Green synthesis of silver nanoparticles using Pluchea (*Pluchea indica* (L.)) leaf extract and antibacterial activity against *Staphylococcus aureus* and *Propionibacterium acnes*

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ABSTRACT: Nanotechnology is widely used in the biomedical purpose as a drug delivery system, cancer, and tumor biomarkers. Currently, metals are most used as precursor agents to form nanoparticles such as silver, gold, iron, zinc, and metal oxides. Acne is one of the skin problems caused by the growth of *S. aureus* and *P. acnes* bacteria. Treatment of acne using inappropriate antibiotics can lead to resistance. Silver nanoparticles are known to have the ability to kill pathogenic microorganisms. Pluchea leaf extract contains flavonoid, polyphenol, and tannin compounds that can work as natural bioreductors in the formation of silver nanoparticles while inhibiting bacterial growth. This study aims to synthesize silver nanoparticles using Pluchea leaf extract and testing antibacterial activity against *Staphylococcus aureus* and *Propionibacterium acnes*. Extraction of Pluchea leaves was carried out using the infusion method with the water solvent. The synthesis of silver nanoparticles was used a shaker incubator at a speed of 150 rpm and a temperature of 37 °C for 48 hours. Characterization of silver nanoparticles using uv-vis spectrophotometry, particle size analyzer (PSA), zeta potential analyzer, and FESEM-EDX. Antibacterial activity test using microdilution method. The characterization results of silver nanoparticles showed a particle size of 20.50 nm, a zeta potential of -38.6 mV, and a spherical morphological shape. Silver nanoparticles have antibacterial activity against *S. aureus* and *P. acnes* with a Minimum Inhibitory Concentration (MIC) value of 62.5 ppm and Minimum Bactericidal Concentration (MBC) value >250 ppm.

KEYWORDS: Antibacterial; Pluchea indica (L.); Propionibacterium acnes; silver nanoparticles; Staphylococcus aureus.

INTRODUCTION

Nanotechnology is widely used in the healthcare as a drug delivery system, cancer, and tumor biomarkers. Currently, many have used metals as precursor agents to form nanoparticles that are silver, gold, iron, zinc, and metal oxides. However, of all these precursor agents, there is one metal that has attracted the attention of researchers, it was a silver [1], [2], [3]. The formation of silver nanoparticles aims to produce better physical properties, chemical properties, and antibacterial properties compared to bulk materials [3], [4]. Although chemical methods are the most common and cost-effective choice for large-scale silver nanoparticle (AgNP) synthesis, biological methods (green synthesis) are considered the most appropriate choice due to their non-toxic, environmentally friendly nature and safety for biomedical applications, overcoming the inherent toxicity drawback of chemical approaches [3], [5].

The green synthesis method is a method that utilizes biological materials such as plants, bacteria, algae, fungi, and molds as reducing agents in the formation of silver nanoparticles [5]. Plants contain several secondary metabolite compounds with -OH groups such as polyphenols, flavonoids, tannins, saponins, and terpenoids which have the potential as stabilizers and reducing agents for the synthesis of silver nanoparticles [5], [6]. Some plant extracts that have proven capable as bioreductors in the synthesis of silver nanoparticles are breadfruit leaf extract (*Atrocarpus altilis*) [7], cherry leaf extract (*Phyllanthus acdus*) [8], noni leaf extract (*Morinda citrifolia*) [4] and Moringa leaf extract (*Moringa oleifera*) [9].

There are several studies that have shown the ability of silver nanoparticles (AgNPs) as antibacterial agents that are bacteriostatic or bactericidal against various types of bacteria like *Propionibacterium acnes* [10], *Escherichia coli* and *Pseudomonas aeruginosa* [7], *Micrococcus, Vibrio cholerae, Salmonella typhi, Staphylococcus aureus* and *Proteus vulgaris* [9]. The potential of AgNPs as antibiotics is related to their various mechanisms of

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action, which attack microorganisms in multiple structures at a time and give them the ability to kill various types of bacteria [11].

Pluchea leaf are known to contain several secondary metabolite compounds such as polyphenols, flavonoids, and tannins [12]. These compounds are thought to have the potential as stabilizing agents and natural reducing agents for the synthesis of silver nanoparticles [5], [12]. The total phenolic compounds contained in Pluchea leaves are 47.862 mg/g [13], in addition the ethanol extract of Pluchea leaves has antibacterial activity against *Propionibacterium acnes* and *Staphylococcus aureus* bacteria [14], [15]. *Staphylococcus aureus* and *Propionibacterium acnes* are one of the factors causing acne. In general, *Propionibacterium acnes* bacteria can cause acne on human skin by producing lipase which works by breaking down free fatty acids from skin lipids, then these fatty acids can cause tissue inflammation when associated with the immune system and support the occurrence of acne [16]. This study aims to synthesize silver nanoparticles using Pluchea leaf extract, characterize the final product, and evaluate the antibacterial activity against *S.aureus* and *P.acne*.

MATERIALS AND METHODS

Materials

Pluchea indica (L.) leaves purchased from Research Center for Medicinal and Aromatic Plants, Bogor, Indonesia; Nutrien Agar (Himedia®, India); Nutrien broth (Himedia®, India); Mueller Hinton Agar (Himedia®, India); Mueller Hinton Broth (Himedia®, India). Shaking incubator (Maskot®, China), Spektrofotometri UV-Vis (Thermo Scientific Quattrol®, USA); Biology Safety Cabinet (Elisa V60®, Indonesia); centrifuge (Kobuta®, Japan); oven (Thermo®, USA); Particle Size Analyzer (Horiba®, Japan); zeta potensial (Horiba®, Japan); FESEM EDX (Thermo Scientific Quattrol®, Japan).

Methods

Preparation and extraction of Pluchea leaves

Extraction of Pluchea leaves was conducted by boiling (infuse) method. Previously, Pluchea leaf samples were washed thoroughly under tap water, and then cut into small pieces. Samples and water solvent were put into a pot in a ratio of 1:10. The extraction process was carried out for 15 minutes starting after the temperature reached 90 °C. Then the extract was allowed until it reached room temperature and filtered [17].

Synthesis of silver nanoparticles

The synthesis of silver nanoparticles was carried out by mixing the Pluchea leaf extract that had been made with 1 mM AgNO₃ solution. Mixing the extract with AgNO₃ solution was made with a volume ratio of 1:90, then the mixture was incubated using a shaking incubator for 48 hours at 37 °C with a rotation speed of 150 rpm. After that, the color of the solution was observed and analyze uv-vis to see the absorbance. If the sample has formed nanoparticles by observed color and absorbance, the sample is centrifuged for 2 hours at 3500 rpm. The precipitate formed was then dried using an oven at 70 °C to produce a dry powder of nanoparticles.

Characterization of silver nanoparticles

Characterization of silver nanoparticles was carried out using UV-Vis spectrophotometry at 400-500 nm. Particle size was observed using a PSA (Particle Size Analyser) instrument. The stability of silver nanoparticles was observed using a zeta potential instrument. The morphological shape and composition of silver nanoparticles were observed using the FESEM-EDX instrument.

Antibacterial activity test using microdilution method

Antibacterial activity of silver nanoparticles against *Staphylococcus aureus* and *Propionibacterium acnes* bacteria are tested by looking at the MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration). Testing the MIC and MBC of silver nanoparticles was carried out using the microdilution method, with the principle of two folds dilution using Mueller Hinton Broth (MHB) liquid media in 96-well microplates. Bacterial suspensions of *S. aureus* and *P. acnes* turbidity were made equivalent to the 0.5 McFarland standard (108 CFU/mL), then diluted 100 times until the bacterial suspension had a concentration of 106 CFU/mL. Positive control and negative control were using tetracycline and DMSO. Furthermore, the microplate was incubated at 37 °C for 24 hours. Then the microplate can be observed visually

by looking at the turbidity that arises in the wells. Clear wells mean that it has antibacterial activity. The lowest concentration that shows no bacterial growth is the MIC value (18). MBC testing is done by scratching the test suspension that has a MIC value on a Petri dish containing MHA agar. Furthermore, incubation was carried out for 18-24 hours at 37 °C. The part that does not show any bacterial growth on the plate is determined as the MBC value [18].

RESULTS

Extraction of Pluchea leaf using the infusa method produces a yellow color shown in Figure 1. The synthesis of silver nanoparticles began after reacting 1 mM $AgNO_3$ solution and Pluchea leaf extract, produced a yellow color. After incubating using a shaking incubator for 72 hours there was a change in color from yellow to reddish brown (Figure 2). The precipitate then dried using an oven at 70 $^{\circ}$ C until dries. The dried silver nanoparticle precipitate will produce a shiny gray color. The results of the precipitate before and after drying can be observed in Figure 3.



Figure 1. Water extract of Pluchea leaf

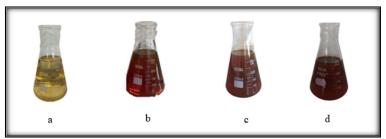


Figure 2. Color changes of biosynthesis silver nanoparticles.

(a) 1-hour (b) 24-hours (c) 48-hours (d) 72-hours



Figure 3. (a) Precipitate of silver nanoparticles (b) Dry silver nanoparticles

An indication of the formation of silver nanoparticles is seen from the appearance of a strong and distinctive absorbance peak at maximum wave length of 400-500 nm. Measurements using a uv-vis spectrophometer were carried out during the incubation period every 1, 24, 48, and 72 hours (Figure 4). The average particle size produced was 20.5 nm (Figure 5) and potential zeta was -38.6 mV.

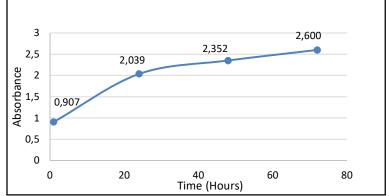


Figure 4. Absorbance of silver nanoparticles after incubation 1, 24, 48, and 72 hours.

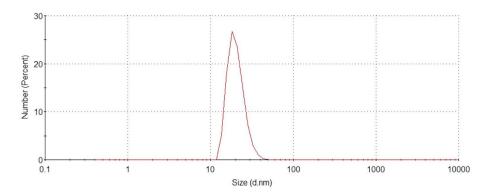


Figure 5. Size distribution curve of silver nanoparticles.

The morphological of silver nanoparticles is in the form of a sphere. The results of FESEM analysis using 3 different magnifications are shown in the figure 6 and the EDX analysis are shown in Figure 7.

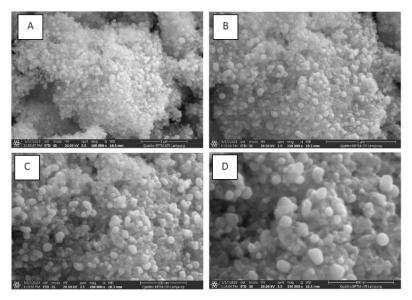


Figure 6. Morphology of silver nanopartciles. 100.000x (A), 150.000x (B), 200.000x (C), 350.000x (D) Magnification

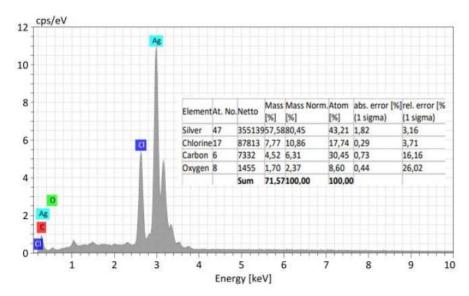


Figure.7 EDX analysis on silver nanoparticles.

EDX map is used to mapping distribution of Ag (silver), Cl (Chlorine), C (Carbon), and O (Oxygen) elements contained in silver nanoparticle samples. The results of EDX mapping data are shown in the figure 8.

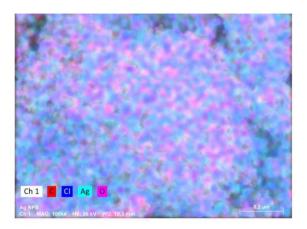


Figure 8. EDX mapping of silver nanoparticles.

The results of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests demonstrated that silver nanoparticles exhibited antibacterial activity against both *Staphylococcus aureus* and *Propionibacterium acnes*. The MIC value for silver nanoparticles was 62.5 μ g/mL for both bacterial strains, indicating their ability to inhibit bacterial growth at this concentration. However, the MBC values were greater than 250 μ g/mL, suggesting that the nanoparticles were bacteriostatic rather than bactericidal at the tested concentrations.

Table 1. Antibacterial activity of silver nanoparticles.

Sample	S. aureus		P. acnes	
	MIC (μg/ml)	MBC (µg/ml)	MIC (μg/ml)	MBC (μg/ml)
Silver Nanoparticles	62.5	>250	62.5	>250
Positive control	< 0.98	< 0.98	< 0.98	< 0.98
Negative control	>250	>250	>250	>250

DISCUSSION

The extraction process in this method was done at 90 °C for 15 minutes in consideration of that condition is the most optimal method for attracting flavonoid compounds in large quantities [19]. The synthesis of silver nanoparticles used a Pluchea leaf extract and AgNO₃ solution with a ratio of 1:90. Furthermore, in the preparation of AgNO₃ solution, silver nanoparticles were synthesized using 2 different concentrations of AgNO₃ solution; 1 mM and 2 mM. This experiment aims to determine the optimum concentration to form silver nanoparticles in the size of 10-100 nm. After the silver nanoparticles were characterized using a PSA (Particle Size Analyzer), it was found that the silver nanoparticles using a concentration of 1 mM AgNO₃ solution produced a smaller particle than 2 mM. Eventually, 1 mM AgNO₃ solution was chosen as a precursor to form silver nanoparticles.

Variations in the concentration of AgNO₃ solution can affect the size of silver nanoparticles. The higher the concentration of AgNO₃ solution cause the faster reduction process so that over time it will form silver nanoparticles in large sizes. The size of silver nanoparticles >100 nm will reduce its effectiveness in killing bacteria because the antibacterial properties of silver nanoparticles will be influenced by particle size [20].

The silver nanoparticle solution showed a color change from a light yellow to a reddish brown solution after 24 hours of incubation. After incubating 48-72 hours, it turned dark reddish brown indicating that silver nanoparticles (AgNPs) had formed. The color change to dark brown in the synthesis of AgNPs indicates that the reduction process between the bioreductor and AgNO₃ has taken place and silver nanoparticles have been formed [21]. This silver nanoparticle synthesis process is produced from silver compounds that undergo oxidation, with the reducing agent from metabolite compounds in Pluchea leaf extract that reduce Ag⁺ ions to Ag⁰. Ion reduction ability depends on the presence of compounds such as proteins, enzymes & coenzymes, lipids, carbohydrates, alkaloids, flavonoids, phenols, terpenoids, and others substances in plants, which play

an important role in the production of nanoparticles [22]. The active ingredient responsible for Ag⁺ ion reduction varies depending on the organism or biological extract. For the nano-transformation of AgNPs, electrons are believed to be derived from the dehydrogenation of acids and alcohols in hydrophytes, keto to enol conversions in mesophytes, or both mechanisms in xerophytes. Similar reduction processes can be performed by microbial cellular and extracellular oxidoreductase enzymes [23].

Based on figure 4, that the longer of incubation period, it will produce higher absorbance. The absorbance at 1-hour shows the lowest absorbance value, because the synthesis process has not run perfectly thus the silver nanoparticles produced are still in very small quantities [24]. Whereas the synthesis of silver nanoparticles at the 48-hours and the 72-hours produces the highest absorbance indicating silver nanoparticles produced in large quantities [25]. The amount of silver nanoparticles formed will increase significantly by increasing of time progress. However, if the reaction time is excessive, the formation of silver nanoparticles can result in a larger particle size [26].

Particle size

The particle size analysis indicated that the nanoparticles were successfully synthesized, with an average size of 20.5 nm. The use of leaf extracts in the synthesis of silver nanoparticles shows several results including silver nanoparticles synthesized from *Spondias pinnata* leaf producing a particle size of 30.56 nm, silver nanoparticles synthesized from star fruit leaves (*Averrhoa bilimbi* L.) producing a size distribution of 112.8 nm [25]. The size of silver nanoparticles formed can be influenced by several factors, such as reaction time, PH concentration, temperature and reductant [27], [28].

In addition to particle size data, the PSA test also contains a polydispersity index (PI), which is a parameter used to measure the level of particle size heterogeneity in a sample. Polydispersity can occur due to non-uniform particle size distribution in the sample, or due to agglomeration or aggregation of particles during the synthesis or storage process. Polydispersity index greater than 1.0 means that the particle size is not homogeneous, while a polydispersity index value smaller than 1.0 means that the particle size is homogeneous. The results showed a polydispersity index value of 0.398 in other words the particle size that has been formed is homogeneous (uniform) because the polydispersity index value is less than 1.0. it can be concluded that the size of silver nanoparticles using Pluchea leaves extract has good quality, namely at a size of 20.50 nm with a polydispersity index value that shows uniform particle distribution. The greater of the polydispersity index value it will form a non-uniform particle size distribution and will cause particles to agglomerate easily, while the smaller the particle size it will provide a stronger antibacterial effect [20], [21]

Zeta potential

The zeta potential value is generally used to determine particle charge and nanoparticle stability [29]. Nanoparticles with zeta potential values smaller than -30 mV and greater than +30 mV have higher stability [30], [31]. The positive and negative values of zeta potential indicate the type of charge present on the surface of the nanoparticles [32]. The zeta potential value obtained was -38.6 mV. This reduction process converts silver ions, which initially have a positive charge (Ag^+) into silver particles (Ag^0) with a neutral charge. The result shows that Pluchea leaf extract acts as a reducing agent capable of producing silver nanoparticles with a negative charge on their surface. This negative charge can help maintain the stability of silver nanoparticles by avoiding attraction interactions that can cause particle aggregation in solution [32].

Field scaning electron microscope energy dispersive xray spectroscopy (FESEM-EDX)

Field Scaning Electron Microscope Energy Dispersive XRay Spectroscopy (FESEM-EDX) is an instrument used to identify morphology on the surface of particles by providing information related to the distribution of particles in the sample and is able to detect the composition of the elements contained in a sample [33].

The EDX has been integrated with a FESEM instrument that allows for both qualitative and semiquantitative microanalysis in detecting elements contained in silver nanoparticle samples. The mechanism of EDX is done by firing X-rays at the particle to know the composition, then the results will be displayed on the computer in the form of certain peaks representing an element accompanied by the percentage content of each element in a sample [34]. Ion reduction in the silver nanoparticle synthesis process will produce several biosynthesized elemental subtituents, such as the Ag (Silver), Cl (chlorine), C (carbon), and the O (Oxygen) [35]. In addition, the Ag element generally absorbs energy in the 3 keV region due to the plasmon resonance on its surface [36].

Figure 7 showed the percentages elements of the Ag (silver), Cl (Chlorine), C (Carbon), and O (Oxygen) with percentage of 80.45%, 10.86%, 6.31% and 2.37% respectively. The Ag (Silver) peak shows strong energy absorption at 3 KeV and has the highest concentration among other elements. The EDX mapping image shows the distribution of the turquoise color that indicating Ag (silver) element is relatively more than the other elements, indicating that the samples that have been made are silver nanoparticles.

Antibacterial activity

In this study, the size of silver nanoparticles is an important factor affecting antibacterial activity against *S. aureus* and *P. acnes*. Silver nanoparticles size of 20,50 nm showed a MIC of 62.5 ppm. The MIC value is higher when compared to nanoparticles synthesized using Carya illinoinensi leaf extract and Salvadora persica with MIC values of 128 ppm, 100 ppm, respectively [37], [38], [39]. Furthermore, the MBC value from *S. Aureus* and *P. acnes* bacteria is >250, which means at 250 ppm, *Pluchea* leaf extract nanoparticles have not been able to kill *S. aureus*. The previous study, pine bark extract nanoparticles whose particle size is 20-25 nm have a MBC value of 340 ppm against *S. aureus*. The MBC on *P. acnes* has also not been able to kill these bacteria at 250 ppm. The previous study shown that nanoparticles with a smaller size (11,7 nm) have antibacterial ability at concentration of 12 ppm, it can explained that small silver nanoparticles have a larger surface area to interact with various types of bacteria compared to larger silver nanoparticles [40]. When silver nanoparticles interact with bacteria, they play a role in altering cell metabolism and inhibiting cell growth. Silver nanoparticles will penetrate the cell membrane, prevent protein synthesis, further cause a decrease in membrane permeability, and ultimately lead to cell death [20].

CONCLUSION

Pluchea (*Pluchea indica* (L.)) leaf infusion extract can reduce silver nitrate to form silver nanoparticles characterized by a change in color in AgNPs solution. Silver nanoparticles of Pluchea leaf infusion extract (*Pluchea indica* (L.)) has a particle size of 20.5 nm with a polydispersity index (PI) of 0.398, zeta potential of -38 mV, and aggregated spherical particles. Silver nanoparticles of Pluchea (*Pluchea indica* (L.)) leaf extract were inhibited on *S. aureus* and *P. acnes* at a concentration of 62.5 ppm. However, at this concentration, silver nanoparticles of Pluchea (*Pluchea indica* (L.)) leaf extract could not kill S. aures and P. acnes bacteria.

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