Walisongo Journal of Chemistry
Walisongo Journal of Chemistry Vol. 7 Issue 2 (2024), 168-180
ISSN: 2621-5985 (online); 2549-385X (print)
DOI: https://doi.org/10.21580/wjc.v7i2.22996

BIOSYNTHESIS OF SILVER MICROPARTICLES USING Spondias dulcis FRUIT PEEL EXTRACT AND ITS ANTIBACTERIAL ACTIVITY

Thereshia Margaretta Girsang[1], Elisabeth Purba[2], Adela Rosalia Simamora[1], Chatrina Yohana Sihombing[1], Marchella Inabuy[1], Candra Yulius Tahya[1,2*]

[1]Biology Education Study Program, Faculty of Education, Universitas Pelita Harapan, Tangerang, Indonesia
[2]Chemistry Education Study Program, Faculty of Education, Universitas Pelita Harapan, Tangerang, Indonesia

***Corresponding author:** candra.tahya@uph.edu

Abstract

In recent decades, the overuse and misuse of antibiotics, along with various social and economic factors, have accelerated the spread of antibiotic-resistant bacteria, including Klebsiella pneumoniae *and* Pseudomonas aeruginosa*, both of which are Gram-negative pathogenic bacteria. Silver particles (AgPs) have garnered significant research interest over the years due to their diverse biological activities, particularly their antibacterial properties. The green synthesis method for silver particles involves synthesizing silver metal particles using natural materials derived from organisms such as plants, resulting in particles that are less harmful to human cells but highly toxic to pathogenic bacteria.* Kedondong (Spondias dulcis) *is a tropical fruit widely grown in South and Southeast Asia. The peels of this fruit often become organic waste with limited utility. To explore the potential of* kedondong *fruit peels, this study investigated the synthesis of silver particles using their extract. The ethanolic extract of* kedondong *fruit peels was analyzed using LC-MS/MS-QTOF, identifying 5 alkaloids, 21 flavonoids, and 17 terpenoid compounds. The total flavonoid and phenolic contents of the extract were determined to be 1.8918 and 12.8104 mg/g of extract, respectively. The silver particles synthesized in this study had an average size of 4641.97 micrometers and a zeta potential of 40.2 mV, as determined by PSA, and were confirmed as silver particles through P-XRD phase analysis. These silver particles exhibited strong antibacterial activity against* P. aeruginosa*, with an inhibition zone diameter of 19.43 mm, and moderate activity against* K. pneumoniae*, with an inhibition zone diameter of 11.50 mm, at a suspension concentration of 10 mg/mL. Notably, the* P. aeruginosa *strain used in this experiment was resistant to the antibiotic amoxicillin.*

Keywords: *Antibacterial,* Spondias dulcis*, Silver particles.*

Introduction

Antibiotics have been instrumental in treating several bacterial infection-related disorders, including meningitis and bacteremia, which were previously incurable and lethal. However, societal and economic factors, coupled with the abuse and misuse of medicines, have accelerated the spread of antibiotic-resistant bacteria in recent decades. In response to the rise in antibiotic resistance, the World Health Organization (WHO) published a list of pathogens under the acronym ESKAPE (where K represents *Klebsiella pneumoniae* and P stands for *Pseudomonas aeruginosa*). These pathogens hold "priority status" because they seriously threaten human health (Mancuso et al., 2021; Varela et al., 2021). *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are Gram-negative pathogenic bacteria. *P. aeruginosa* is a common pathogen responsible for acute and chronic nosocomial infections, including severe respiratory tract infections in patients with compromised host defenses. Meanwhile, *K. pneumoniae*, particularly in individuals with weakened immune systems, can cause a range of nosocomial and community-acquired illnesses, such as bloodstream infections, pneumonia, liver abscesses, urinary tract infections, and surgical site infections (Chellaiah, 2018; Irawati et al., 2022; Tahya & Ratnaningsih, 2015).

Silver particles (AgPs) have garnered significant research interest over the past decades due to their diverse biological activities, including antibacterial, antiviral, anticancer, anti-inflammatory, and antifungal properties (Almatroudi, 2020; Bruna et al., 2021; Dakal et al., 2016; Jun et al., 2010; Kumari G & S, 2016; Lu et al., 2020; Mathur et al., 2018). As an antibacterial agent, AgPs show potential for treating infectious bacterial diseases and combating multidrug-resistant pathogens, a growing concern responsible for numerous deaths annually. This effectiveness is related to the mechanism of action of AgPs, which target multiple bacterial structures simultaneously (Dakal et al., 2016). The synthesis of AgPs with desired shapes and sizes can be achieved using physical, chemical, and biological methods. However, physical and chemical methods often present environmental challenges due to the use of toxic chemicals and high production costs.
Biological methods for AgP synthesis emphasize green synthesis, which is faster, environmentally safe, easy, energy-efficient, and cost-effective, offering a promising alternative to physical and chemical methods. Green synthesis of AgPs has demonstrated lower solubility, yield, stability, and toxic potential compared to conventional methods. It can be conducted under milder conditions, such as low temperatures, safer solvents, and less harmful reagents, enhancing the biocompatibility and adaptability of AgPs in healthcare applications. The synthesis of AgPs from silver ions using biological extracts can be facilitated by various functional groups, such as carbonyl, amine, urea, and phenolics found in terpenoids, alkaloids, flavonoids, polyphenols, pigments, proteins, polysaccharides, fatty acids, and other reducing agents. These reactions occur both intercellularly and extracellularly (Asmathunisha & Kathiresan, 2013; Castro et al., 2015; Coblenz & Wolf, 1994; Elamawi et al., 2018; Gurunathan et al., 2009; Harada & Misawa, 2009; Ismail et al., 2021; Tahya et al., 2024; Urnukhsaikhan et al., 2021).

Kedondong (*Spondias dulcis*) is a tropical fruit that grows widely in South and Southeast Asia. In Indonesia, it is readily available and commonly consumed on a daily basis. *Kedondong* contains vitamin B, antioxidant flavonoids, polyphenols, and compounds with antibacterial and anticancer properties. One of the potential applications of *kedondong* peel is as a source of pectin (Clarissa et al., 2019). It is a natural polysaccharide extracted from fruits, primarily composed of chains of *D-galacturonic acid* units linked by glycosidic bonds. A more detailed composition also includes small amounts of covalently bound rhamnose, with branches of *L-arabinose*, *D-galactose*, *D-xylose*, and *L-rhamnose* (Clarissa et al., 2019; Rakhmawati & Yunianta, 2015). The alcohol functional group in pectin is an efficient Ag^{+} reductant (Gurunathan et al.,

 | ISSN 2621-5985 (online) | ISSN 2549-385X (print)
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2015; Ndikau et al., 2017; Pallavicini et al., 2017).

The green synthesis method for silver particles is a technique that involves synthesizing silver metal particles using natural materials derived from organisms such as plants and microorganisms. Various plants contain chemical compounds with the potential to act as reducing agents. Recent research has been conducted on synthesizing silver particles using plant extracts as a reducing medium for silver ions. Correspondingly, *Kedondong* fruit peel is one such reducing agent that can be used to synthesize silver particles.

Methodology

Time and place of research

This research was conducted over four months, starting from receiving the *PKM* (Student Creativity Program) funding, precisely from April to July 2024. The process included proposal development, final report writing, and preparation of a publication draft. The research took place at the Chemistry Laboratory and the Food Technology Quality Control (QC) Laboratory of Universitas Pelita Harapan, Tangerang.

Tools and materials

The materials used in this research included *kedondong (Spondias dulcis)* fruit obtained from local vendors. The bacteria *P. aeruginosa* and *K. pneumoniae* were sourced from the Microbiology Laboratory of Universitas Pelita Harapan. Additional materials contained ethanol p.a (Merck), Folin-Ciocalteu reagent (Merck), gallic acid (Merck), Na_2CO_3 (Merck), $AgNO_3$ (Merck), aluminum chloride (Merck), quercetin (Merck), and Luria Broth Agar (LBA). Laboratory equipment included glassware such as test tubes, Erlenmeyer flasks, Petri dishes, and beakers from various brands, as well as non-glass equipment such as spatulas, magnetic stir bars, micropipettes, tips, 2 mL microtubes, 15 mL centrifuge tubes, ovens, hotplates, FTIR (Bruker Alpha II), P-XRD (Rigaku Miniflex 600, Cu, 1D-DteX Ultra detector, Kβ filter), PSA (HORIBA SZ-100), and LC-MS/MS-QTOF (Waters Acquity UPLC I-Class with Xevo G2-XF QTOF).

Spondias dulcis fruit peel extraction

Approximately 5 kg of *kedondong* fruit peel waste was dried in an oven at 60°C for three days and then ground using a dry blender. Approximately 200 g of the dried powder was mixed with 1000 mL of ethanol in a flask, sealed, stirred for 2 × 6 hours, and left to stand for two days at room temperature. The mixture was then filtered to obtain the ethanol extract. The solvent was completely evaporated to produce a concentrated extract, which was further dried at 65°C for 2 × 24 hours and then weighed. Approximately 2.0 g of the crude extract was set aside for LC-MS/MS analysis and total flavonoid-phenolic content analysis.

LC-MS/MS-QTOF analysis

To prepare the test sample, 0.1 g of extract was placed into a 10 mL volumetric flask, ethanol solvent was added to the mark, and the solution was sonicated ultrasonically for 30 minutes and homogenized. The solution was then filtered using a 0.22 µm GHP/PTFE filter membrane before being processed into the LC-MS/MS-QTOF instrument. The LC setup utilized a C1 column at 40°C, with an autosampler temperature of 15°C and an injection volume of 10 µL. The mobile phase consisted of 0.1% formic acid in acetonitrile (mobile phase A) and 0.1% formic acid in distilled water (mobile phase B), with a gradient flow rate of 0.6 mL/min.

The MS settings for the ToF mode used ESI (-)/ESI (+) ionization, with an acquisition range of 50–1200 Da. The UNIFI software, equipped with a mass spectrum library, was employed to screen for active compounds in the natural ingredients. Active compounds were detected if the criteria were met, including a mass error ≤ 5 ppm, MZ Isotope Match RMS % and PPM < 10%, and analyte intensity ≥ 300. The data collected was analyzed with reference to literature reviews.

Total phenolic content

 | ISSN 2621-5985 (online) | ISSN 2549-385X (print)
Volume 7, Nomor 2, 2024

The total phenolic content was calculated using the Folin-Ciocalteu reagent and a spectroscopic technique. Total phenolic content was expressed as milligrams of gallic acid equivalents (mg GAE) per gram of dry sample. Various concentration series were prepared using gallic acid. To prepare the solutions, 0.3 mL of each gallic acid solution was combined with 1.5 mL of 10% Folin-Ciocalteu reagent and 1.2 mL of 7.5% Na_2CO_3, vortexed, and kept at room temperature for 30 minutes in a dark area. A blank solution was also prepared. Absorbance was recorded at 765 nm using a spectrometer. A 1000 ppm extract solution was prepared and subjected to the same process as the gallic acid solutions to determine absorbance in a triplicate experiment. Subsequently, data analysis was performed using the MS Excel program.

Total flavonoid content

The total flavonoid content of the ethanol extract was determined using the aluminum chloride method. A 2 mL sample was mixed with 2 mL of 2% aluminum chloride solution and vortexed. Serial concentrations of quercetin were used to create a calibration curve. The extract was dissolved in ethanol to a concentration of 1000 ppm. The absorbance of the mixture was measured at 415 nm using a spectrophotometer in triplicate experiments. Total flavonoid content was expressed in quercetin equivalents (mg QE/g sample). Afterward, data analysis was performed using the MS Excel program.

Synthesis of silver particles

The crude extract was dissolved in ethanol at various concentrations. The ethanol extract solution was added to a 0.1 mM silver nitrate solution in a 250 mL volumetric flask and stirred for 6 hours daily over 2 days using a magnetic stirrer at room temperature. A color change after 2 days indicated the reduction of silver nitrate to silver nanoparticles (denoted as AgP). The AgP suspension was centrifuged at 10,000 rpm for 5 minutes, and the resulting pellets were collected (Tahya et al., 2024). The pellets were dried in an oven at 70˚C for 3 hours. The AgP powder was characterized using X-ray Diffraction (P-XRD) and Fourier Transform Infrared (FTIR) spectroscopy. The Particle Size Analyzer (PSA) was then used to determine the particle size and zeta potential of the AgP suspension.

Antibacterial activity test

Antibacterial activity was tested using the disc diffusion method. A total of 6 g of Luria Broth Agar (LBA) was dissolved in 150 mL of distilled water and sterilized by autoclaving at 121°C for 15 minutes. 20 mL of LBA liquid was poured into a sterile petri dish and allowed to solidify. The LBA medium was then inoculated with 20 µL of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* suspensions. 20 µL of samples, including a 10,000 ppm crude extract solution and AgP suspension, were dropped onto paper discs. The plates were incubated at 30°C for 24 hours. The antibacterial activity was assessed by measuring the diameter of the inhibitory zones (DDH) around the paper discs using a digital caliper (Elemike et al., 2017).

Results and Discussion

Analysis of total phenolics and total flavonoids

The weight of the extract used for the total flavonoid and phenolic tests was 0.2533 g, dissolved in 25 mL of ethanol, resulting in a total extract concentration of 10,132 ppm. The results of the total phenolic and total flavonoid tests are presented in Tables 1 and 2. Based on these tests, the average phenolic concentration obtained from the three samples was 12.8104 mg/g of extract, while the flavonoid concentration was 1.8918 mg/g. The presence of phenolic and flavonoid compounds indicates various medicinal properties, including the ability to reduce excessive Reactive Oxygen Species (ROS). This reduction highlights their potential as antibacterial and anticancer agents.

The LC-MS/MS-QTOF chromatogram of the extract is shown in Figure 1.

Table 1. Total phenolic test results of *S. dulcis* fruit peel extract samples

Sample Code	Concentration of Total Phenolics of the Extract	
	ppm	mg/g
S1	127.6154	12.5953
S2	132.2307	13.0507
S3	129.5384	12.7851
Average	129.7948	12.8104

Table 2. Total flavonoid test results of *S. dulcis* fruit peel extract samples

Sample Code	Concentration of Total Flavonoid of the Extract	
	ppm	mg/g
S1	19.0546	1.8806
S2	19.2557	1.9005
S3	19.1928	1.8943
Average	19.1677	1.8918

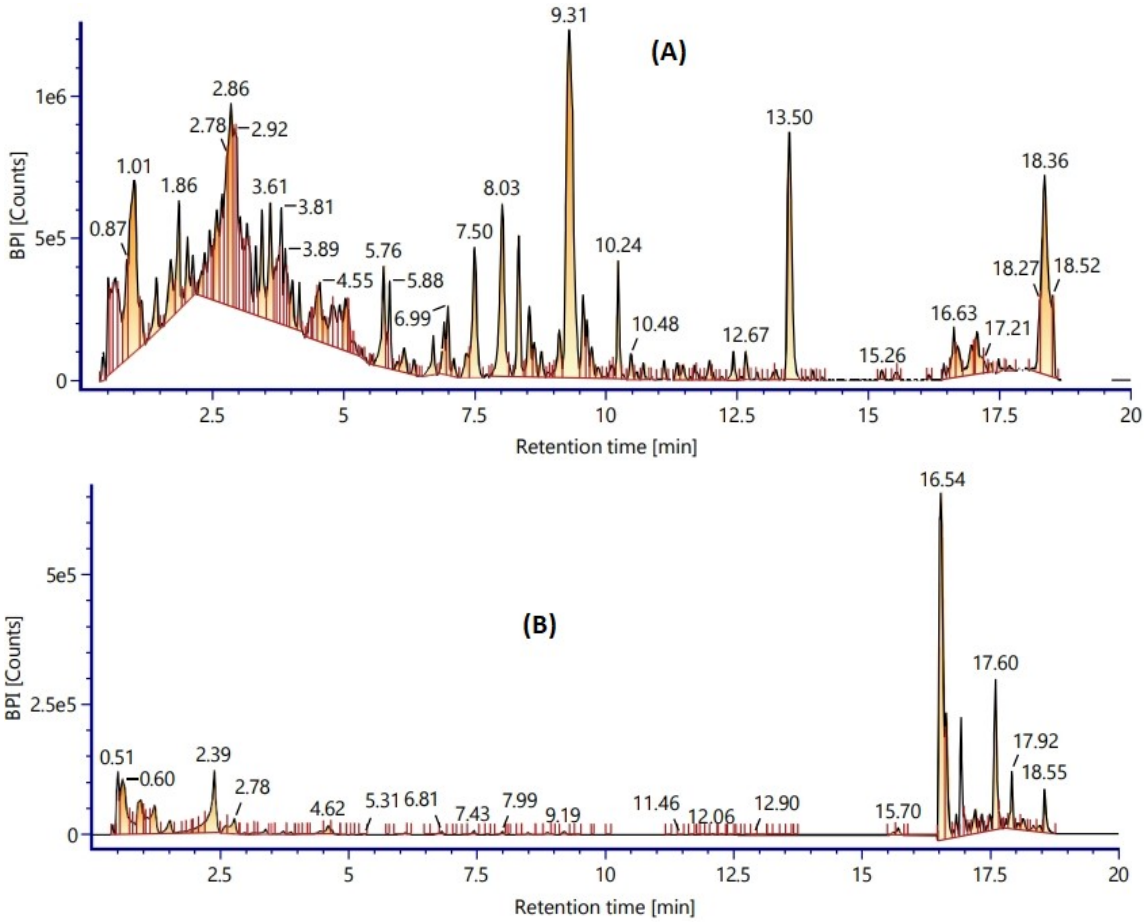

Figure 1. (A) Chromatogram of LC-MS/MS-QTOF ESI (-) mode and (B) Chromatogram of LC-MS/MS-QTOF ESI (+) mode for the ethanolic crude extract of *S. dulcis* fruit peels

Table 3. List of compounds identified in the ethanolic crude extract of *S. dulcis* fruit peels based on LC-MS/MS-QTOF analysis

No.	ESI Mode	Identified Compounds	Analysis Methods
		ALKALOIDS	
1	(+)	6-Isoinosine	18-16-2/MU/SMM-SIG (LC-MS/MS-QTOF)
2	(+)	Dihydro-N-methylisopelletierine	
3	(+)	Gentiatibetine	
4	(+)	Hypoxanthine	
5	(+)	Vitamin B2	
		FLAVONOIDS	
1	(-)	2′,7-Dihydroxy-4′,5′-dimethoxyisoflavone	18-16-2/MU/SMM-SIG (LC-MS/MS-QTOF)
2	(-)	5-Hydroxy-7,8-dimethoxyflavone	
3	(-)	6-Formyl-isoophiopogonanone A	
4	(-)	6-Hydroxykaempferol-3-O-glucoside	
5	(-)	7-Hydroxy-1-methoxy-2-methoxyxanthone	
6	(-)	Daidzin_1	
7	(-)	Dihydrokaempferol-5-O-β-D-glucopyranoside	
8	(-)	Dihydromorin	
9	(-)	Genistein	
10	(-)	Irisflorentin	
11	(-)	Jaceosidin	
12	(-)	Kaempferol	
13	(-)	Malvidin 3,5-diglucoside	
14	(-)	Myricetin	
15	(-)	Nevadensin-7-O-[α-L-rhamnosyl(1→6)]-β-D-glucoside	
16	(-)	Onjixanthone II	
17	(-)	Quercetagetin	
18	(-)	Quercetin-7-O-[β-D-glucopyranosyl(1→6)-β-D-glucopyranoside]	
19	(-)	Rhamnocitrin-3-O-rhamnoside	
20	(-)	Silandrin	
21	(-)	Undulatoside A	
		TERPENOIDS	
1	(+)	(24S)-Pseudoginsenoside RT4	18-16-2/MU/SMM-SIG (LC-MS/MS-QTOF)
2	(+)	E-p-Coumatic acid	
3	(-)	10-O-Acetylgeniposidic acid	
4	(-)	20(R)-Ginsenoside Rh2	
5	(-)	3-O-α-L-Rhamnopyranosyl-(1→2)-α-L-arabinopyranosylgypsogenin	
6	(-)	9-Hydroxylinalool-9-β-D-glucopyranoside	
7	(-)	Andrographatoside	
8	(-)	Anemonin	
9	(-)	Aucubin	
10	(-)	Bruceine I	
11	(-)	E-p-Coumatic acid	
12	(-)	Genipin	
13	(-)	Mudanpioside G	
14	(-)	Pseudosantonin	
15	(-)	Schizonepetoside B	
16	(-)	Semiaquilegoside A	
17	(-)	trans-Carveol-6-β-glucopyranoside	

The LC-MS/MS-QTOF instrument successfully identified 5 alkaloids, 21 flavonoids, and 17 terpenoid compounds, as listed in Table 3.

Synthesis results of silver particles

The synthesis of silver particles was conducted using two different solvent conditions. In the initial treatment, $AgNO_3$ was dissolved in distilled water to produce an Ag^+ solution with a concentration of 0.1 mM, which was then mixed with a solution of *kedondong (S. dulcis)* bark ethanol extract at a concentration of 5000 ppm in ethanol solvent (p.a.). The mixture was stirred for approximately six hours per day over two days. During this process, the color changed from brown to blackish brown. The resulting mixture was centrifuged to obtain a black solid, which was subsequently tested for particle size using a Particle Size Analyzer (PSA) HORIBA SZ-100. The analysis revealed a particle size >10,000 nm (>10 micrometers) and a zeta potential (see Table 4). This sample was coded as AgP_W. To reduce the particle size, modifications were made by adjusting the concentration of the extract and changing the solvent used to dissolve the $AgNO_3$. In this step, $AgNO_3$ was dissolved in hot ethanol until fully dissolved, producing an Ag^+ solution with a concentration of 0.1 mM. *Kedondong* fruit peel extract was also dissolved in ethanol (p.a.). The raw extract was used at a concentration of 1000 ppm. During the reaction process for synthesizing silver particles, two variations of the final extract concentration were employed: 750 ppm (coded AgP_Et_750) and 500 ppm (coded AgP_Et_500). The resulting silver particles were finer than those synthesized with distilled water as the solvent. The AgP_Et_750 silver particles were subsequently tested for particle size using PSA, as shown in Figure 2. The measurement results are presented in Table 4.

Based on the PSA data, the synthesized silver particles had an average particle size of 4641.97 micrometers (μm), qualifying them as silver microparticles. These silver microparticles were then analyzed using P-XRD and FTIR instruments. The results of the XRD analysis confirmed that the particles formed were silver. The FTIR analysis identified the functional groups present, as shown in Figures 3 and 4. The XRD 2-theta diffraction peaks observed around 20–40° (peaks [2] and [3] in Figure 3) were unique peaks corresponding to silver particles (Rautela et al., 2019; Vanaja & Annadurai, 2013). Meanwhile, the FTIR spectrum exhibited the presence of strong peaks corresponding to O–H stretch vibrations (3425–3418 cm^{-1}), C–H stretch vibrations (3000–2850 cm^{-1}), C=O stretch vibrations (1785–1710 cm^{-1}), C=C/C=N stretch vibrations (1700–1605 cm^{-1}), and C–O stretch vibrations (1320–1000 cm^{-1}) (Zhang et al., 2017) in both the silver particles and the crude extract.

Table 4. PSA test results for silver particle samples

Parameter	AgP_W			Average	AgP_Et_750			Average
	1	2	3		1	2	3	
Z-Average (nm)	>10000	>10000	>10000	>10000	4253.9	5037.7	4634.3	4641.97
Polydispersity Index (PI)	nd	nd	nd	-	1.368	1.437	1.417	1.407
Zeta Potential Mean (mV)	-25.7	-26.5	-25.7	25.97	32.0	41.2	47.4	40.2

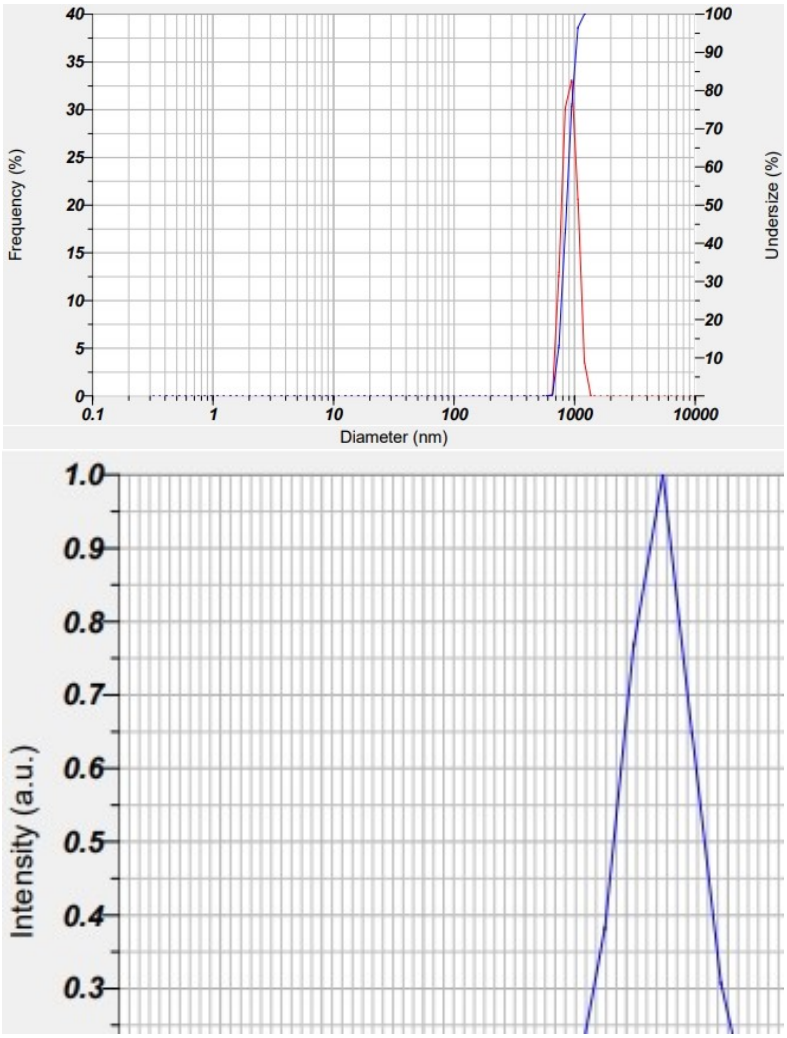

Figure 2. Particle size analysis (top) and zeta potential (bottom) of silver microparticles in the AgP_Et_750 sample

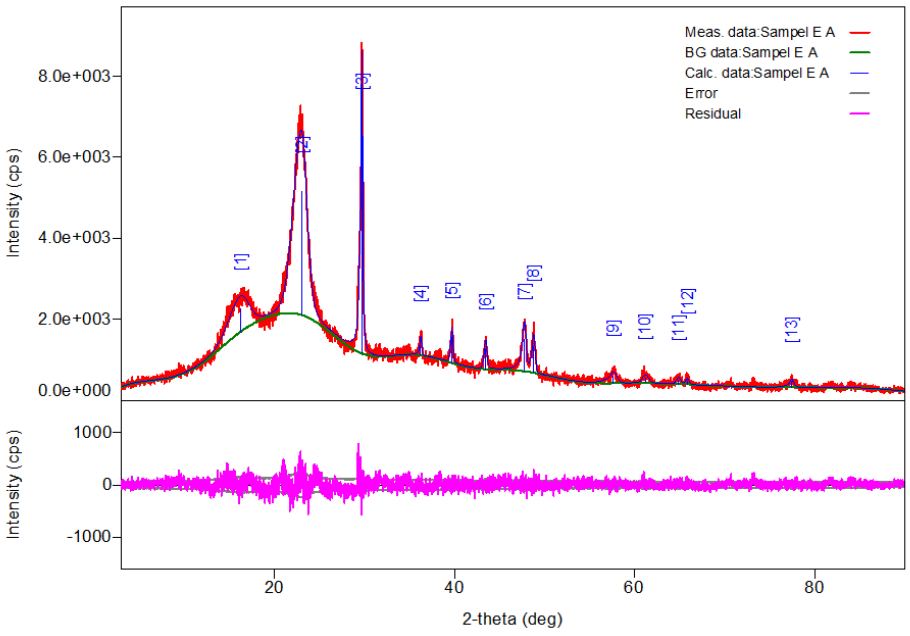

Figure 3. P-XRD diffractogram of the silver microparticle

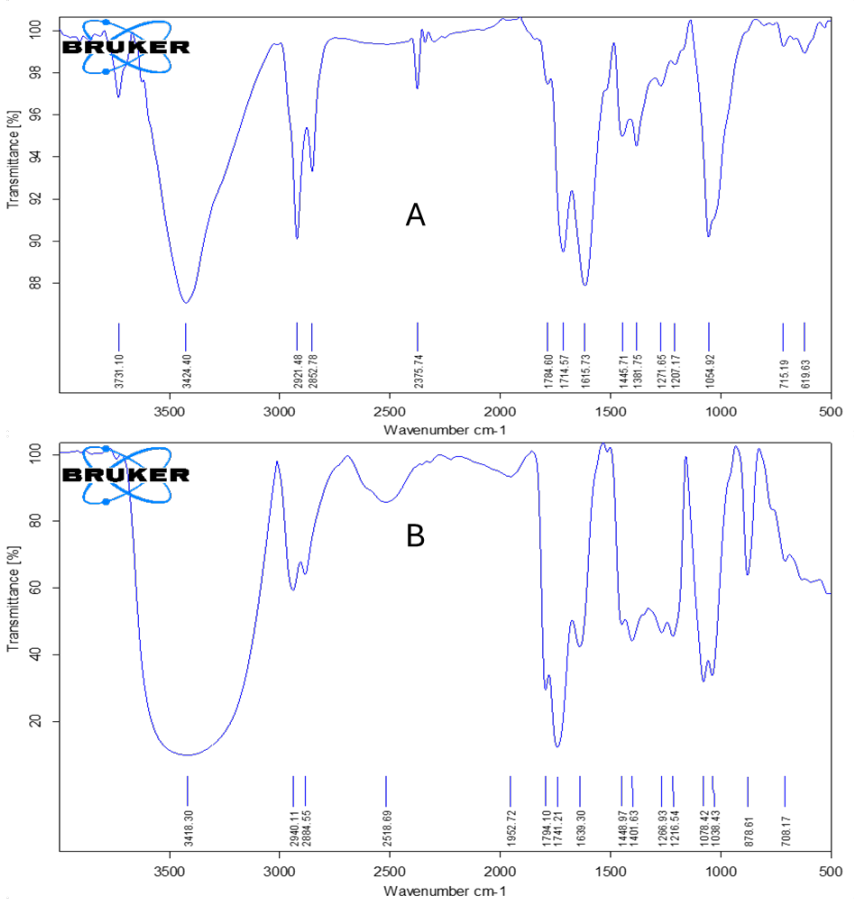

Figure 4. FTIR spectrum of the silver microparticle (A) and crude extract (B)

Table 5. Antibacterial test results of silver microparticles from *kedondong* fruit peel extract

Bacteria	Inhibition Zone Diameter (mm)			
	Extract (10,000 ppm)	**AgP_W Suspension (10 mg/mL)**	**AgP_Et_750 Suspension (10 mg/mL)**	**Ag_Et_500 Suspension (10 mg/mL)**
Klebsiella pneumoniae	5.84	7.44	11.15	10.10
Pseudomonas aeruginosa	8.19	14.05	19.34	18.38

Table 6. Effect of silver microparticle suspension concentration on inhibition zone diameter

Bacteria	Inhibition Zone Diameter (mm)			
	10 m/mL AgP_Et_750 suspension	1 mg/mL AgP_Et_750 suspension	0.1 mg/mL AgP_Et_750 suspension	0.01 mg/mL AgP_Et_750 suspension
Klebsiella pneumoniae	11.50	10.32	7.14	7.08
Pseudomonas aeruginosa	19.43	17.69	11.36	6.51

Antibacterial test results

This study evaluated the antibacterial activity of silver particles synthesized from *kedondong* fruit peel extract against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. The disc diffusion method was employed, where silver particles derived from *kedondong* fruit peel extract were applied to paper discs and subsequently placed on agar inoculated with *P. aeruginosa* and *K. pneumoniae*. These bacterial strains were previously isolated and identified in studies conducted by our lecturers. All silver microparticles were suspended in DMSO to achieve a concentration of 10 mg/mL. The results are presented in Figure 5 and Table 5. Additionally, the concentration of the silver suspension was varied to evaluate its effect on the inhibition zone diameter; these results are detailed in Table 6.

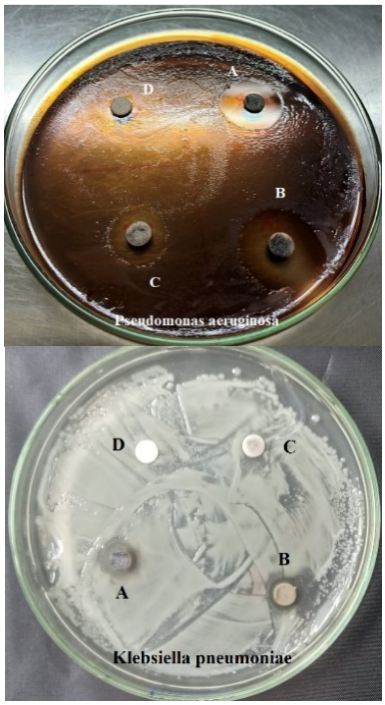

Figure 5. Antibacterial activity of silver particles against *P. aeruginosa* and *K. pneumoniae*: (A) AgP_Et_750, (B) AgP_Et_500, (C) AgP_W silver microparticles, and (D) 10,000 ppm *kedondong* peel extract.

The silver particles developed in this study exhibited strong inhibitory effects on the growth of *P. aeruginosa* and moderate inhibitory effects on the growth of *K. pneumoniae*. The *P. aeruginosa* strain used in this experiment was known to be resistant to the antibiotic amoxicillin, as demonstrated in previous studies (Tahya et al., 2024).

Conclusion

Using LC-MS/MS-QTOF analysis, the ethanolic extract of *kedondong (Spondias dulcis)* fruit peel revealed the presence of 5 alkaloids, 21 flavonoids, and 17 terpenoids. The average size of the silver particles produced in this study was 4641.97 micrometers, with a PSA-based zeta potential of 40.2 mV. P-XRD phase analysis confirmed that the particles were indeed silver. At a suspension concentration of 10 mg/mL, the silver particles significantly inhibited the growth of *P. aeruginosa*, producing an inhibition zone diameter of 19.43 mm, and moderately inhibited the growth of *K. pneumoniae*, with an inhibition zone diameter of 11.50 mm. These findings confirmed the strong antibacterial activity of the silver particles against *P. aeruginosa* and their moderate activity against *K. pneumoniae*. The *P. aeruginosa* strain used in this study was resistant to amoxicillin, as shown in prior experiments. Reducing the size of the silver particles to the nanometer scale might further enhance their antibacterial potential.

Acknowledgment

The authors extend sincere gratitude to the 2024 Student Creativity Program (*PKM*), funded by the Directorate General of Higher Education, Research, and Technology through the Directorate of Learning and Student Affairs, for supporting this research. The authors also thank the Student Life Department of Universitas Pelita Harapan for providing additional funding. Furthermore, the authors are grateful to the Center for Research and Community Service for their assistance in disseminating and publishing this article.

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