoparticles from Lime Peel Extract

a. Color Characterization

Figure 1. Changes in solution color during the synthesis process of silver nanoparticles (AgNPs)

Based on visual observation, the samples arranged from right to left consisted of lime peel extract, $AgNO_3$ solution, the pre-synthesis solution, and the synthesised silver nanoparticle solution (Citrus-AgNPs). The lime peel extract exhibited a dark greenish-brown colour, indicating the presence of natural bioactive compounds that potentially act as reducing and stabilising agents in the synthesis process. The $AgNO_3$ solution appeared clear and colourless, indicating that the silver ions remained dissolved and had not yet undergone reduction. Meanwhile, the pre-synthesis solution appeared clear to pale yellow, suggesting that silver nanoparticle formation had not yet occurred optimally. In the synthesised solution, the colour changed to yellowish-

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brown to dark brown, indicating the formation of silver nanoparticles due to the reduction of Ag^+ ions to Ag^0. This colour change served as an initial indicator of the successful synthesis of AgNPs using lime peel extract.

b. UV-Vis Spectrophotometer Analysis

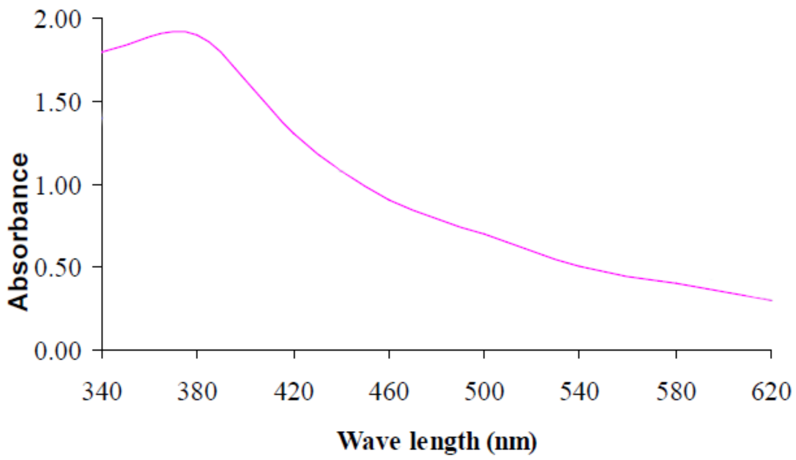

Figure 2. Results of the Ultra Violet-Visible (UV-Vis) Test

The UV-Vis analysis showed a maximum absorbance at approximately 380 nm, with an absorbance of 2.00. This peak is characteristic of the *Surface Plasmon Resonance* (SPR) phenomenon, indicating that Ag^+ ions were successfully reduced to silver nanoparticles (Ag^0). The peak below 400 nm indicates that the synthesised nanoparticles were small and spherical. Meanwhile, the slightly broadened absorption band indicates a variation in particle size distribution (polydispersity). Overall, these findings demonstrate that lime peel extract effectively acted as a reducing agent in the biosynthesis of silver nanoparticles.

c. Fourier Transform Infrared (FT-IR) Analysis

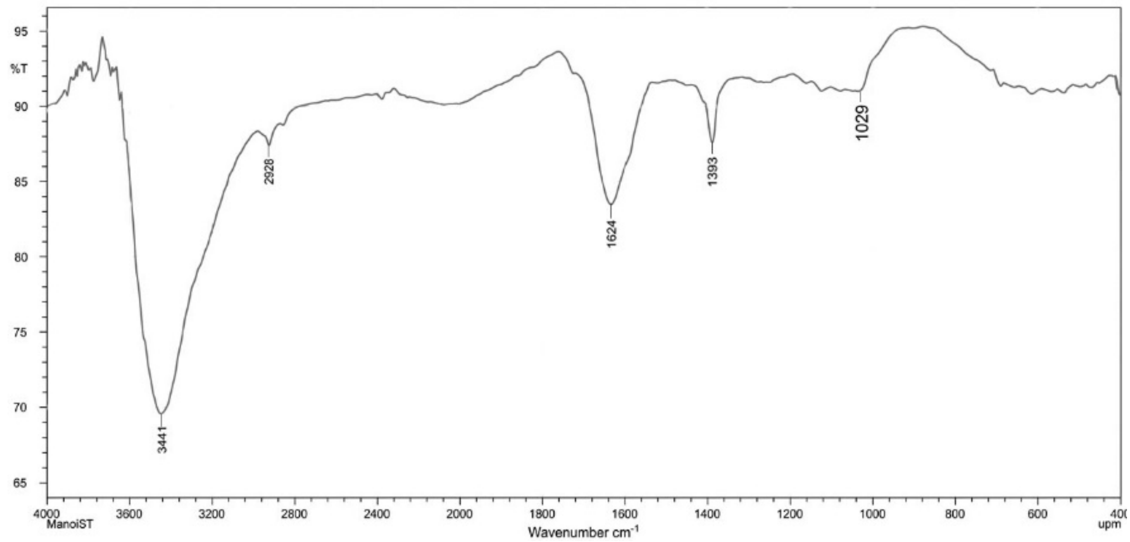

Figure 3. Results of the Fourier Transform Infrared (FT-IR) Test

Fourier Transform Infrared (FT-IR) testing was carried out to identify the biomolecular functional groups in lime peel extract that act as reducing agents and stabilizers (capping agents) in the synthesis of silver nanoparticles (AgNPs). The FT-IR spectrum showed five main absorption peaks representing these interactions. The broad peak at 3441 cm^{-1} indicates the stretching vibration of hydroxyl groups (–OH) from polyphenol or flavonoid compounds, while the peak at

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2928 cm^{-1} indicates aliphatic C–H bond stretching. Furthermore, the absorption at 1624 cm^{-1} is characteristic of the stretching vibration of carbonyl groups (C=O) or aromatic double bonds (C=C). The peak at 1393 cm^{-1} is associated with the symmetric stretching of carboxylate groups ($-COO^-$) from organic acids, and the absorption at 1029 cm^{-1} indicates C–O stretching vibrations generally originating from alcohols, ethers, and carbohydrates.

Based on the identification of these functional groups, it can be concluded that silver nanoparticles were successfully synthesized and coated by secondary metabolites from lime peel extract. Compounds such as flavonoids, polyphenols, and organic acids act synergistically through their hydroxyl and carbonyl group interactions, not only as agents for reducing silver ions but also as effective stabilizing agents that prevent agglomeration (clumping) of the nanoparticles.

d. Scanning Electron Microscopy (SEM) Analysis

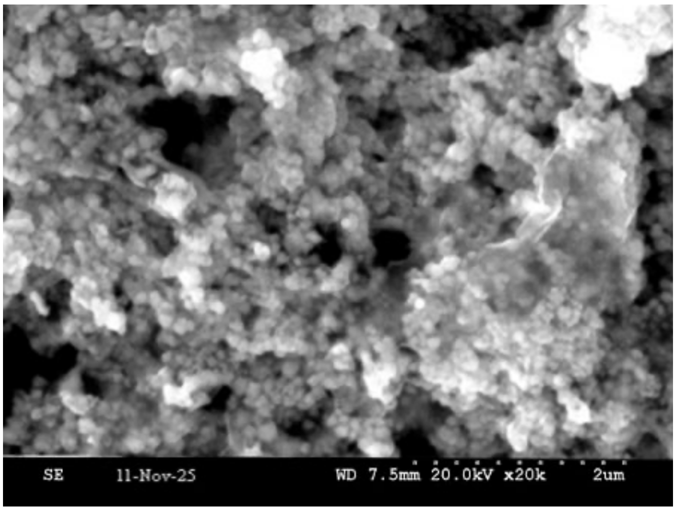

Figure 4. Results of the Scanning Electron Microscopy (SEM) Test

Testing using Scanning Electron Microscopy (SEM) was carried out to observe the morphology, physical shape, and size distribution of silver nanoparticles (AgNPs) synthesized using lime peel extract. Based on the SEM micrograph, the silver nanoparticles formed predominantly had a spherical morphology. This spherical shape is consistent with the single Surface Plasmon Resonance (SPR) absorption profile observed in the UV-Vis test. Quantitative measurements showed that the particles had a size distribution ranging from 13 to 53 nm, with an average size of 31 nm. These dimensions visually and mathematically confirm that the biosynthesis process successfully reduced silver ions into solid material that is purely on the nanometer scale.

Furthermore, the micrograph clearly also showed the phenomenon of agglomeration or particle clustering in several areas. This tendency of nanoparticles to agglomerate is common in green synthesis and is generally caused by the high surface area and surface energy of the particles, which leads them to attract one another in order to reach a more stable thermodynamic state. In addition, interactions among the phytochemical coating compounds (capping agents) from lime peel extract may also trigger intermolecular interactions that strengthen this accumulation, especially during the sample-drying process prior to analysis. Overall, the SEM results validate that lime peel extract effectively facilitates the formation of nanoscale silver nanoparticles with spherical morphology, although partial agglomeration activity was still observed.

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2. Antibacterial Activity of Silver Nanoparticles Synthesized from Lime Peel Extract

Table 1. Inhibition zone diameters of lime peel extract AgNPs against the test bacterium P. aeruginosa

Concentration	Inhibition zone diameter (mm) ± SD	
	Mean	Category
AgNPs 25 μg/mL	11.37 ± 0.78	Strong
AgNPs 50 μg/mL	13.08 ± 0.83	Strong
AgNPs 75 μg/mL	14.81 ± 0.65	Strong
AgNPs 100 μg/mL	15.45 ± 0.70	Strong
AgNPs 125 μg/mL	18.20 ± 0.81	Strong
Positive control	35.53 ± 2.13	Very strong
Negative control (5% DMSO)	0.0	None

Antibacterial activity testing was carried out to evaluate the bioactivity potential of silver nanoparticles (AgNPs) biosynthesized using lime peel extract against the test bacterium Pseudomonas aeruginosa, which represents a Gram-negative pathogenic bacterium. The effectiveness of this antibacterial agent was assessed based on the measurement of the inhibition zone diameter (clear zone) formed around the test disc.

Based on the data presented in the table, it can be observed that all AgNP concentration variations were able to effectively inhibit the growth of P. aeruginosa. The test results showed a positive correlation (dose-dependent effect), in which the magnitude of the inhibition zone diameter was directly proportional to the increase in the concentration of silver nanoparticles administered. At the lowest concentration of 25 μg/mL, AgNPs were already able to produce an inhibition zone of 11.37 ± 0.78 mm. This inhibitory value continued to increase gradually as the test dose increased, becoming 13.08 ± 0.83 mm (50 μg/mL), 14.81 ± 0.65 mm (75 μg/mL), and 15.45 ± 0.70 mm (100 μg/mL). The maximum inhibition diameter produced by the AgNP preparation was 18.20 ± 0.81 mm at the highest concentration, namely 125 μg/mL. Referring to the standard categorization of antibacterial inhibitory strength, the entire range of AgNP concentrations tested (25 μg/mL to 125 μg/mL) consistently fell into the "Strong" inhibition category.

As a validation instrument, this test involved a negative control in the form of 5% DMSO solvent, which was proven not to produce any inhibition zone at all (0.0 mm). This confirms that the lytic activity in the bacterial cells was induced by the bioactivity of the AgNPs, not by solvent toxicity. On the other hand, the positive control (generally using a standard antibiotic) showed an inhibition zone of 35.53 ± 2.13 mm, which falls into the "Very Strong" category.

The results of this study revealed that the effectiveness of lime peel AgNPs in inhibiting the growth of P. aeruginosa increased proportionally with increasing concentration. These findings highlight the prospect of AgNPs as a promising alternative antibacterial agent in addressing the current challenge of antibiotic resistance. A visual representation of the inhibition zones produced by the AgNP preparation and the control groups against the test bacterium is presented in Figure 5.

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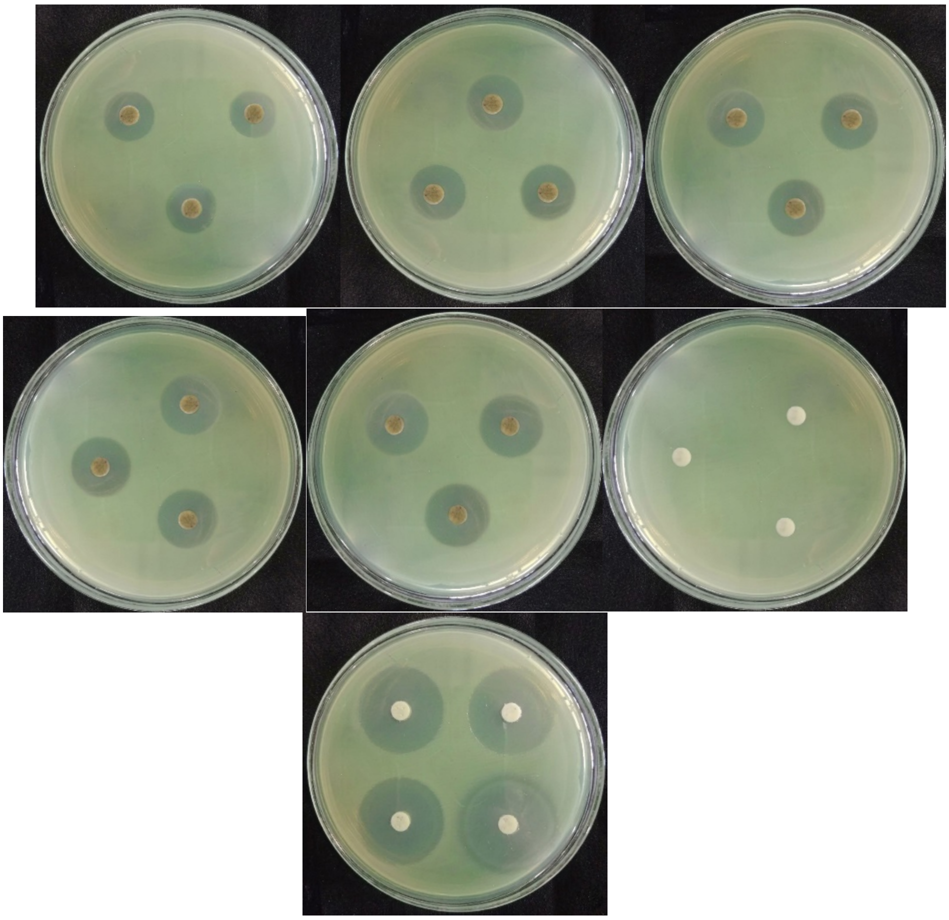

Figure 5. Inhibition zone diameters. (1) AgNPs 25 µg/mL P. aeruginosa (2) AgNPs 50 µg/mL P. aeruginosa (3) AgNPs 75 µg/mL P. aeruginosa (4) AgNPs 100 µg/mL P. aeruginosa (5) AgNPs 125 µg/mL P. aeruginosa (6) Positive control P. aeruginosa (7) Negative control P. aeruginosa

3. Analisis Statistik

Table 2. Normality test results of the inhibition zones of lime peel extract AgNPs against the test bacterium P. aeruginosa

Concentration	*Shapiro Wilk*		
	Statistik	df	Sig.
AgNPs 25 µg/ml	.928	3	.543
AgNPs 50 µg/ml	.993	3	.873
AgNPs 75 µg/ml	.997	3	.935
AgNPs 100 µg/ml	.972	3	.638
AgNPs 125 µg/ml	1.000	3	.967
Positive control	.998	3	.936

Table 3. Homogeneity test results of the inhibition zones of lime peel extract AgNPs against the test bacterium P. aeruginosa

Levene Statistic	df1	df2	Sig.
.640	4	13	.667

Table 4. One-Way ANOVA test results of the inhibition zones of lime peel extract AgNPs against the test bacterium P. aeruginosa

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Levene Statistic	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.647	4	.139	146.582	.000
Within Groups	.011	13	.001		
Total	.658	17			

Discussion

The UV-Vis spectrophotometry results showed a maximum absorption peak at around 380 nm with an absorbance approaching 2.00. This peak confirms the formation of silver nanoparticles because the characteristic Surface Plasmon Resonance (SPR) absorption of AgNPs generally appears in the visible wavelength range, and several recent reports have shown that biosynthesized AgNP peaks are found in the range of 400 to 445 nm, depending on particle size, shape, and synthesis conditions. The appearance of a peak at a relatively low wavelength in this study indicates that the particles formed were small in size and possessed optical characteristics consistent with nanoscale silver nanoparticles (Dung et al., 2021).

Scientifically, the SPR phenomenon occurs due to the collective oscillation of free electrons on the surface of metal particles when they interact with light. Therefore, the presence of a clear absorption peak indicates that Ag+ ions were successfully reduced to Ag0 by the active compounds in lime peel extract. Recent literature also confirms that the intensity and position of the UV-Vis peak can be used as an initial indicator of AgNP formation while also providing a general picture of colloidal stability and particle size. Thus, the UV-Vis data in this study support the successful biosynthesis of AgNPs using lime peel extract (Dung et al., 2021; Willian et al., 2023).

The FT-IR spectrum showed several main peaks at 3441 cm^{-1}, 2928 cm^{-1}, 1624 cm^{-1}, 1393 cm^{-1}, and 1029 cm^{-1}. These peaks indicate the presence of hydroxyl groups, aliphatic C-H, carbonyl or aromatic double bonds, carboxylate, and C-O groups originating from the secondary metabolite compounds in the extract, such as flavonoids, polyphenols, organic acids, alcohols, ethers, and carbohydrates. This absorption pattern is consistent with reports on AgNP biosynthesis using plant extracts, which show that hydroxyl and carbonyl groups play an important role in reducing silver ions while also stabilizing the particle surface (Ghani et al., 2022; Willian et al., 2023). The presence of these functional groups strengthens the assumption that the bioactive compounds in lime peel act not only as reducing agents, but also as capping agents that coat the nanoparticle surface. This coating function is important because it can reduce particle surface energy and suppress the tendency toward agglomeration. These findings are in line with recent studies stating that natural biomolecules in green synthesis play a dual role, namely reducing metal ions and stabilizing particles so that they remain at the nanoscale. Therefore, the FT-IR results in this study prove that the biosynthesis of AgNPs occurs through the active contribution of phytochemical compounds in lime peel extract (Ghani et al., 2022; Willian et al., 2023).

Based on the results of this study, silver nanoparticles (AgNPs) synthesised using lime peel extract exhibited predominantly spherical morphology, with a size range of 13–53 nm and an average size of 31 nm, and showed partial agglomeration. In terms of biological activity, all tested AgNP concentrations inhibited the growth of Pseudomonas aeruginosa, with a consistent increase in inhibitory effect as concentration increased. At a concentration of 25 μg/mL, the inhibition zone diameter was 11.37 ± 0.78 mm, and it increased to 18.20 ± 0.81 mm at 125 μg/mL. This pattern indicates a dose-dependent relationship, meaning that higher AgNP concentrations correspond to greater inhibitory effects on the test bacterium. In a biological context, this evidence confirms that biosynthesis using lime peel extract not only successfully produced

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nanoparticles at the nanoscale but also generated a material that retained antibacterial activity against opportunistic Gram-negative bacteria, such as *P. aeruginosa*.

Compared with previous studies, the average AgNP size in this study falls within a competitive range and is even smaller than that reported in several studies that also used lime-based materials. Mulyana et al. (2024) reported that the synthesis of AgNPs using lime (Citrus aurantifolia) juice powder produced an optimum particle size of approximately 68.4 nm under conditions of 1 mM $AgNO_3$, 1% extract concentration, and pH 9. In contrast, the average particle size of 31 nm obtained in this study indicates that the reduction process and nanoparticle nucleation growth occurred quite effectively. However, the results of this study are still larger than those reported by Mickky et al. (2024), who, through the optimisation of physicochemical variables using a Plackett–Burman design with Citrus sinensis peel, successfully obtained nanoparticles with an approximate size of 11.44 nm and a more uniform morphology. This difference indicates that the final size of AgNPs is strongly influenced by synthesis conditions, particularly pH, the ratio of extract to silver precursor, $AgNO_3$ concentration, incubation time, and system steadiness during particle formation. Thus, the similarity between this study and previous research lies in the successful formation of spherical AgNPs from citrus-based biomaterials, whereas the differences mainly lie in the degree of optimisation of the synthesis conditions, which ultimately determines particle size and homogeneity (Mulyana et al., 2024; Mickky et al., 2024).

The presence of partial agglomeration in the SEM micrographs is also an important aspect in interpreting the findings of this study. Theoretically, small AgNPs have a high surface area-to-volume ratio, allowing for more intensive interactions with bacterial cell walls, cytoplasmic membranes, and intracellular components. However, when particles aggregate or agglomerate, the effective surface area available to interact with bacteria decreases. Recent reviews have shown that agglomeration is a major challenge in the development of AgNPs, as it can reduce stability, alter dispersion behaviour, and affect both their toxicity and biological effectiveness. Eker et al. (2025) emphasised that physicochemical properties such as size, shape, agglomeration, concentration, and surface coating are closely related to the biological performance of AgNPs, including their antimicrobial activity and cytotoxicity. Therefore, the partial agglomeration observed in this study may help explain why, although the antibacterial activity was categorised as strong, its effect still did not approach that of the positive control (Eker et al., 2025).

The increase in antibacterial activity with increasing concentration observed in this study is consistent with trends reported in the literature over the past five years. For example, Bemis et al. (2023) reported that green-synthesised AgNPs using areca peel as a bioreductant produced inhibition zones of 11.2 mm against *Escherichia coli* and 13 mm against *Staphylococcus aureus* under certain conditions. Compared with the present study, the inhibition zone diameter of 11.37–18.20 mm against *P. aeruginosa* is quite favourable, especially since *P. aeruginosa* is generally more difficult to inhibit than *E. coli* or *S. aureus* due to its high adaptability and virulence. On the other hand, Al-Momani et al. (2023), in a study on clinical isolates of *P. aeruginosa*, reported that biosynthesised AgNPs had an MIC of 15.6 µg/mL and an MBC of 31.25 µg/mL, and were also able to reduce both planktonic growth and biofilm formation at certain subinhibitory concentrations. These data support the interpretation that the findings of this study are not merely incidental, but rather part of a consistent biological pattern: the greater the exposure to AgNPs, the stronger the antimicrobial pressure exerted on bacterial cells (Bemis et al., 2023; Momani et al., 2023).

The similarity between this study's findings and those of previous studies can also be explained by the relatively universal mechanisms of action of AgNPs. Recent studies have shown that AgNPs act through several simultaneous pathways, including disrupting cell membrane integrity, increasing membrane permeability, inducing the generation of reactive oxygen species (ROS), interfering with cellular respiration, inactivating essential proteins, and disrupting the

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replication of genetic material. In *P. aeruginosa*, these mechanisms are particularly relevant because this bacterium is well known for its ability to form biofilms and regulate virulence through the quorum-sensing system. Rodrigues et al. (2024) emphasised that AgNPs can reduce various virulence factors and quorum-sensing-related genes in *P. aeruginosa*, including decreases in biofilm formation, pyocyanin production, protease activity, and other signalling molecules. Therefore, although this study did not evaluate biofilm formation or gene expression, the increase in inhibition zone diameter across all concentration groups remains consistent with the antimicrobial mechanisms of AgNPs described in recent international studies (Rodrigues et al., 2024; Mikhailova , 2024).

However, the results of this study also showed that the inhibition zones produced by AgNPs were still considerably smaller than those of the positive control. This difference can be interpreted from several perspectives. First, the positive control, in the form of a standard antibiotic, is an agent that has been pharmacodynamically and pharmacokinetically optimised to achieve good diffusion in agar media. In contrast, nanoparticles, particularly those that partially agglomerate, exhibit limited diffusion in solid media, so inhibition zone values do not always fully reflect their actual bactericidal potential. Second, *P. aeruginosa* possesses a complex outer membrane, efflux pump systems, and a high capacity for biofilm formation, making it inherently more difficult to inhibit than many other bacteria. Third, the biomolecular layer derived from lime peel extract, which functions as a capping agent, may exert a dual effect: stabilising the particles while also potentially limiting the release of free Ag^+ ions, which play a crucial role in antimicrobial activity. Therefore, the differences between the findings of this study and some previous studies are most likely not simply due to whether the test material was “stronger or weaker,” but rather to a combination of particle size, degree of agglomeration, properties of the test medium, and the intrinsic characteristics of *P. aeruginosa* as a complex Gram-negative pathogen (Mickky et al., 2024; Eker et al., 2025; Khalifa et al., 2025).

A deeper interpretation should also acknowledge the disk diffusion method as a limiting factor in interpreting the results. In this method, the diameter of the inhibition zone does not solely reflect the intrinsic antibacterial potential of the tested agent, but is also strongly influenced by the particles' ability to diffuse through the agar medium. Therefore, AgNPs with smaller particle sizes and better stability tend to produce larger inhibition zones than larger or agglomerated particles, even though they are chemically based on the same silver composition. This explains why the present study resembles other studies in demonstrating a dose-dependent pattern, yet may still differ in the magnitude of the inhibition zone observed. In other words, the similarity in pattern reflects the consistency of the biological activity of AgNPs, whereas differences in the magnitude of the effect are more closely related to the physical characteristics of the particles and the testing methodology (Mickky et al., 2024; Eker et al., 2025).

From the perspective of developing antibacterial candidates, the first important limitation to acknowledge is the absence of MIC and MBC testing in this study. Without MIC and MBC data, this study cannot yet clearly determine the minimum concentration required to inhibit bacterial growth and the minimum concentration required to actually kill the bacteria. This distinction between bacteriostatic and bactericidal effects is particularly important, especially if AgNPs are to be developed as alternative antibacterial agents against resistant pathogens. Al-Momani et al. (2023) demonstrated that MIC and MBC data for *P. aeruginosa* provide a much stronger understanding of the biological translational potential of AgNPs than inhibition zone measurements alone. Therefore, future studies should include MIC and MBC testing to comprehensively evaluate the antibacterial activity of AgNPs synthesised from lime peel (Al-Momani et al., 2023).

The second limitation is the lack of cytotoxicity testing in mammalian cells. This aspect is critically important because high antibacterial efficacy does not automatically imply safety for biomedical applications. A study by Dell'Annunziata et al. (2024) on *Citrus limon*-based AgNPs showed that cytotoxicity evaluation using VERO and HaCaT cells can provide important insight into the safety margin of nanoparticles, with CC50 values remaining well above several biologically active concentration ranges. On the other hand, the review by Eker et al. (2025) emphasised that the cytotoxicity of AgNPs is influenced by particle size, agglomeration, surface charge, concentration, and particle stability; smaller particles tend to release more Ag^{+} ions and may therefore increase toxicity. Thus, although the findings of this study are promising for antibacterial activity, it cannot yet be concluded that lime peel-derived AgNPs are safe for biomedical use until cytotoxicity testing has been conducted (Dell'Annunziata et al., 2024; Eker et al., 2025).

The third limitation is that the antibacterial testing was restricted to only one bacterial species, namely *P. aeruginosa*. This limitation means that the antibacterial spectrum of the AgNPs cannot yet be broadly established. Previous studies have shown that responses to AgNPs may vary among bacterial species, even within the Gram-negative group itself. Mickky et al. (2024) tested citrus peel-mediated AgNPs against various microorganisms, including *Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhimurium*, and *Candida albicans*, and found that the sensitivity of each organism varied. The results confirm that this study's findings are robust for *P. aeruginosa* but cannot yet be directly generalised to other bacteria. Therefore, future studies should expand the scope of testing to include additional Gram-positive and Gram-negative bacteria to map the activity spectrum of lime peel extract-mediated AgNPs more comprehensively (Mickky et al., 2024).

Overall, the findings of this study demonstrate that AgNPs biosynthesised using lime peel extract possess physical characteristics that support antibacterial activity, particularly their spherical morphology and nanometer-scale particle size, and are capable of inhibiting the growth of *P. aeruginosa* in a concentration-dependent manner. These outcomes are consistent with previous studies regarding the successful green synthesis of AgNPs and the tendency for antibacterial activity to increase with concentration. Meanwhile, the differences observed compared with other studies may mainly be explained by variations in synthesis parameters, particle size, degree of agglomeration, testing methods, and the microbial species used. By acknowledging the limitations of the present study, including the absence of MIC/MBC testing, cytotoxicity analysis, and broader bacterial-spectrum testing, this work nevertheless provides a strong scientific basis for the further development of lime peel extract-derived AgNPs as a natural product-based alternative antibacterial candidate (Mickky et al., 2024; Eker et al., 2025; Khalifa et al., 2025).

Conclusion

Based on the results of this study, it can be concluded that lime peel extract (Citrus aurantifolia Swingle) was successfully used as both a bioreducing and stabilising agent in the green synthesis of silver nanoparticles (AgNPs). The success of the synthesis was confirmed by physicochemical characterisation, which revealed that the nanoparticles formed had a predominantly spherical morphology, with a size distribution of 13–53 nm and an average size of 31 nm, although partial agglomeration was still observed. These data confirm that lime peel extract can facilitate the formation of AgNPs at the nanoscale. The biosynthesised AgNPs exhibited strong antibacterial activity against Pseudomonas aeruginosa at all tested concentrations (25, 50, 75, 100, and 125 µg/mL). The diameter of the inhibition zone increased gradually from 11.37 ± 0.78 mm at 25 µg/mL to 18.20 ± 0.81 mm at 125 µg/mL, showing a dose-dependent relationship. The negative control showed no inhibitory effect, whereas the positive control produced a larger inhibition zone, confirming that the observed antibacterial activity was attributable to the synthesised AgNPs. Statistical analysis showed that the data were normally distributed and homogeneous, and that there were significant differences among the treatment groups based on the One-Way ANOVA test (p = 0.000). This indicates that changes in AgNP concentration significantly affected the magnitude of inhibitory activity against P. aeruginosa. Therefore, biosynthesised AgNPs using lime peel extract have the potential to be developed as a natural product-based alternative antibacterial agent, particularly against opportunistic Gram-negative bacteria. However, this potential still needs to be further supported by future studies that include MIC/MBC testing, cytotoxicity analysis, and evaluation against a broader spectrum of bacteria.

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