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Author(s): Johan Sukweenadhia, Kezia Irianti Setiawanb, Christina Avantib, Kartini Kartinib, Esrat Jahan Rupac, Deok-Chun Yang

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Scale-Up of Green Synthesis and Characterization of Silver Nanoparticles Using Ethanol Extract of *Plantago major* L. Leaf and Its Antibacterial Potential: A Recent Study

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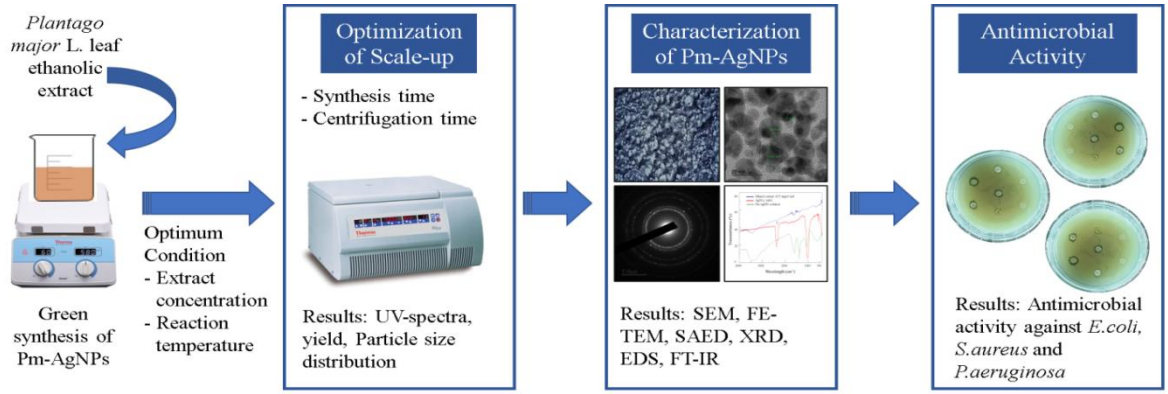
ABSTRACT

Green synthesis of silver nanoparticles (Ag NPs) utilizing plant extracts has been widely optimized these days because of its eco-friendly, simple, and cost-effective manner. This present study was performed to evaluate the scale-up trial on synthesis parameter and centrifugation duration of *Plantago major* L. leaf ethanolic extract in the green synthesis of Ag NPs. Leaf extract concentration of 0.25%, 70^{o}C of temperature, 60 min of synthesis time, and 30 min of centrifugation time were concluded as the optimized scale-up condition of green synthesis. The scale-up trial resulted in spherical silver nanoparticles with an average size of 12.2±5.11 nm, proven from various spectroscopic (UV-Vis, EDS, FTIR), microscopic observations (SEM, FE-TEM), and other observations (SAED, DLS, XRD). The synthesized Ag NPs exhibit good antibacterial activity against several tested bacteria at 20 µg mL^{-1} of dosage. This study offers a nine times higher yield of the synthesized Ag NPs (107.2±6.82 mg) at about the same time as a smaller scale of green synthesis. Sufficient nanoparticles will provide flexibility to carry out its characterization efficiently and further bioactivity test and formulation experiments, such as in cosmetics, medical ointments, and other pharmaceutical products.

GRAPHICAL ABSTRACT



Keywords: Eco-friendly; higher yield; optimized scale-up; synthesized Ag NPs.

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HIGHLIGHTS

- Scale-up optimization was done for green synthesis of Ag NPs from *Plantago major* L.
- Optimization process yielded almost nine times higher Ag NPs.
- Well-dispersed spherical and Ag NPs with sizes ranging from 10-20 nm were obtained.
- Synthesized Ag NPs show promising antibacterial activities.

ABBREVIATIONS

DLS : Dynamic Light Scattering;
EDS : Energy-dispersive X-ray spectroscopy;
FE-TEM : Field Emission Transmission Electron Microscope;
FTIR : Fourier Transform Infra-Red spectroscopy;
Pm-Ag NPs : green synthesized silver nanoparticles using Plantago major L. leaf extract;
SAED : selected area diffraction pattern;
SEM : Scanning Electron Microscope;
UAE : Ultrasonic-Assisted Extraction;
XRD : X-ray diffraction.

1. INTRODUCTION

The benefits of *Plantago major L.* (after this called *P. major*) as folk medicines have been acknowledged globally for years. This plant has several bioactive compounds, including alkaloids, fatty acids, flavonoids, terpenoids, phenolic acid derivatives, vitamins, etc., which contribute to its specific therapeutic effects [1,2,3]. Leaf of *P. major* (called as "*Daun Sendok*" in Indonesia) has been widely known for its efficacy on wound healing empirically [4,5,6] among many other efficacies [1,7]. The plants, which are usually considered weeds on a daily basis, also have antibacterial [8] and antioxidant activities [9]. Based on the phytochemical analysis, it was found that plantamajoside and polyphenol content were high in its ethanol extract [7,10]. Polyphenol content is thought to be a responsible component in the wound healing process [3], whereas plantamajoside, a caffeic acid derivative, is known to have biological activities as an anti-inflammatory, antioxidant and antibacterial agent [5,10].

Nanoparticles are commonly used to increase several characteristics such as target specificity, permeability, drug activity, and bioavailability, solubility, stability, and pharmacological effects [11]. The current trend is metal nanoparticles, of which the most common are silver nanoparticles. Silver nanoparticles (Ag NPs) are widely used for antibacterial, anticancer, anti-inflammatory, and wound treatment [12]. The synthesis of nanoparticles can be done by physical, chemical, and biological methods. Physical and chemical methods were avoided due to environmental issues, while the biological method or so-called green synthesis is preferred because it is eco-friendly.

Moreover, the nanoparticle produced by the biological method is more valuable than the chemical method due to the non-existence of toxic organic residuals, minimum wastages, high volume of production, and repeatability [13]. The biological method can be conducted via plants, bacteria, yeasts, and viruses. It is known that proteins, amino acids, organic acids, vitamins, and plant secondary metabolites such as flavonoids, alkaloids, terpenoid, heterocyclic components, and polysaccharides have an important role in the synthesis of metal nanoparticles, acting as reducing and capping agents [14]. The use of plants for the synthesis of green nanoparticles is more beneficial for the resources of microorganisms [15,16].

Several previous results reported the green synthesis of nanoparticles from *P. major* with various extraction methods, solvent, plant parts, metal nanoparticles, and optimization conditions. Poor et al. [17] synthesized around 432 nm of Ag NPs utilizing water extract of Plantago seeds and tested on its cytotoxic activity. Lohrasbi et al. [18] made predominantly spherical Ag NPs with sizes ranging 4.6–30.6 nm from Plantago leaf extract and checked its decolorization activity. They used the boiling method and reflux on their way to prepare the extract, respectively. Küünal et al. [19] investigated two different conditions during the extraction of Plantago aerial parts. They concluded aqueous extraction

with the thermal condition and ethanol extraction with UV radiation, resulting in Ag NPs with a 50-100 nm range of size with antibacterial activities. Nikaeen et al. [20] confirmed the central composite design (CCD) prediction to optimize the Ag NPs synthesis from aqueous extract of Plantago seeds and yielded nanoparticles on 11 to 32.3 nm range of size.

One of the challenges that arise with green synthesis is controlling the shape and size of nanoparticles [21]. Those factors strongly affect the physical, optical, and catalytic properties of Ag NPs hence determining the application [22]. All the previous work reported various but limited *in vitro* applications of the Ag NPs from Plantago. Increasing the production scale needs to be done to fulfill the need to carry out further activity tests and toxicity tests. However, the scale-up trial of green synthesis is not reported yet. Ag NPs have been synthesized without any toxic reducing agent using ethanol extract of P. major leaf extract in this study. Up-scaling was carried out by increasing the synthesis volume and the centrifugation volume. The initial stage of the research was to optimize several synthesis parameters. The goal was to obtain the minimum time to produce an optimal amount of silver nanoparticles yield on upscale process. The final products (Pm-Ag NPs) were characterized by several spectroscopic and microscopic methods, and its antibacterial activity against Gram-positive and Gram-negative bacteria was studied by well diffusion technique. Recent study of similar green synthesis silver nanoparticles from *Plantago lanceolata* extract showed outstanding antioxidant and antibacterial activities [23]. According to the best of our knowledge, this is the first work reported the optimized scale-up green synthesis of silver nanoparticles from Plantago ethanolic leaf extracts. Sufficient amounts of nanoparticle will give more resource to do further experiments, such as bioactivity test add/or formulation experiments.

2. MATERIALS AND METHODS

2.1 Plant Materials and Plant Extract Preparation

The dried leaves of *P. major* were obtained from the Center for Research and Development of Medicinal and Traditional Medicinal Plants ("*Balai Besar Penelitian dan Pengembangan tanaman Obat dan Obat Tradisional*," Tawangmangu, Central Java, Indonesia) with Certificate of Plant Identification (certificate number 1258/D.T/IX/2017). The dried leaves were powdered and sorted using a mixer and 20-mesh sorting net to obtain a uniform size of powder particles. The fine leaf powder was extracted through 50% ethanol (10% w/v) using Ultrasonic-Assisted Extraction (UAE) with a frequency of 37 kHz for 20 min. The Whatman filter filters the extract and is stored at the refrigerator (4°C) for further synthesis [24].

2.2 Green Synthesis of Silver Nanoparticles and its Scale-Up Optimization

Silver nitrate ($AgNO_3$; Sigma-Aldrich, St. Louis, US) and various concentrations (w/v) of *P. major* leaf extract were used for the bio-reduction process. Each *P. major* leaf extract with concentrations of 0.25%, 0.50%, and 1.00% was mixed with $AgNO_3$ stock solution until reaching the final concentration of 1 mM. To find the optimal process of particle reduction, the synthesis process took place at various temperatures (60°C, 70°C, and 80°C) for 60 min. The heating and stirring process used a *hot plate magnetic stirrer* (Thermo Scientific Cimarec, Barnstead Thermolyne, NH, USA). The pH of the solution was adjusted to 10 by gently adding the NaOH 0.2 M. Several green synthesis parameters were modified from the previously reported condition (Nikaeen et al., 2020). Three independent replicates did all measurements.

Observations were done by looking at the color change into reddish-brown that marked the formation of silver nanoparticles. Plasmon resonance of silver nanoparticles was detected using a double beam ultraviolet-visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). As modified from previous work, the maximum absorbance was recorded at a wavelength range between 300-500 nm [25]. Then, the purification and collection process of synthesized silver nanoparticles from *P. major* extract (Pm-Ag NPs) were done by using centrifugation at 2,000 rpm for 10 min (Hettich® Zentrifugen D-78532, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany) followed by centrifugation at 11,000 rpm for 15 min (Sorvall™ Legend™ Micro 17R Microcentrifuge, Thermo Fisher Scientific, Waltham, MA, USA). Upscaling trial was done by increasing the synthesis volume from 100 mL to 1000 mL (10x

up-scale) with the optimized condition from a small scale. First, Pm-Ag NPs were collected by centrifugation at 2000 rpm for 10 min to remove impurities. The supernatant was read for absorbance at a wavelength of 300-500 nm with a spectrophotometer. For silver nanoparticle collection, the last centrifugation step was carried out with 8 tubes. Each tube was filled with 35 ml (35x up-scale) of the purified supernatant at 11,000 rpm (Sorvall™ Biofuge Stratos centrifuge, Thermo Scientific Nalgene, Rochester, NY, USA). The duration of centrifugation was optimized by doing centrifugation at various duration, namely 5, 15, 30, and 45 min. The Pm-Ag NPs obtained after 11,000 rpm centrifugation were washed using de-ionized water to remove undesired components. Pellets were dried at room temperature till reaching their constant weight and used for further characterization and antibacterial activity tests.

2.3 Characterization of Green Synthesized Silver Nanoparticles

Estimation of particle size and its distribution were performed through the Dynamic Light Scattering technique (DLS; Delsa™ Nano C Particle Analyzer, Beckman-Coulter, USA). Data were analyzed based on three replicated tests. X-ray diffraction (XRD) was done to determine the phase distribution, crystallinity, and purity of the synthesized silver nanoparticles. XRD analysis was performed by using an X-Ray spectrometer (D8-Advance, Bruker, Germany) operated at 40 kV,40 mA, with Cu(Kα) radiation (λ= 1.54 Å), observation speed: 4°min, step size 0.02, in range of the diffraction angle (2θ) from 10° to 90° [18]. The shape, morphology, and elemental distribution of the Pm-Ag NPs were analyzed by using Scanning Electron Microscope (SEM; JSM-6510, JEOL Co. Ltd., Japan) with the coating (Coater JFC-1600, JEOL Co. Ltd., Japan) and Field Emission Transmission Electron Microscope (FE-TEM) with a JEM-2100F (JEOL Co. Ltd., Japan) to check its elemental mapping. The instrument operated at 200 kV [19]. Further, the elemental mapping, selected area diffraction pattern (SAED), and energy-dispersive X-ray spectroscopy (EDX) of nanoparticles have been performed using FE-TEM (JEOL, Tokyo, Japan). Tests using Fourier Transform Infra-Red spectroscopy (FTIR; Jasco FT / IR 4200, Germany) were performed to determine the functional groups of plants present in Pm-Ag NPs.

2.4 Antibacterial Test of Synthesized Silver Nanoparticles

The antibacterial activity test was performed using the Disk Diffusion method on Muller Hinton Agar (MHA) medium. This test was performed against *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 9027, and *Escherichia coli* ATCC 25922. Chloramphenicol (250 µg mL^{-1}) and Gentamicin (100 µg mL^{-1}) were used as a positive control against Gram-Positive and Gram-Negative bacteria, respectively. All tested bacteria were refreshed by sub-culturing it on Nutrient Broth (NB) and incubated at 37^{o}C overnight. 100 µl bacteria suspension culture was spread over the surface of the MHA medium before the disk paper was given in a certain location at the top of MHA. 20 µl of several samples were dropped at the top of each disk. The culture was incubated for 48 hours, and then the diameter of the clear area was documented. Each treatment was done as three individual replications.

2.5 Statistical Analysis

Statistical analysis All values were presented as mean±SEM or mean±SD, n = 3. For multiple variables comparison, data were analyzed by ANOVA followed by Tukey test using GraphPad Prism statistical software (GraphPad Software Inc. Windows Version 5.01). Differences were significant at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Optimization of Green Synthesis and its Scale-Up Condition

The silver nanoparticle (Pm-Ag NPs) synthesis was done using ethanol extract of *Plantago major* L. leaf. The formation of silver nanoparticles usually could be suspected to happen by naked eye observation, that is, by changing the color of the solution into reddish-brown. Although it needs confirmation through other tests, the color change to reddish-brown is an early sign of the presence of

silver nanoparticles, as shown in previous work [26]. Surface plasmon resonance (SPR) is a characteristic of a metal that can be observed using a UV-Vis Spectrophotometer. Pm-Ag NPs from 0.25% *P. major* extract provide a high-intensity reddish-brown aqueous solution, followed by 0.5% and 1% extract concentration. On the other hand, Pm-Ag NPs from 2% and 3% *P. major* extract gave the color of the black solution. The black color usually indicates that many agglomerations have happened, and fewer nanoparticles were formed. This phenomenon was also reported in a previous study which stated that green synthesis in aqueous media under thermal heating produces larger nanoparticles that tend to agglomerate [19]. Therefore, the 2% and 3% concentrations of *P. major* extracts were not used in this research. At 60^{o}C temperature, Pm-Ag NPs solution was showed the maximum absorbance wavelengths at 436.6 nm, 382.3 nm, and 383.3 nm, for 0.25%; 0.5%; and 1.0% *P. major* extract, respectively (Fig. 1a). At 70^{o}C temperature, Pm-Ag NPs solution was showed the maximum absorbance wavelengths at 420.4 nm, 381.2 nm and 382.4 nm for 0.25%; 0.5%; and 1.0% *P. major* extract, respectively (Fig. 1b). Meanwhile, at 80^{o}C temperature, Pm-Ag NPs solution was showed the maximum absorbance wavelengths at 426.4 nm, 383.8 nm and 390.4 nm for 0.25%; 0.5%; and 1.0% *P.major* extract, respectively (Fig. 1c). The characteristic of silver nanoparticles is to provide an SPR absorbance peak at wavelength 400-450 nm [27,28,29]. Other reports suggest that the peak at ~420 nm corresponds to the surface plasmon resonance of silver nanoparticles [12]. SPR shifting can be caused by increased particle size and plant extract [15,16], as shown on silver nanoparticles from *Passiflora caerulea* extract, which has been shown in silver nanoparticles a peak close to 379 nm [30].

Variations in the concentration of plant extracts can cause certain nanoparticles to differ from one another, as indicated by the various color changes of the reaction. This is due to the excitation of surface plasmon echoes in the silver nanoparticles. Due to the free conduction electrons induced by the electromagnetic field, the SPR for silver nanoparticles will receive a dark brown to reddish-brown color from colloidal silver. The conducting particles oscillate at a certain wavelength due to the SPR [31]. The increasing *P. major* extract concentration influences the amount of yield, as shown in Table 1. This may be happened by a rapid and continuous agglomeration process. Without agglomeration, particles will be dispersed very slowly and cannot form nanoparticles [32]. However, if the aggregation happened excessively, nanoparticles yield could also decrease because of eliminating the larger agglomeration at the first centrifuge step, as shown on Pm-Ag NPs yield of green synthesis using 0.50% 1.00% *P. major* leaf extract (Table 1). The decrease in absorption intensity at the optimum SPR wavelength may also be due to some silver nanoparticle aggregation [29]. 0.25% *P. major* extract at 70^{o}C gave the strongest reddish-brown appearance, proper maximum wavelength, and highest absorbance and yield results; thus, it was selected as the synthesis parameter for an up-scale trial. The temperature and duration of the syntheses were slightly adjusted from the procedures described in previous studies [33].

For the up-scaling trial, 10x synthesis volume (1L) was applied using 0.25% *P. major* extract at 70^{o}C with the same $AgNO_3$ concentration and pH condition of a smaller scale. Then, the absorbance of this solution was observed every 15 min using a spectrophotometer at a wavelength of 300-500 nm to observe the formation of silver nanoparticles (t_0, t_{15}, t_{30}, t_{45}, and t_{60}). A color change indicates the formation of silver nanoparticles, which is a change in color from light yellow to reddish-brown (Supplementary Fig. S1a). The change in its absorption peak indicates the stability of the colloidal silver nanoparticle solution. The shift of the peak to a larger wavelength indicates less stability of the nanoparticles and tends to agglomerate. Like small-scale results, t_{60} showed the largest absorbance peak, in coherence with its solution color appearance (Supplementary Fig. S1b). The maximum absorbance was 421.8 nm, 423.3 nm, 424.2 nm, and 425.9 nm for t_{15}, t_{30}, t_{45}, and t_{60}, respectively (Supplementary Table S1).

Meanwhile, no maximum absorbance was observed for the initial extract (0.25% *P. major* extract, namely "E") and t_0, at a range of 400-450 nm. During the scale-up trial, the stirring speed was adjusted from 120 rpm (small-scale) to 190 rpm (large-scale) by using the same device. The solution volume for the last step centrifugation was increased to 35 mL (35x up-scale) for the silver nanoparticles purification step. While using the same speed, various centrifugation duration (5, 15, 30, and 45 min) were applied. The UV-spectroscopy of its supernatant was observed as shown in Supplementary Fig. S1c. The absorbance and maximum wavelength of the supernatant resulting from

centrifugation were measured to obtain the minimum time to obtain optimal silver nanoparticles (Supplementary Table S2). Selection of the best centrifugation time determined by lowest absorbance value of the supernatant (Pm-Ag NPs solution). Within the same maximum wavelength range (420-430 nm), absorbance decreased as the duration of centrifugation increased. Still, by the 30 and 45 min, there was no longer a decrease in absorbance value. Thus, it can be concluded that the optimum centrifugation time to obtain optimal silver nanoparticles is 30 min. The yield from the up-scale trial was 107.2±6.82 mg (4.29±0.27 %). The result indicated that the larger scale green synthesis was done efficiently to yield a larger yield.

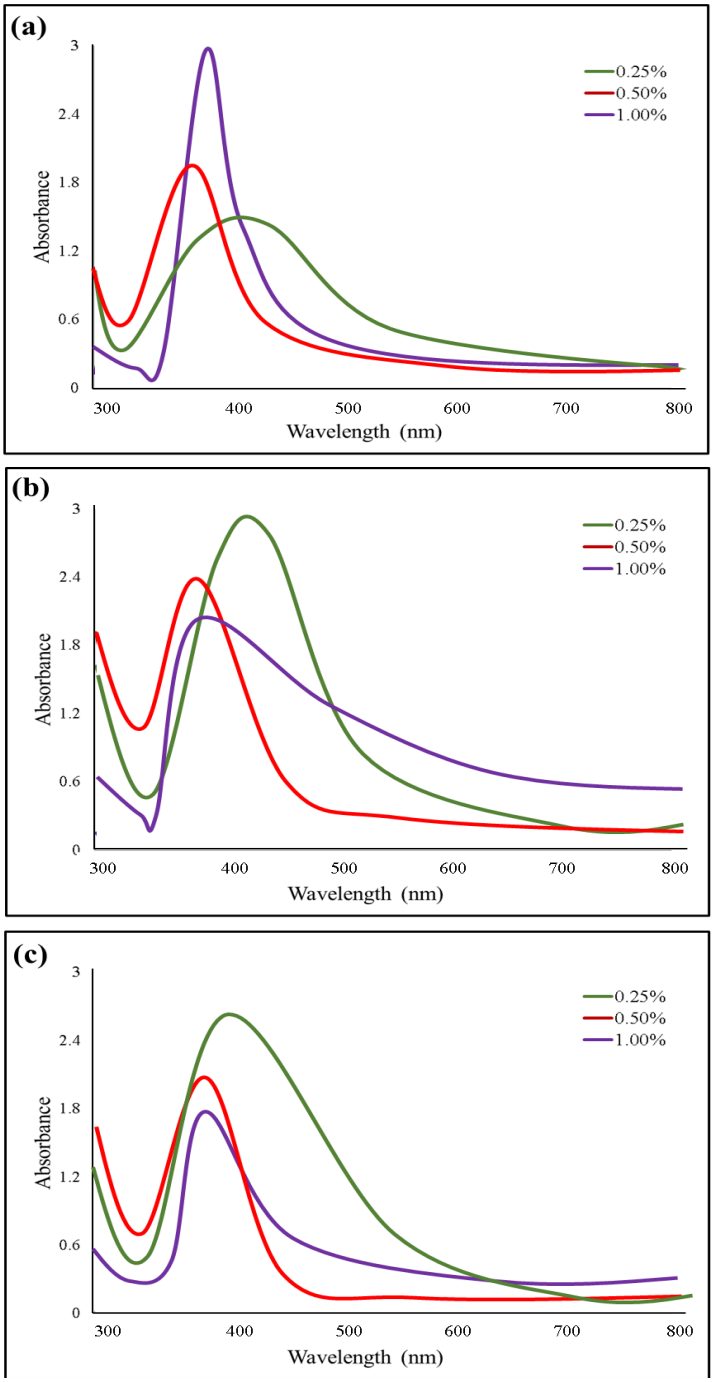

Fig. 1. UV-Vis spectra of Pm-Ag NPs solutions obtained from green-synthesis using various ethanol extract (w/v) at a different temperature: (a) 60°C, (b) 70°C, and (c) 80°C, respectively

Table 1. The yield of Pm-Ag NPs on small scale synthesis

The concentration of *P. major* Leaf Extract	Green synthesis Temperature		
	60°C	**70°C**	**80°C**
0.25%	11.2±0.87 mg [a]	11.9±0.57 mg [a]	10.8±0.93 mg [a,b]
	4.48±0.35 % [a]	4.75±0.23 % [a]	4.33±0.37 % [a]
0.50%	8.5±0.82 mg [b]	8.9±0.85 mg [b]	8.1±0.98 mg [b]
	3.40±0.33 % [b]	3.56±0.34 % [b]	3.25±0.39 % [b]
1.00%	5.9±0.91 mg [c]	6.1±0.97 mg [c]	5.6±0.91 mg [c]
	2.37±0.36 % [c]	2.43±0.39 % [c]	2.33±0.36 % [c]

Values are means ± SD of three determinations. Means within each column with different letters differ significantly (P < 0.05)

3.2 Particle Size and Distribution Study

Particle Size Analysis was used to determine the particle size and its distribution. The particle size distribution can be seen from the Polydispersity Index (PDI) value. Table 2 shows the particle size of Pm-Ag NPs and their polydispersity index. The *P. major* extract at a concentration of 0.25% yielded Ag NPs with the overall smallest particle size, whereas 0.50% and 1.00% produced overall larger sizes. Furthermore, the dispersity of Pm-Ag NPs was also measured as a PDI value. The polydispersity index (PDI) is a parameter that describes the spread or uniformity of the particle size distribution. PDI value can range between 0 to 1. The values of PDI≤ 0.1, 0.1–0.7, and >0.7 are related to the highly monodispersed, moderately, and highly polydisperse distributions, respectively [34]. The other report stated that PDI less than 0.1 implies monodisperse distributions while the values more than 0.1 may imply polydisperse distributions. Polydisperse particles have poor particle size uniformities [35]. Meanwhile, the optimized scale-up synthesis condition of Pm-Ag NPs resulted in the average particle size at 12.2±5.11 nm (PDI = 0.18). It is similar to the average particle size from the previous smaller scale of green synthesis, which resulted in 10.3±3.98 nm (PDI = 0.15). The synthesized silver nanoparticles are nearly monodisperse due to the effective binding ability of secondary metabolites compounds with silver nanoparticles. It acts as a phyto-reductant and capping agent, resulting in uniformity on metallic nanoparticle size and minimalizing its aggregation [36,37] The plant extract concentration needs to be optimized initially, not too little or too much to achieve that point. This result is coherent with the previous report on green synthesis of cooper [38], gold [39], and silver nanoparticles [39,40,41]. Furthermore, the optimization process of scaling up needs to be done because increasing the synthesis scale can change the rate of heat per mass transfer, which may cause differences in nanoparticle growth and the nucleation process. Therefore, it affects the yield of the green synthesis [36,42].

3.3 Shape, Morphology, and Elemental Distribution Study

The SEM results (20,000x) indicate that the nanoparticle has a spherical shape (Supplementary Fig. S2a). The Pm-Ag NPs produced through the bottom-up method will generally have a spherical shape [43,32]. Another report also supports this result, saying that Surface Plasmon Resonance (SPR) at 400-450 nm indicates that the particles have a spherical shape [26,44]. As shown in Supplementary Fig. S2b, the results of FE-TEM analysis revealed that the biosynthesized Pm-Ag NPs were spherical, with sizes ranging from ~10 nm to ~20 nm. It shows well-dispersed synthesized nanoparticles similar to previous work [15,39]. Furthermore, the FE-TEM results are coherent with the PSA results, as it shows similar spherical Pm-Ag NPs at ~10 nm to ~20 nm range of a size (Supplementary Fig. S2c). FE-TEM is the preferred method to directly observe the nanoparticles' morphology, size, and distribution [45].

SAED was used to identify the crystallite structure of the nanosized particles. There were four diffraction rings referred to [111], [200], [220], and [311] lattice plane of Bragg's reflections (Fig. 2a), which corresponds to the elemental silver, thus confirming the crystalline nature of Ag NPs. The obtained SAED patterns of Pm-Ag NPs also agreed with previous literature [45,13,32]. However, another report showed that its silver nanoparticles have five diffraction rings, referred to as the lattice pattern of [111], [200], [220], [311] and [222] [46]. The EDS spectra confirmed the silver nanoparticles

are in metallic form (Ag), with no presence of Ag_2O, which indicates the yields are free from any other impurities (Fig. 2b). The XRD pattern shows four intense peaks (as SAED results) in the whole spectrum of 2θ values ranging from 30° to 80° confirmed the presence of silver compounds in the synthesized nanoparticles (Fig. 2c). The Pm-Ag NPs analyzed by elemental mapping were visible in the electron image and were observed to be the major component in the nanoparticles (Fig. 2d).

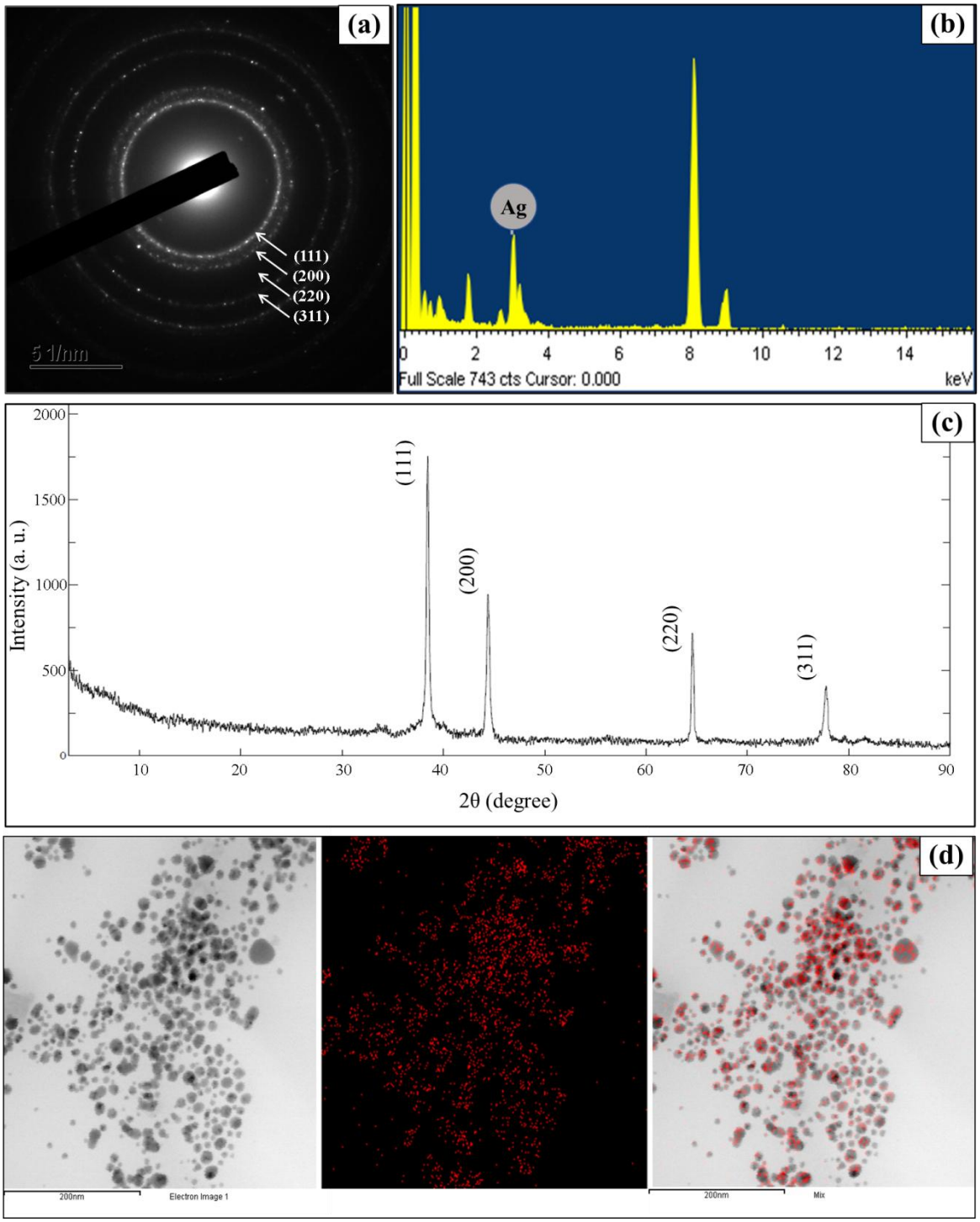

Fig. 2. (a) SAED pattern, (b) EDS spectrum, (c) XRD patterns of Pm-Ag NPs, and (d) elemental distribution mapping of Pm-Ag NPs

3.4 FTIR Analysis

FTIR analysis was used to determine the presence of functional groups in the *P. major* leaf extract and the yield of silver nanoparticles (Fig. 3). Vibration band at 3468.35 cm-1 is happened due to O–H bonds, 1786.72 cm-1 for bonds C=O, which is commonly found in the proteins [47], 1630.52 cm-1 and 1612.2 cm-1 for C–N bonds, which can be a sign of aromatic and aliphatic amines, 1408.75 cm-1 for C–C or N–H bonds. The broadest peak designated to the hydroxyl group implies that this group plays a major role in the reduction of Ag+ and has a strong ability to bind with Ag NPs. The existence of the O–H bond, presumably derived from phenolic compounds in the *P. major*. These results also suggest a possibility of alcohol, phenol and proteins involved in the green synthesis of Ag NPs [36,17]. It is concurrent with the previous report on the observation of Pm-Ag NPs peak during FTIR analysis, and its predicted compound is shown in Supplementary Table S3. These plant metabolites act as capping and stabilizing agents for synthesized silver nanoparticles [48,12].

Table 2. Particle size average and polydispersity index of pm-ag nps on small scale synthesis

The concentration of *P. major* Leaf Extract	Green synthesis Temperature		
	60°C	**70°C**	**80°C**
0.25%	8.9±3.56 nm ^{d}	10.3±3.98 nm ^{c,d}	22.1±9.34 nm ^{c}
	PDI = 0.16	PDI = 0.15	PDI = 0.18
0.50%	49.3±21.56 nm ^{b,c}	62.7±28.3 nm ^{b}	76.8±33.2 nm ^{a,b}
	PDI = 0.19	PDI = 0.20	PDI = 0.19
1.00%	74.1±34.2 nm ^{a,b}	82.2±44.1 nm ^{a}	91.8±49.8 nm ^{a}
	PDI = 0.21	PDI = 0.29	PDI = 0.29

Values are means ± SD of three determinations. Means within each column with different letters differ significantly (P < 0.05)

Table 3. Antibacterial properties of Pm-Ag NPs

Code	Sample	Clear Zone Diameter (mm) against		
		S. aureus	***P. aeruginosa***	***E. coli***
1	Blank	0 ^{f}	0 ^{f}	0 ^{f}
2	Aquadest	0 ^{f}	0 ^{f}	0 ^{f}
3	0.25% *P. major Extract*	0 ^{f}	0 ^{f}	0 ^{f}
4	$AgNO_3$ 1mM	5.73±0.164 ^{e}	6.85±0.393 ^{d}	9.56±0.351 ^{ab}
5	Pm-Ag NPs 10 µg mL^{-1}	5.76±0.127 ^{e}	7.42±0.252 ^{bc}	9.12±0.152 ^{b}
6	Pm-Ag NPs 20 µg mL^{-1}	7.31±0.532 ^{bc}	8.59±0.582 ^{b}	9.91±0.222 ^{ab}
7	Positive control	7.14±0.310 ^{c}	8.13±0.267 ^{bc}	10.54±1.084 ^{a}

Values are means ± SD of three determinations. Means within each column with different letters differ significantly (P < 0.05)

3.5 Antibacterial Activity Test

The mechanism of antibacterial activity of Ag NPs remains unclear to date. Meanwhile, the mechanism of action of silver itself is by disrupting the cell membrane, causing ROS, penetration of cell membrane, and will bind to DNA and protein. However, based on existing literature, the principle of the antibacterial mechanism of Ag NPs is divided into oxidative stress, metal ion release, and non-oxidative mechanism. Oxidative stress is a very important antibacterial mechanism. Ag NPs can produce superoxide radicals or reactive oxygen species (ROS) and hydroxyl ions, which can cause acute microbial death. The action that occurs is by disrupting the cell membrane, causing ROS, penetration of cell membrane, and binding to DNA and protein. The mechanism activity of Ag NPs as antibacterial is probably due to the surface charge of Ag NPs that bind to the negatively charged bacteria [49]. In our research, Gram (-) bacteria look more susceptible to Pm-Ag NPs than Gram (+) bacteria. This is due to the differences in the structure of cell membrane and cell wall between Gram (+) and Gram (-) bacteria [50]. The diameter of the clear zone region confirmed Pm-Ag NPs from either at 10 µg mL^{-1} or 20 µg mL^{-1} had a significantly different antibacterial activity with $AgNO_3$ and differed not significantly with tested antibiotics as a positive control (Fig. 4). It means that the efficacy

of synthesized silver nanoparticles was superior to that shown by $AgNO_3$ solution but equal compared with the chloramphenicol and Gentamicin.

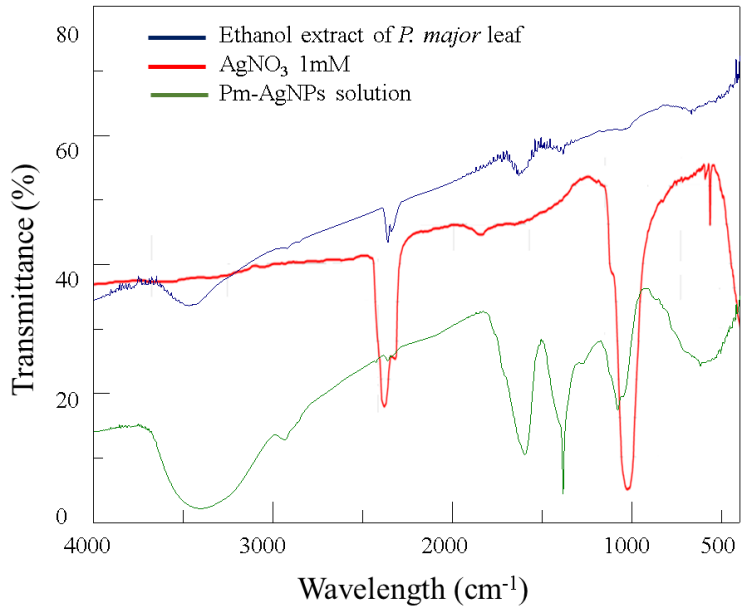

Fig. 3. FT-IR spectra of Pm-Ag NPs solutions compared with $AgNO_3$ and ethanol extract of *Plantago major* leaf

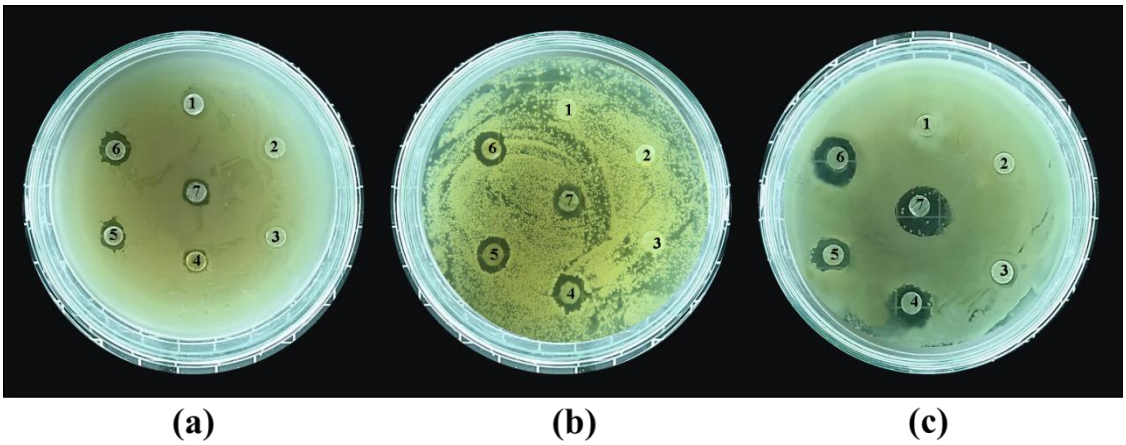

Fig. 4. Representative results of antibiotic activity test of Pm-Ag NPs against (a) *S.aureus*, (b) *E. coli* and (c) *P. aeruginosa.* Labeling Information as follows: 1 = Blank (no sample); 2 = Aquadest; 3 = 0.25% *P. major* Extract; 4 = $AgNO_3$ 1mM; 5 = Pm-Ag NPs 10 µg mL^{-1}; 6 = Pm-Ag NPs 20 µg mL^{-1}; 7 = Positive control (Chloramphenicol 250 µg mL^{-1} or Gentamicin 100 µg mL^{-1})

4. CONCLUSION

An efficient scale-up and eco-friendly synthesis method of silver nanoparticles from *Plantago major* L. leaf extract have been conducted. The scale-up trial with 10x synthesis volume and 35x purification volume showed resemblant Ag NPs characteristics with the smaller scale. The spherical nanoparticles with a size ranging from 10-20 nm were obtained. All the UV-spectra and elemental analyses confirmed the presence of silver nanoparticles. The Pm-Ag NPs also exhibit potential antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* at a concentration of 20 µg mL^{-1}. This study suggests that ethanolic leaf extract of P. major at a concentration of 0.25%, 70oC of temperature, and 60 min of synthesis can be done on a larger scale,

which efficiently offers nine times bigger yields. Therefore, there will be enough Pm-Ag NPs to be used on further bioactivity and formulation works.

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SUPPLEMENTARY MATERIALS

Supplementary material is available in the following link: https://www.bookpi.org/wp-content/uploads/2021/12/Supplementary_2021_BP_2314C.pdf

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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