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Green Synthesis of Gold Nanoparticles Using Bioreductors of Bilimbi Fruit Extract (Averrhoa bilimbi L.) as an Antioxidant

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Abstract: The rapid development of technology today affects the birth of renewable technologies such as nanotechnology. This study reports the results of synthesizing gold nanoparticles with a more environmentally friendly method (green synthesis) using bilimbi fruit extracted through the infusion process. This synthesis method approach can be widely used in biological preparation. This study aims to determine the characteristics of synthesized gold nanoparticles and their potential as antioxidants shown through IC_{50} intensity. UV-Vis spectrophotometer and TEM were used to analyze the quantitative formation of gold nanoparticles. The analysis used 20, 10, 5, and 2.5 ppm concentration variations. The UV-Vis spectrum characterization results showed a *surface plasmon resonance* (SPR) of 534-536 nm. The average diameter of the synthesized nanoparticles was 7.98 nm withΩΩ, an approximate overall particle size of 7-12 nm characterized using TEM. Antioxidant activity was carried out using 1,1-diphenyl-2-picrylhydrazyl (DPPH) silencing assay. The percent attenuation at each gold nanoparticle concentration of 20, 10, 5, and 2.5 ppm was 99.4%, 90.7%, 79.5%, and 71.9%, respectively. So, the IC50 value can be obtained at 0.514 ppm, which is included in the strong antioxidant. This shows that gold nanoparticles mediated by bilimbi fruit extract are effective antioxidant agents.

Keywords: Antioxidant Activity; Bilimbi; Gold Nanoparticles; Green Synthesis.

Introduction

Nanotechnology supports the scientific growth of new materials with unique properties that differ from bulk materials [1]. The National Nanotechnology Initiative (NNI) defines nanotechnology as a science, engineering, and technology development implemented at the nanoscale (1 to 100 nm), which allows applications in various fields, ranging from chemistry, physics, biology, medicine, engineering, and electronics. Nanoparticles are a foundation in the application of nanotechnology [2]. Among nano-sized particles, gold metal deserves more attention because it is one of the unique metals among other metals. Gold is highly resistant to corrosion and oxidation and has ease of synthesis, stabilization, functionalization, low toxicity, and ease of detection, making it most suitable for biological/medical applications [3]. Two methods for synthesizing gold nanoparticles are top-down (physics) and bottom-up (chemistry). Both methods produce negative impacts, including toxic waste products, and the energy required is significant [4]. Recent scientific developments have led to the emergence of environmentally friendly production processes (biogenic, green synthesis) due to the advantage of producing more biocompatible nanoforms with a more efficient process. [5]

The principle of the green synthesis method of metal nanoparticles utilizes natural materials that have free electron pairs (PEBs), such as hydroxyl (OH), amine ($-NH_2$), and carboxyl (-COOH) groups that act as reducing agents and capping agents [6], [7]. Reduction reaction process of Au^{3+} ions into Au^0 (gold nanoparticles) with the addition of reducing agents from natural material extracts [8]. Bilimbi fruit has shown its potential as antimicrobial, antioxidant, hepatoprotective, anticancer, wound healing, antidiabetic, anti hyperlipidemia, antihypertensive, and antithrombotic [9]. Bilimbi extract contains several phytochemical compounds, such as flavonoids, alkaloids, phenolics, saponins, and tannins [10]. The main content of citric acid as a reducing and buffering agent [11], [12]. As much as 92.6-133.8 mEq of citric acid content in 100 grams of star fruit extracts [13].

The antioxidant activity of nanoparticles has a strong and long-lasting antioxidant intensity, including the most effective antioxidants in reducing free radicals [14]. Free radicals are byproducts of energy metabolism, so they can develop cumulative cell damage and relieve inflammation, resulting in losing an organism's ability over time [15]. One of the tests to determine antioxidant activity can use synthetic radicals such as DPPH [16]. The mechanism of action of antioxidants in reducing DPPH radicals by donating hydrogen radicals through antioxidants, inhibiting the beginning of the reaction chain, preventing the occurrence of continued reactions, and the ability to reduce DPPH radicals [17]. The research focused on an environmentally friendly, simple, inexpensive method with biocompatible results for synthesizing gold nanoparticles using star fruit. This study aims to determine the characteristics of gold nanoparticles resulting from green synthesis using star fruit extract (*Averrhoa bilimbi* L.) and the potential antioxidant activity at several concentrations of gold nanoparticles formed. UV-Vis and TEM characterized the synthesized gold nanoparticles. The antioxidant activity of nanoparticles resulting from synthesis using star fruit is reported.

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Research Methods

Material

Fresh bilimbi (*Averrhoa bilimbi* L.), 1000 ppm $HAuCl_4$, mother liquor, distilled water, filter paper, 96% ethanol solution, and DPPH powder.

Extraction of Bilimbi Fruit.

The extraction process was carried out using the infusion method. Starfruit was mashed with a ratio of 3:4 (sample ratio: distilled water). Put it into a 250 mL Erlenmeyer flask, which was heated in a 1000 mL beaker above a water bath and heated for 15 minutes at a temperature of 90ºC with occasional stirring. The heating results were filtered, and the bilimbi extract filtrate was obtained. The filtrate was used as a bioreductor in the synthesis process. The filtrate results were tested for pH and an extract with an acidity level of around 2-3 to be used as a benchmark for the results of nanoparticles that will later be formed or response variables. The sequential flow of the extraction process is explained in Figure 1.

Figure 1. Schematic diagram showing the steps for infusion methods

Synthesis of Gold Nanoparticles

The synthesis begins with preparing 2 mL of $HAuCl_4$ solution, which is diluted using distilled water solvent to the boundary mark into a 100 mL measuring flask and homogenized. The solution is reduced using 1 mL of starfruit extract at a temperature of 90ºC while stirring using a magnetic stirrer at a speed of 500 rpm until it changes colour to red wine and cools to room temperature. The detailed synthesis process is shown in Figure 2.

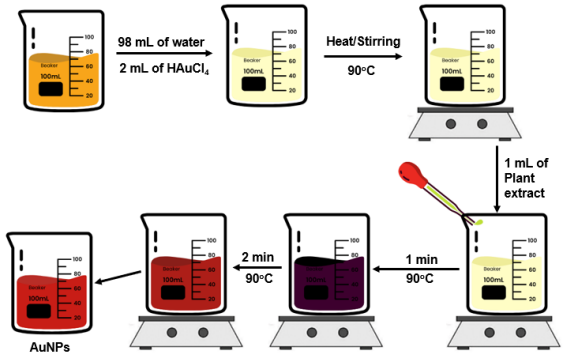

Figure 2. Schematic diagram showing the steps for gold nanoparticle synthesis

Characterization of Gold Nanoparticles

The sample's UV-Vis spectra were analyzed using a Shimadzu double-beam spectrophotometer (Model UV-1800) with a resolution between 200 and 800 nm. The blank used was distilled water. Thus, the maximum λ data and absorbance of gold nanoparticles were obtained. An analysis using the *Transmission Electron Microscope* (TEM) used is JEOL JEM-1400. In this study, the initial step is to prepare a TEM grid cleaned with an organic solvent using ethanol. Then, a little sample of gold nanoparticle synthesis is dropped on the TEM grid and left to dry. Then, they are observed using TEM at the appropriate magnification to observe the distribution of gold nanoparticles formed.

Antioxidant Activity

This antioxidant activity test was carried out using a UV-Vis spectrophotometer instrument. The first step in making a 0.003% DPPH solution is to weigh DPPH powder in the form of black powder as much as 3 mg or 0.003 grams. Then, put it into a 100 mL measuring flask with aluminium foil. Then, it was added with ethanol solution p.a. as a colourless solution to the limit mark and homogenized. The DPPH solution will change to dark purple and be left for 30 minutes in a dark place. After that, the DPPH solution is measured using a UV-Vis spectrophotometer at a wavelength of 400-600 nm. So that the maximum λ DPPH data is obtained, which will be used to measure the absorbance of the sample. The 20 ppm gold nanoparticle colloidal solution was diluted to get variations of 20, 10, 5, and 2.5 ppm and measured as much as 2 mL of each variation as a control variable for this process. A total of 2 mL of sample solution was reacted with 2 mL of 0.003% DPPH solution and incubated in a dark room for 30 minutes. The incubated sample was analyzed using a UV-Vis spectrophotometer at λ max DPPH. The absorbance value was recorded, and the % free radical quenching was calculated.

Results and Discussion

Extraction of Bilimbi Fruit

The extraction process is carried out using the infusa method; the infusa method was chosen because the manufacturing process is short and simple with low operational costs [18]. This method uses water as a solvent and produces infusions through liquid preparations [19]. The content of citric acid in star fruit will be maximally attracted due to the suitability of the solvent's polarity level [20]. The distilled water solvent was chosen because it does not leave harmful residues with synthesized results that are more biocompatible when applied in biomedical preparations than using other polar solvents [20], [21].

75 grams of fruit was pulverized using a copper/blender. The fine sample was added with distilled water in the ratio of 3:4 (sample and solvent). This method was carried out at 90ºC for 15 minutes with occasional stirring [18]. Controlling temperature and time in the extraction process helps accelerate and optimize extract results and avoid changes in bioactive compounds if the temperature is too high and too long in the extraction process [22]. The extract is filtered using filter paper to separate the

filtrate and residue until a greenish-yellow infusion is obtained, which is used as the base material for the bioreductor, as shown in Figure 1.

Synthesis of Gold Nanoparticles

$HAuCl_4$ solution was obtained at the Analytical Laboratory of the Chemistry Department of Surabaya State University. $HAuCl_4$ solution is clear yellow. The solution was obtained by dissolving gold metal with king water (aquaregia), as in the following reaction equation [23]:

$$Au_{(s)} + HNO_{3(aq)} + 6HCl_{(aq)} \rightarrow HAuCl_{4(aq)} + NO_{2(g)} + 2H_{2(g)} + 2Cl_{(g)} + H_2O_{(l)}$$

Furthermore, the solution resulting from the above reaction is heated until the NO_2 and H_2 gases and acid residues still in the solution are released [14]. According to Taufikurohmah (2013), the results of the synthesis of gold nanoparticles have concentration variations of 5-40 ppm optimum at a concentration of 20 ppm. A total of 2 mL of 1000 ppm $HAuCl_4$ mother solution was put into a 100 mL volumetric flask, diluted with distilled water, and then homogenized. They obtained 20 ppm colloidal solution. Then, the solution was put into a 250 mL beaker and heated to 90°C. When the required temperature was reached, 1 mL of star fruit extract was added. 1 mL of extract was chosen to prevent the agglomeration process because many side compounds still do not play a role in reducing Au^{3+}. The synthesis process was carried out for 1-5 minutes and stirred continuously using a magnetic stirrer (500 rpm) with temperature-controlled heating of 90 °C until a burgundy color change occurred, as shown in scheme 2. Precise temperature control can increase the rate of reduction of gold ions and efficiency in the synthesis process. It can be used as a determining parameter for increasing the number of particles by decreasing the size of the gold nanoparticles obtained [24].

The color change to burgundy occurs following the collective oscillation of electrons on the surface of gold nanoparticles [25]. The content of citric acid compounds in bilimbi fruit extract acts as a reducing agent and capping agent. The hydroxyl group in citric acid will reduce trivalent gold metal ions (Au^{3+}) into uncharged metal elements (Au^0). The gold surface will adsorb the negatively charged ions possessed by citric acid. Between gold particles, there is a dynamic process where they will collide because there is a negative charge from the citrate on the surface area of the gold nanoparticles, thus preventing the aggregation process of the gold nanoparticles from being formed [26]. The reaction that occurs in synthesizing gold nanoparticles is shown in Figure 3 [27].

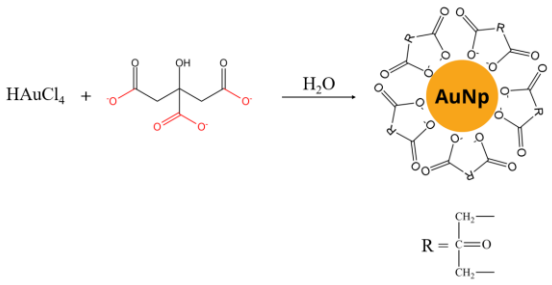

Figure 3. The reaction mechanism of gold nanoparticles using citric acid

Reduction-oxidation reactions carried out by the same compound as oxidizer and reductant will have a disproportionation process. Citric acid will reduce Au^{3+} ions from $AuCl_4^-$ to gold monochloride while oxidizing to alpha-ketoglutaric acid. Furthermore, a disproportionation process will convert AuCl into gold nanoparticles (Au^0). The mechanism of the growth reaction into gold nanoparticles using plant-based biosynthesis goes through three phases: (1) Activation phase, where there is a reduction of metal ions Au^{3+} to Au^0 by free electrons in plants; (2) Growth phase, where small nanoparticles are close together and spontaneously form larger particles; (3) Termination phase, where gold nanoparticles are shaded and form gold nanoparticles of a specific diameter [8], [28], [29].

Characterization of Gold Nanoparticles

In addition to being seen from the color change, indicators of nanoparticles can be evidenced from the results of the shift in the peak of surface plasmon resonance (SPR). $HAuCl_4$ solution results of characterization using UV-Vis spectrophotometer Shimadzu UV-1900. The maximum absorption peak of the $HAuCl_4$ solution obtained was 309 nm. The 20 ppm gold nanoparticle colloidal solution was characterized using a UV-Vis spectrophotometer so that the data received from the bathochromic shift of $HAuCl_4$ solution, which initially 309 nm to 533 nm, indicates the formation of gold nanoparticles, as shown in Figure 4. Gold nanoparticles' surface plasmon resonance peak is in the 520-550 nm absorption range compared to optical theory and plasmonic properties. [7], [30].

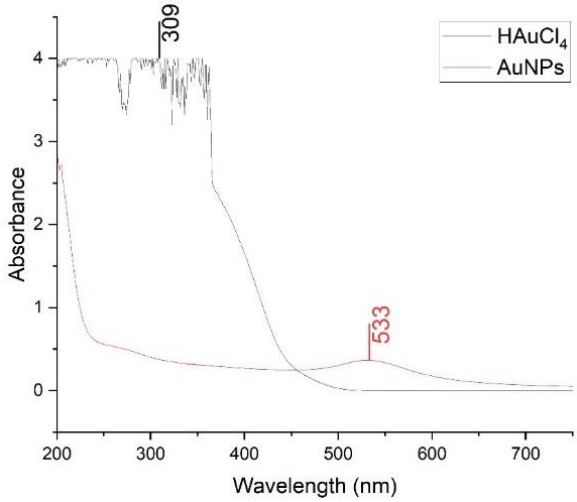

Figure 4. The shift of the bauchromic absorption spectrum of $HAuCl_4$ to synthesized gold nanoparticles

After analyzing the SPR peak, the 20 ppm colloidal solution was characterized using TEM to determine the diameter of the synthesized gold nanoparticle cluster, as shown in Figure 5. The characterization results were processed using OriginPro software, and data was obtained, as shown in Figure 6.

The particle size distribution of gold nanoparticles obtained in Figure 6 shows the smallest gold nanoparticle cluster diameter size ranging from 2-3 nm and the most significant cluster diameter size ranging from 11-12 nm, with the highest frequency of cluster sizes in the 8-9 nm range. Thus, the average diameter of the gold nanoparticle cluster size is 7.98 nm.

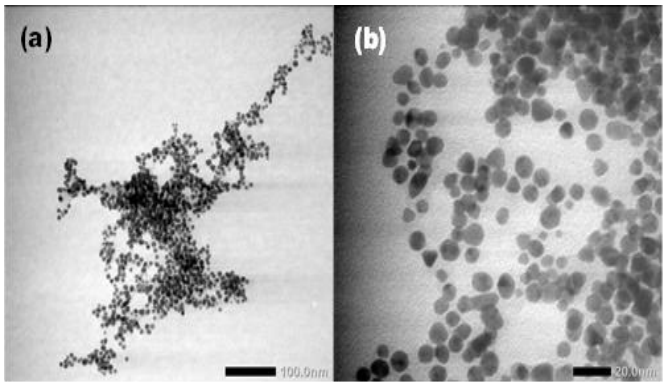

Figure 5. Characterization at magnifications of 80,000 (a) and 150,000 (b)

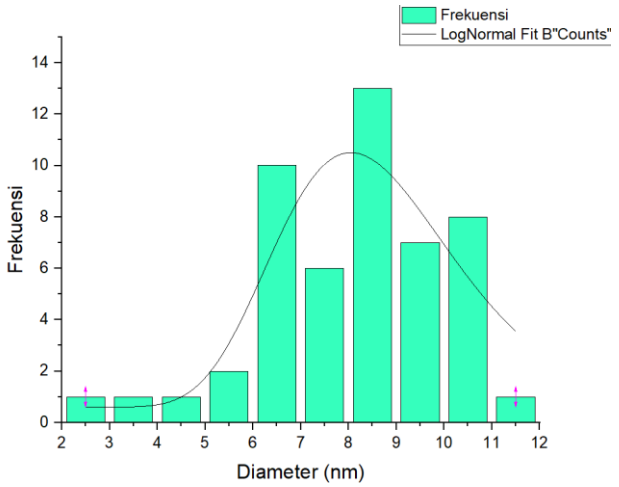

Figure 6. OriginPro data processing results

Antioxidant Activity

It began with preparing a DPPH solution with a concentration of 0.003%. 3 mg of black DPPH powder was dissolved using ethanol solvent in a 100 mL volumetric flask that had been coated with aluminium foil and obtained a 0.003% DPPH solution—coating the volumetric flask using aluminium foil because the DPPH solution is easily degraded by light and oxygen, so incubation in a dark room is necessary [31]. The incubation process is carried out for 30 minutes so that DPPH can dissolve completely until a purple solution is produced [32]. After the incubation process, the DPPH solution was analyzed using a UV-Vis spectrophotometer in the absorption range of 400-600 nm to obtain the maximum absorption of DPPH, which was later used in measuring the absorbance of gold nanoparticles. The maximum absorbance of DPPH was reported at 518 nm with an absorbance value of 0.557, which was used as the initial absorbance value.

The solution of colloidal gold nanoparticles was synthesized at 20 ppm, and the diluted nanoparticle concentration variations were 20 ppm, 10 ppm, 5 ppm, and 2.5 ppm. Diluting colloidal gold nanoparticles 20 ppm is done using a measuring flask. 50 mL of 20 ppm gold nanoparticles were diluted in a 100 mL volumetric flask, and 10 ppm gold nanoparticles were. The nanoparticle concentration of 5 ppm and 2.5 ppm is obtained with the same process, as shown in Figure 7.

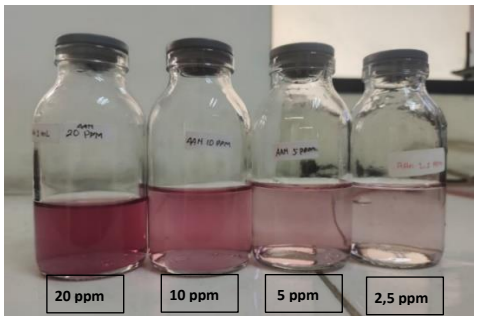

Figure 7. Gold nanoparticles variety 20; 10; 5; 2.5 ppm

Each colloidal solution of gold nanoparticles 20; 10; 5; 2.5 ppm was reacted with DPPH after 30 minutes of incubation in a ratio of 1:1. A total of 2 mL of gold nanoparticles 20; 10; 5; 2.5 ppm with 2 mL of 0.003% DPPH was put into four different test tubes that had been coated with aluminium foil and then homogenized. The process was incubated for 30 minutes in a dark room to provide the reduction reaction process of antioxidant compounds in the sample with DPPH radical compounds to the maximum [33]. Furthermore, the incubation solution was analyzed using a UV-Vis spectrophotometer by measuring the decrease in absorption at a wavelength of 518 nm so that the data obtained is in Table 1.

Table 1. Percent silencing of DPPH free radicals by gold nanoparticles

Conc. of AuNPs (ppm)	Abs.of AuNPs + DPPH at λ518 nm (A)	Abs.of AuNPs at λ518 nm (B)	A-B	% Inhibition
2.5	0.250	0.094	0.156	72%
5	0.243	0.129	0.114	79%
10	0.254	0.203	0.051	91%
20	0.363	0.36	0.003	99%

Based on the data of the percent silencing of DPPH free radicals by gold nanoparticles, antioxidant activity can be expressed by IC_{50} (inhibitory concentration), where the antioxidant concentration is described as 50% silencing of DPPH free radicals. The less the silencing value, the stronger the antioxidant activity, which is then calculated into the inhibition curve [34]. This is in line with research conducted by Yanti & Taufikurohmah (2013), which states that the greater the concentration of gold nanoparticles, the more gold particles can reduce the activity of DPPH radicals. A linear regression equation determines the IC50 value for each concentration of gold nanoparticles, y = ax + b. This equation is obtained from the curve between the concentration of the solution (on the x-axis) and the percent silencing of DPPH radicals (on the y-axis) [35] as presented in Figure 8.

Based on the %damping curve above, the equation y = ax + b is processed using the following formula [36]:

$$IC_{50} = \frac{(50 - a)}{(b)}$$

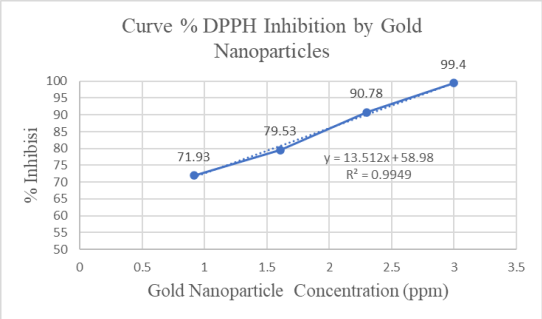

Figure 8. Graph of the percent reduction of DPPH free radicals by gold nanoparticles at several concentration variations

Based on the %reduction curve graph above, the percent of DPPH free radical silencing with 20 ppm nanoparticles was 99.4% and decreased significantly while the smaller the concentration of nanoparticles added ten ppm; 5 ppm and 2.5 ppm respectively by 90.78% 79.53% and 71.93%. The results of this research data are the research conducted by Sari and Taufikurohmah (2019) that the greater the concentration of gold nanoparticles, the more gold particles will reduce (reduce) DPPH free radicals so that the increasing percent of DPPH radical silencing, then free radical silencing will be more effective. From the % inhibition curve in Figure 8, a linear equation y = 13.512x + 58.98 is obtained with a regression value of 0.9949. Furthermore, processed into the IC_{50} formula, the value of DPPH free radical silencing by antioxidants (gold nanoparticles) is 0.514 ppm so that the antioxidant activity of the synthesized gold nanoparticles has an intensity that is classified as very strong, where the IC_{50} value is below 50 ppm.

Conclusion

The green synthesis process successfully synthesized gold nanoparticles. Gold nanoparticles synthesized using the bioreductor of star fruit (Averrhoa bilimbi L.) produced a burgundy color. The hydroxyl group in the star fruit extract causes the reduction process and the anchoring of the nanoparticles. The characteristics of gold nanoparticles were observed using a UV-Vis spectrophotometer and TEM. The average diameter of the prepared nanoparticles was about 8.48 ± 0.501 nm. The antioxidant activity of gold nanoparticles reduced silencing by 99.4%, 90.7%, 79.5%, and 71.9%. Optimal radical suppression was shown at a concentration of 20 ppm. IC_{50} value obtained 0.514 ppm classifies very strong antioxidant intensity so that the gold nanoparticles synthesized using the bioreductor of star fruit extract can be applied as an antioxidant preparation.

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