Screening of antibacterial activity from biosynthesized silver nanoparticles using *Diospyros discolor* Willd. extract

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Abstract. Silver nanoparticles (AgNPs) have a specific property for antibacterial agents. This research aims to explore the antibacterial activity from the AgNPs synthesized using *Diospyros discolor* Willd. extract. We determined that the leaf extract solution of *D. discolor* can provide reducing properties for AgNPs synthesis. The result showed a maximum absorbance peak wavelength between 420–450 nm in the produced AgNPs. The Transmission Electron Microscope (TEM) image supported the presence of AgNPs, with size between 10–50 nm. Antimicrobial assays using *E. coli* produced an inhibition zone of 7.4 ± 0.7 mm from the centrifuged AgNPs, whereas those using *S. aureus* produced an inhibition zone of 7.66 ± 0.9 mm. An antimicrobial activity index (AI) assay against *E. coli* resulted in 0.2 ± 0.1 mm, whereas the activity against *S. aureus* resulted in an activity index of 0.40 ± 0.19 mm. AI values that were lower than 1.0 was considered had less significant activity. We also tested the growth of bacteria in the medium added with AgNPs using the dilution method. The growth of bacteria colony from the streak line showed a decline in growth for the bacteria that had been exposed to biosynthesized AgNPs with the increasing of time exposure for 5, 10 and 15 minutes, respectively.

Keywords: Silver nanoparticles (AgNPs); *E. coli*; *S. aureus*; *Diospyros discolor*, antimicrobial

1. Introduction

Nanotechnology research involves particles that are 10⁻⁹ m in size (Nano-sized). Synthesis of nanoparticles can be achieved via physical, chemical, or biological processes. Plant extracts can be a reducing agent that facilitate the reduction of a metal solution to form nanoparticles. Because it requires less hazardous chemicals, biosynthesis of nanoparticles is an environmentally friendly process than the physical or chemical synthesis of nanoparticles.

Nanoparticles are used in a variety of disciplines. For example, Nano-carbon has been used to build optic fiber systems [1]; zinc and titanium oxide enables cosmetics to provide UV-protection [1], and silver nanoparticles (known as AgNPs) are frequently used as antimicrobial agents in medical fields. It is widely believed that AgNPs inhibit microbial growth by disrupting the integrity of cell membranes [2].

Synthesis of AgNPs (using reductive plant extract) has become widespread in recent years owing to their antimicrobial properties. Crude extracts derived from various plant species have been investigated...
for their biosynthesis potential. For example, the genus *Diospyros* comprises several species that exhibit antimicrobial and antiviral properties [3, 4]. Thus far, crude extracts from several *Diospyros* species have been used to efficiently synthesize AgNPs, including extracts from *D. kaki* [5], *D. anisandra* [6], *D. peregrina* [7], *D. melanoxylon* [8], and *D. ferrea* [9]. This study examines the AgNPs biosynthesis potential of *Diospyros discolor* Willd., known as “Bisbul” in West Java for synthesized silver nanoparticles then screening for their antimicrobial potential.

2. Materials and method

2.1. Preparation of leaf extract

*D. discolor* leaves were collected from the Mathematics and Natural Sciences Faculty area at University of Indonesia, Depok, West Java. The leaves were washed with distilled water and then air-dried before being heated in an oven. The plant material dried overnight with the oven temperature set at 40°C. The oven-dried leaves were coarsely powdered using a blender. After that, 2% (w/v) leaf powder was prepared by boiled for 15 min and then air-cooled until it attained room temperature then filtered the extract through a Whatman filter paper No. 1. The filtrate was kept in refrigerator at 4 ºC until used.

2.2. Silver nanoparticles synthesis and characterization

Synthesize of silver nanoparticles were done by mixed the 2% of leaf extract with 1 mM AgNO₃ solution (1:10, v/v). We examined reactions in this solution at set time intervals (15 min, 1 h and 24 h) to determine the optimal time for AgNPs formation. After 24 hours, the AgNPs divided into two samples, based on non-washing (AgNPs) and washing (AgNPs-C) which is through centrifugation process. The AgNPs-C were washed by centrifugation at 10,000 rpm for 10 minutes [10, 11]. All the AgNPs obtained were characterize using UV-Vis spectroscopy (from 200–700 nm). The color changes observed throughout our time series (15 min, 1 h and 24 h), to determine whether AgNPs were formed. After that, the shape and size of AgNP observed using TEM (FEI Tecnai G2 SuperTwin).

2.3. Antimicrobial assay using the cylinder diffusion method

The antimicrobial screening activity used the AgNPs after synthesized for 24 hours of reaction time. After centrifugation AgNPs and AgNPs-C were sonicated to homogenize the colloid and used for antimicrobial activity test. We used Gram negative *E. coli* (NBRC3301) and Gram positive *S. aureus* (NBRC100910). Both cultures were obtained from the Microbiology Laboratory of the Biology Department, University of Indonesia. The assay conducted using two different methods: cylinder diffusion agar and dilution method. We tested the antimicrobial activity of the following four solutions: (1) Bisbul Extract, (2) AgNO₃ 1 mM, (3) centrifuged AgNPs solution (AgNPs-C) and (4) non-centrifuged AgNPs. We diluted the bacteria for precultured in nutrient broth for 16–18 h until the optical density of the broth reached 0.1. The culture then inoculated in a sterile nutrient agar which contain 0.1 ml of the bacteria. Assay cylinders were placed in four physically divided and symmetrical areas of the petri dishes and each divided area was filled with one type of antimicrobial test solution which contain 240 μL in each cylinder. Each tested bacterium had three replicates. After that, the Petri dishes were incubated for 24 h at 30 ºC. The diameter of inhibition zones was recorded and tabulated. We measured an activity Index for each treatment using this equation [12, 13]:

\[
\text{Activity index (AI)} = \frac{\text{Clear zone diameter (mm)} - \text{Cylinder diameter (mm)}}{\text{Cylinder diameter (mm)}}
\]

(1)

2.4. Antimicrobial assay using the tube dilution method

The dilution method was performed to observe antimicrobial activity of synthesized AgNPs towards *E. coli* and *S. aureus* over a short time frame, followed by a Minimum Inhibitory Concentration test
toward time exposure to the treatment (reaction times: 5, 10 and 15 min). The tested bacteria that had been inoculated on nutrient agar and incubated for 16–18 h. After that the bacteria were resuspended by adding 5 ml of sterile physiological saline stirred with a vortex. Four sterile vials were filled with 0.1 ml of the suspended test organisms mixed with 5 ml of four different solutions: (1) Bisbul Extract, (2) AgNO₃ 1 mM, (3) centrifuged AgNPs solution (AgNPs-C), and (4) non-centrifuged AgNPs. These four mixtures allowed to expose the bacteria for three different time periods (5, 10 and 15 min) before inoculation by streaking the exposed bacteria to AgNPs on nutrient agar plates. The plates were incubated for 24 h at 32 ºC and observed for the abundance of the bacteria colony from the streak lines. This assay was performed in triplicate to test data reliability. We also tabulated visual observations of growth on the agar media.

3. Results and discussion

3.1. Characterization of silver nanoparticles

Differences in reaction times between the Bisbul, AgNPs, and AgNO₃ solutions are provided in figure 1. The Bisbul solution changed color over time from yellowish brown to dark brown (an image of each solution’s color is displayed in boxes labelled alphabetically from A to E). Peak wavelengths for treatments C, D, and E (sonicated treatments) occurred at 400–420 nm, in contrast to the non-sonicated treatments of Bisbul extract (line A) and AgNO₃ solution (line B), which peaked at shorter wavelengths. These differences in peaks indicates that AgNPs form inside the solutions [14], presumably caused by a strong Surface Plasmon Resonance (SPR) [15]. The AgNPs colloid (with the 24 h reaction time) had a slightly wider peak than the solutions provided with only 15-min or 1 h reaction times (figure 1), indicating that the AgNPs might vary widely in morphology and size [16]. Some studies have shown that the approximate sizes of AgNPs vary from 420 to 435 nm and from 10 to 60 nm [17, 18].

Figure 2a showed the UV-Vis spectroscopy data, wherein the centrifuged AgNPs (line C) shows a slightly higher peak than either the non-sonicated, non-centrifuged treatment (line A) or the sonicated, non-centrifuged treatment (line B). The observed peak wavelength was still about 400–420 nm. A curved line with a high peak indicates a large number of particles in the AgNPs colloid [19, 20].

![Figure 1. Spectrum absorbance from biosynthesized silver nanoparticles (AgNPs); UV-Vis absorption spectra for the following: (A) Bisbul extract, (B) AgNO₃ 1 mM, (C) AgNPs with a 15-min reaction period, (D) AgNPs solution with a 1-hour reaction period, and (E) AgNPs solution with a 24-h reaction period. Colors of solutions are depicted below the figures.](image-url)
This situation may occur if during the centrifugation process, the supernatant is discarded and replaced with another batch of AgNPs solution/destilled water or if sonication disperses large particles into smaller ones.

TEM image further confirmed AgNPs formation in the solution (figure 2b). Non-centrifuged AgNPs (figure 2c) show a variety of AgNPs particle sizes (range: 10–50 nm). In addition, the AgNPs have tended to have a bold capping that surrounded the particles. These caps may be originated from molecules in the plant extract used during the biosynthesis of AgNP [16]. The centrifuged AgNPs (figure 2b) display more similarly sized particles, ranging from 20 to 50 nm. There are also fewer capping agent surrounding the particles, which might be due to supernatant discarding during centrifugation, leaving AgNPs in suspension with various biomolecules.

3.2. Antimicrobial assay of silver nanoparticles

The synthesized silver nanoparticles (AgNPs) showed activity against *E. coli* and *S. aureus* in both the tests (cylinder diffusion and tube dilution). Data obtained from cylinder diffusion shows that all four solutions [Bisbul extract, AgNO₃ 1mM, and two AgNPs solutions (centrifuged and non-centrifuged)] inhibited both the test microbes (figure 3). We observed clear zones around the AgNPs in both treatments. The antimicrobial activity against the two microbial species did show any different,
where the clear zone diameter around 7 mm (table 1). Meanwhile, table 2 showed activity index from the treatment, the higher the activity index value it will showed stronger antimicrobial activity. The result showed that Bisbul leaves extract was more prominent against S. aureus while 1 mM AgNO₃ showed more significant inhibitory effect than synthesized AgNPs (table 2).

We performed antimicrobial assays (using the tube dilution method) in order to determine the short-term effect of AgNPs treatment against microbes (figure 4). The assays using E. coli and S. aureus showed that microbial growth was inhibited in all four treatments (i.e., all showed microbe-free zones for all tested solutions). These visual data were reinforced by quantitative measurements of the diameters of the microbe-free zones and the antimicrobial activity index (table 2). Although the Bisbul extract failed to inhibit either microbe even after 15 min of exposure, the AgNO₃ solution inhibited microbial growth within 5 min (table 3). The non-centrifuged AgNP inhibited S. aureus growth after 15 min of exposure. The centrifuged AgNP inhibited S. aureus growth within 5 min of exposure. Overall, synthesized AgNPs was more successful in preventing microbial growth in S. aureus than in E. coli. The AgNPs activity for antimicrobial activity affected by size, stability, and their ROS activity.

![Figure 3](image)

**Figure 3.** Antimicrobial assay of silver nanoparticles against: (a) E. coli and (b) S. aureus.

| Test strains | Bisbul extract | AgNO₃ 1 mM | AgNPs | AgNPs-C |
|--------------|----------------|------------|-------|---------|
| E. coli      | 8.3 ± 1.1       | 8.5 ± 1.8  | 7.0 ± 1.0 | 7.4 ± 0.8 |
| S. aureus    | 12.3 ± 2.4      | 7.9 ± 1.0  | 7.7 ± 1   | 7.7 ± 0.6 |

**Table 1.** Average diameters of the clear zones obtained with the cylinder diffusion antimicrobial assay method.

| Test strains | Bisbul extract | AgNO₃ solution | AgNPs | AgNPs-C |
|--------------|----------------|----------------|-------|---------|
| E. coli      | 0.4 ± 0.2      | 0.5 ± 0.3      | 0.2 ± 0.2 | 0.2 ± 0.1 |
| S. aureus    | 1.0 ± 0.4      | 0.3 ± 0.1      | 0.4 ± 0.2 | 0.3 ± 0.1 |

**Table 2.** Average activity indexes (AI) obtained with the cylinder diffusion antimicrobial assay method.
Figure 4. Antimicrobial assay of silver nanoparticles (AgNPs) using the streak method against *E. coli* (top row) and *S. aureus* (bottom row) after 15 minutes exposure.

### Table 3. Antimicrobial activity of synthesized silver nanoparticles (AgNPs) using the tube dilution method.

| Test solution | *E. coli* Exposure time (min) | *S. aureus* Exposure time (min) |
|---------------|-------------------------------|--------------------------------|
|               | 5 | 10 | 15 | 5 | 10 | 15 |
| Bisbul extract| +++ | +++ | +++ | +++ | +++ | ++ |
| AgNO₃ solution| - | - | - | - | - | - |
| AgNPs         | +++ | ++ | + | ++ | + | - |
| AgNPs-C       | + | + | + | - | - | - |

+++ : High growth density, +++ : Medium growth density, ++ : Low growth density, + : Very low growth density, - : No growth.

4. Conclusion
Our research demonstrates the potential use of a natural, non-hazardous, low-cost biological reducing agent from Bisbul extract to produce metal nanostructures in an aqueous solution at room temperature, thus avoiding the application of potentially hazardous solvents. The extract was capable of synthesizing 10-50 nm-sized silver nanoparticles, which were able to exhibit antimicrobial activity against *E. coli* and *S. aureus* over a 24 h exposure period. The synthesized silver nanoparticles exhibit antimicrobial activity against both microbial species, although they were not as effective as an AgNO₃ solution. This may be because the synthesized AgNPs varied more widely in size and larger AgNPs may have more difficulty penetrating microbial cells than smaller particles. Through this experiment the AgNPs have the potential as antimicrobial agent, but we need to improve their activities by reducing the size, stability and determine their action mechanism.

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