Antibacterial Activity of Synthesized Silver Nanoparticle using *Langsat* Leaf Extract (*Lansium domesticum* var. *pubescen* Kooders et Valeton) as Bioreductor against *Escherichia coli* and *Staphylococcus aureus*

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Abstract: The emerging of antimicrobial resistance has caused the urgency to find new alternative agents. The application of silver in the form of silver nanoparticles (AgNP) began to be studied again. The less toxic and ecofriendly method to synthesize AgNP is through the green synthesis approach using plant extract. *Langsat* is one of the endemic plants of South Kalimantan and its leaf provides secondary metabolites substances to help the synthesize of AgNP. The synthesis of AgNP was prepared and $AgNO_3$ using *Langsat* Leaf (LL) extract as the bioreductor. The synthesized LL-AgNP was then characterized by UV-Vis spectrophotometer on 575 nm. The MIC study was done using broth dilution method with 6 different concentrations ranged from 3.125% to 100% and 2 controls, followed by the MBC study on MHA plates. The LL-AgNP successfully inhibited the growth of *Escherichia coli* and *Staphylococcus aureus* in concentration of 6.25% and 25%, respectively. The LL-AgNP also showed bactericidal activity against *Escherichia coli* in concentration of 25% but showed no activity on *Staphylococcus aureus*. This result indicates that LL-AgNP has potential as an antibacterial agent against *Escherichia coli* and *Staphylococcus aureus.*

1 INTRODUCTION

Infection caused by microorganisms is one of the main causes of chronic infection and even death (Linlin, Chen, & Longquan, 2017). There are more than 200 known diseases that can be transmitted from bacteria, fungi, viruses, and other microbes to human (Ganesan et al., 2017). It increases from time to time and becomes a real threat for the community. Antibiotics are currently used as preferred method for bacterial infection treatment because they are considered to be more effective in cost and have proven to give strong and clear results.

However, nowadays, the use of antibiotics has also started to cause other dangerous phenomena. The emergence of bacteria causing antibacterial resistance has increased quite rapidly throughout the world (Ventola, 2015). The limitation of new antibiotics in nature to replace antibiotic agents that are no longer effective raises the urgency to develop new

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antibiotics to strengthen the effectiveness of existing antibiotic agents (World Health Organization, 2014).

There are many types of precious metals that are used in the medical world, especially in antimicrobial research. One of them is silver metal (Ag). Ag has been used to demonstrate antibacterial effects and has been frequently used in medicine including orthopedics (Castiglioni, Cazzaniga, Locatelli, & Maier, 2017). The advantage of Ag utilization is that it can be formed into silver nanoparticles (AgNP).

AgNP gives promising potential as antibiotic alternatives because AgNP has better physicochemical and biological properties than whole silver (Qing et al., 2018). One of the methods to create AgNP is the green synthesis approach. This approach is seen from the biology perspective, especially the utilization of natural organisms has offered a method that is reliable, simple, non-toxic, and environmentally friendly (Velusamy, Kumar, Jeyanthi, Das, & Pachaiappan, 2016). In green synthesis, many sources from nature such as plants, bacteria, and fungi can be used in the process.

Indonesia is a country which has rich biodiversity. This provides a good opportunity and potential to develop the synthesis of nanoparticles that is more environmentally friendly using plant extracts from various plants in Indonesia. One of the endemic plants found in South Kalimantan, especially Tabalong, is *Langsat*. *Langsat* (*Lansium domesticum* var. *Pubescen* Kooders et Valeton) is a plant that comes from Meliaceae family. Every fruiting season, *Langsat* produces a lot of fruits but people in South Kalimantan only eat the fruits, despite the results of previous research proving that other parts such as bark, fruit skin, and leaf have potential as traditional medicine.

To the best of our knowledge, the synthesis of AgNP using *Langsat* leaf has been hardly explored until now. This fact supports the research of antibacterial activity using silver nanoparticles synthesized by biological methods using a bioreductor from *Langsat* leaf extract (*Lansium domesticum* var. *Pubescen* Kooders et Valeton) in vitro as one of the endeavor to find the potential of new antibacterial agents in the future.

2 MATERIALS AND METHODS

This research is a true experiment and was done with randomized post-test only control group design. There were 6 different LL-AgNP concentrations and 2 controls (positive and negative). Silver Nitrate ($AgNO_3$) 99% from Merck was used for the synthesis of silver nanoparticle. *Langsat* (*Lansium domesticum* var. *pubescen* Kooders et Valeton) leaf were collected from a private plantation in Tabalong District, South Kalimantan, Indonesia. Bacterial strain of Gram-negative *Escherichia coli* ATCC 25922 and Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 were purchased from Center of Health Laboratory (BBLK) Surabaya, East Java, Indonesia. The research was conducted in Microbiology Laboratory Faculty of Medicine, Universitas Airlangga Surabaya, East Java Indonesia.

2.1 Preparation of Leaf Extract

The fresh *Langsat* leaves were collected from *Langsat* trees and wiped gently using dry paper towel to remove dust on surface. The *Langsat* leaves were then washed thoroughly with running water and were tapped gently with paper towels afterward. The leaves were dried using a microwave at medium temperature for 6 minutes. The dried leaves were chopped using blender and were sifted with 60 mesh strainer. The extraction process was following the maceration steps. First, 100 grams of fine dried leaves were weighed and added by ethanol 96% until the volume reached 1000 ml. The mixture was left for 24 hours and was strained after that. The process was repeated for 3 times and was evaporated using *vacuum rotary evaporator* until a thick extract was obtained.

2.2 Silver Nanoparticles Synthesis

Synthesis of silver nanoparticles using *Langsat* Leaf extract (LL-AgNP) was done using the green synthesis method. The ratio of $AgNO_3$ solution and the extract was 1:10. Around 15 ml of the extract was mixed with 150 ml $AgNO_3$ solution inside a beaker glass that has been covered with aluminum foil to prevent light intrusion. The solution was then warmed inside a water bath at 60^{o}C and was stirred once in a while. The discoloration is one of the indications that the synthesis of LL-AgNp has been done.

2.3 Silver Nanoparticles Characterization

The characterization of synthesized LL-AgNP was done using UV-Vis Spectrophotometer. As much as 1 ml of LL-AgNP solution was scanned between 300 - 650 nm for determining the wavelength peak. The scan was done 4 times; right after the synthesis process (D+0) and one month after watching the stability of LL-AgNP (D+37, D+44, D+51, and D+58) with the same steps as mentioned above. The

result from LL-AgNP characterization was then used to calculate its size using a formula described by Amirjani, Firouzi, & Haghshenas, (2020).

$$\text{Nanoparticle size} = 0{,}78\,\lambda_{\text{LSPR}} - 266 \quad (1)$$

2.4 Minimum Inhibitory Concentration Assay

The Minimum Inhibitory Concentration (MIC) assay was done by the broth dilution method. First, 5 ml MHB sterile was pipetted to 8 different test tubes and were given labels; tube 1-5, Control (+), and Control (–). Then, 2 ml of LL-AgNP was added to the first tube and homogenized. After being homogenized, 1 ml of the solution from tube 1 was pipetted and was moved to the second tube and was vortexed. This step was done until the fifth tube and from tube 5, 1 ml of the solution was discharged and became the lowest concentration.

Aquadest was used as negative control, while Meropenem and Vancomycin were used as the positive control for *Escherichia coli* and *Staphylococcus aureus*, respectively. The tubes were vortexed well and were incubated on an incubator for 24 hours at 35ºC. The color of the solution on each tube was observed after the incubation. Tubes with color turbidity imminent with turbidity on positive control were then noted and the lowest concentration was decided as MIC. The tubes were proceeded to MBC assay. This assay was done in triplicate. The result was noted and analyzed descriptively.

2.5 Minimum Bactericidal Concentration Assay

One loop of the solution from tubes that has been selected from previous MIC assay was taken and was streaked to the Mueller-Hinton Agar plates. The plates were then incubated on an incubator for 24 hours at 35ºC. The colony growth was observed after the incubation period. Plates without any colony growth observed were then noted and the lowest concentration of it was decided as MBC. The result was noted and analyzed descriptively.

3 RESULTS

3.1 Silver Nanoparticles Synthesis

The *Langsat* leaf (LL) extract had a dark brown color after the extraction process was done. Meanwhile, $AgNO_3$ had no color with a clear solution just like the solvent used. After both of the components were mixed with ratio 1:10 of $AgNO_3$ 1 mM solution and LL extract, discoloration was observed. The mixed solution showed a yellowish color and slightly turbid. Based on the color changes, it could indicate the formation of silver nanoparticles. However, a further test was done in order to make sure the silver nanoparticles were successfully synthesized.

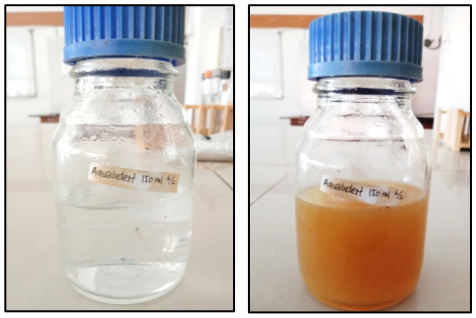

Figure 1: $AgNO_3$ solution before the synthesis process (left) and LL-AgNP after the synthesis process (right).

3.2 Silver Nanoparticles Characterization

The solution was then run through the spectrophotometer UV-Vis reading to get the wavelength peak in order to confirm the formation of LL-AgNP. The reading was done at λ 300 - 650 nm. Based on the reading process, the peak of LL-AgNP was observed at λ 398 nm with an absorbance value of 0.92. This result confirmed that LL-AgNP has been synthesized and reflected on Figure 1.

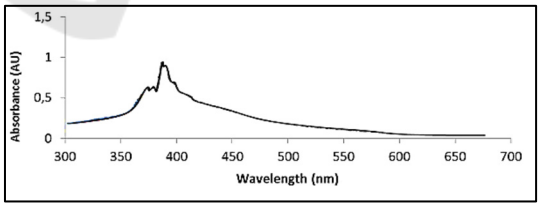

Figure 2: Characterization of LL-AgNP.

The recorded wavelength of LL-AgNP was then calculated with the formula to roughly estimate its size. Based on the calculation, a 44 nm LL-AgNP was synthesized. As stated in Khodashenas & Ghorbani (2019), AgNP shape with the size similar to the synthesized AgNP would be spherical. Biological methods for AgNP synthesis are still under development. Nanoparticles with shapes other than

spherical and cubic could only be synthesized through physical or chemical methods.

One month after the synthesis, another reading on the spectrophotometer was done to measure the current absorbance value and the stability of LL-AgNP. The LL-AgNP absorbance value shifted from 0.92 (D+0) to 1.06 (D+37), 2.66 (D+44), 3.25 (D+51), and 3.57 (D+58) (Figure 3). It could indicate the LL-AgNP has agglomerated and alteration of size and shape has occurred.

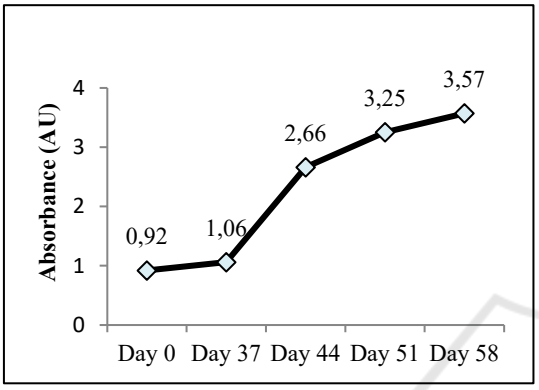

Figure 3: The LL-AgNP stability observation on several days after the initial synthesys.

3.3 Minimum Inhibitory Concentration Assay

The Minimum Inhibitory Concentration (MIC) result of various LL-AgNP concentrations presented on table 1.

Table 1: MIC of LL-AgNP.

Isolates	R	LL-AgNP Concentration (%)							
		+	100	50	25	12.5	6.25	3.125	–
E. coli	1	–	–	–	–	–	–	+	+
	2	–	–	–	–	–	–	–	+
	3	–	–	–	–	–	–	–	+
S. aureus	1	–	–	–	–	+	+	+	+
	2	–	–	–	–	–	–	+	+
	3	–	–	–	–	–	–	+	+

R (replication), + (Meropenem for *Escherichia coli* and Vancomycin for *Staphylococcus aureus*), – (Aquadest)

Based on the data in table 1, LL-AgNP could inhibit the growth of *Escherichia coli* at concentration 6.25% and *Escherichia coli* ESBL at 12.5%. Meanwhile, on the Gram-positive bacteria, the growth of *Staphylococcus aureus* and MRSA inhibited by LL-AgNP at concentration 25%.

3.4 Minimum Bactericidal Concentration Assay

The Minimum Bactericidal Concentration (MBC) result of previously obtained from MIC assay presented on tablr 2.

Tabel 2: MBC of LL-AgNP.

Isolates	R	LL-AgNP Concentration (%)						
		+	100	50	25	12.5	6.25	–
E. coli	1	–	–	–	–	+	+	+
	2	–	–	–	–	–	–	+
	3	–	–	–	–	–	–	+
S. aureus	1	–	+	+	+	+	+	+
	2	–	+	+	+	+	+	+
	3	–	+	+	+	+	+	+

R (replication), + (Meropenem for *Escherichia coli* and Vancomycin for *Staphylococcus aureus*), – (Aquadest)

Based on the data in table 2, LL-AgNP showed bactericidal activity against *Escherichia coli* at concentration 25% and Escherichia coli ESBL at 12.5%. Discrepant with the result of Gram-negative bacteria, there was no bactericidal activity observed against *Staphylococcus aureus* and MRSA.

4 DISCUSSION

Langsat leaf extract was proven can be used as bioreductor on silver nanoparticle synthesis. This could happen because Langsat leaf extract contains secondary metabolites that act as a reduction agent. Phytochemical screening of Langsat leaf was done previously by Mayanti et al. (2015), Yunus, Boddhi, & Queljoe (2018), and Matsumoto et al. (2019). The results showed Langsat leaf posses phenolic compounds, saponin, and triterpenoid/steroid. The phenolic compound on Langsat leaf has the potential as metal salt reducer and agent to stabilize the nanoparticles from agglomeration.

Pal, Rai, & Pandey (2019) explained reduction and stabilization of silver ions by biomolecule combination like protein, amino acid, polysaccharide, secondary metabolites, and vitamin that plant has, will serve as the easiest and cheapest way to synthesize NP. Generally, plant leaf has high polyphenol level. The phenolic compound has hydroxyl and ketonic group that has the ability to bond with metal and reduces metal salt and gives stability from agglomeration. Kawas (2016) described the process of secondary metabolites as a reducer of silver nitrate is $AgNO_3$ will detach into Ag+ and NO_3- form and greatly influenced by

temperature and light. During the process, it is necessary to seal the beaker glass with a cover like aluminum foil to make the beaker dark. The plant extract also gives proteins and enzymes to $AgNO_3$ solution in which the Ag+ ion will combine with enzymes to make enzyme-substrate complex. The enzymes released from plant extract work on silver ion and release nanoparticles as the product (Prasad, 2014). This formation is not just through covalent bond, but also because of the existence of protein attraction through hydrogen bond, electrostatic, or other supramolecular interactions (Ballottin et al., 2016).

Characterization of synthesized nanoparticles is one of the important steps. This has to be done in order to ensure the formation of nanoparticles. Noah (2019) explained that AgNP shows strong absorbance band and specific colors on its solution. The synthesized LL-AgNP color is yellowish and slightly turbid. The color variation occured because of the variety of phytochemical compounds on plant extract that used for the AgNP synthesize process (Ovais, 2016). Moreover, the variety of shape and size of the formed AgNP also will contribute to the AgNP solution color diverseness (González, Noguez, Beránek, & Barnard, 2014). Beside color observation, a more quantitative test needs to be done as part of NP characterization. One of the methods us spectrophotometer Uv-Vis. The LL-AgNP shows a wavelength peak at λ 398 nm. According to Seifipour, Nozari, & Pishkar (2020), AgNP that successfully synthesized has a peak observed at around 370 nm - 500 nm. With this result, it can be considered that LL-AgNP has formed.

Almost all AgNP is prone to agglomeration and it is a commonly found phenomenon. Agglomeration is a process when nanoparticles lose their nano characteristic (Bae, Lee, Kim, Choi, & Yi, 2013). Based on the finding result, one month after the LL-AgNP was formed, agglomeration was observed. AgNP stability can be monitored from time to time. The wavelength will shift and it indicates the change of absorbance spectrum on the UV-Vis area. Badiah, Seedeh, Supriyanto, & Zaidan (2019) explained that large surface tension force causes greater cohesion force. This causes the interaction between AgNPs to become greater as well. Over time, the particles will become larger in size due to the formation of groups amongst AgNPs.

Silver nanoparticles are stabler, more consistent in size, and not toxic to human tissues compared to silver in metal form. It affects the effectiveness of AgNP as antibacterial (Nolan, 2018). AgNP can penetrate to microbe cell wall easier because of its smaller size than the microorganism (Siddiqi, Husen, & Rao, 2018). Based on the obtained result, it is known that synthesized LL-AgNP has potential as an antibacterial agent against Escherichia coli and Staphylococcus aureus. The LL-AgNP could inhibit the growth of said bacteria.

The MIC and MBC assay showed that LL-AgNP works better against Escherichia coli than Staphylococcus aureus. In line with the result, according to Qing et al. (2018), AgNP gives a stronger effect on Gram-negative bacteria. One of the theories that support this finding is because Gram-negative bacteria have thinner peptidoglycan cell walls, while Gram-positive bacteria have thicker cell walls (Sizar & Unakal, 2020). Kailasa, Park, Rohit, & Koduru (2019) also mentioned the amount of Ag+ ions that successfully penetrate into the Gram-positive bacteria is fewer. It shows that there is a strong interaction between AgNP and Gram-negative bacteria.

The specific mechanism of AgNP for each bacteria is still under investigation. Generally, AgNP mechanisms as antibacterial are similar, both on Gram-negative and Gram-positive (Baptista et al., 2018). The observed mechanisms are inhibition of cell wall synthesis, protein synthesis, and nucleic acid synthesis. Furthermore, damage on the cell surface and respiration chin are also known.

There are several mechanisms of synthesized AgNP using plant extract as the reducer that has been explained by experts. The AgNP will release silver ions (Ag+) and it will directly penetrate into the bacteria cell wall (Rajeshkumar & Bharath, 2017). Wong and Liu (2010) in Durán et al. (2016) also explained that AgNP has wide surface area to come into contact with bacteria. This makes AgNP possible to adhere to the cell membrane and easily get into the bacteria. The released Ag+ ion has strong antibacterial properties. The Ag+ ion will interact with the bacteria cell membrane and cell wall components. This is one of the crucial mechanisms of AgNP toxicity towards bacteria (Liu et al., 2020).

Silver nanoparticles have positive charge. It will cause the electrostatic attraction between AgNP and bacteria cell membrane that has negative charge. The bacteria cell wall has negative charge because of electron release that is caused by catalysis activity on cell respiration. This charge interaction will help AgNP to attach to the cell membrane (Abbaszadegan et al., 2015). Hence, the antibacterial effect could be enhanced by altering the surface charge of AgNP so that stronger obstruction will be obtained (Mandal et al., 2016).

5 CONCLUSIONS

Nanoparticles were successfully synthesized using $AgNO_3$ and *Langsat* leaf extract with the recorded wavelength peak at 398 nm. The approximate size of the LL-AgNP is 44 nm. Based on the results that are obtained, we conclude that the LL-AgNP shows antibacterial activity against Gram-negative bacteria *Escherichia coli* and Gram-positive bacteria *Staphylococcus aureus*. The LL-AgNP MIC is observed at 6.25% on *Escherichia coli* and 25% on Staphylococcus aureus. The MBC is observed at 25% on *Escherichia coli* but no bactericidal activity is observed on *Staphylococcus aureus*. Further tests with different strains, concentrations, and methods are suggested to add more diversity from the findings of this study.

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