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Green Synthesis Gold Nanoparticles using Bioreductor of Butterfly Pea (*Clitoria ternatea* L.) Leaf Extract as an Antioxidant

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Abstract: Gold nanoparticles are inorganic metal materials in suspended solutions with a particle size reduction process to the 5-400 nm nanoscale range. Gold nanoparticles can be synthesized using the green synthesis method using a bioreductor of butterfly pea leaf infusion extract, which can be used as an antioxidant. This study aimed to determine the characteristics of gold nanoparticles using a UV-Vis spectrophotometer and TEM and to determine the antioxidant strength of gold nanoparticles through the IC_{50} value. This study used 20, 10, 5, and 2.5 ppm concentration variations. The results of the characterization of gold nanoparticles using a UV-Vis spectrophotometer showed a maximum wavelength of 550 nm, and the results of the TEM test showed a particle diameter of 6.58 nm. The antioxidant activity test was carried out using the 1,1-diphenyl-2-picryhydrazyl (DPPH) quenching method, resulting in % quenching at a concentration of 20, 10, 5 and 2.5 ppm of 76.3; 70.6; 85.5; and 92.9% and an IC_{50} value of 0.25 ppm which is included in strong antioxidants. It can be concluded that gold nanoparticles synthesized using butterfly pea leaf extract have the potential to be an effective antioxidant agent.

Keywords: Antioxidant Activity; Butterfly Pea; Green Synthesis; Gold Nanoparticles.

Introduction

Nanoscience and nanotechnology are science and engineering studies that are developing among world scientists [1]. Nanoparticles are nanoscale materials that have a size between 1-100 nanometers. Nanoparticles can be formed naturally and through synthetic processes [2]. Gold nanoparticles have many health benefits [3]. Gold nanoparticles are among the nanoparticles that are interesting to research. Gold nanoparticles are an inorganic metallic material in the form of a suspended solution that undergoes a particle size reduction process to the nanoscale in the range of 5-400 nm [4]. One of the advantages of gold nanoparticles is a surface that can be modified to be widely used in several fields, such as biomedicine, pharmaceuticals, biosensors, cosmetics, and other biological applications [3]. In general, the synthesis of gold nanoparticles can use top-down methods (physics) and bottom-up methods (chemistry) [5]. However, these methods cause many problems, including toxic solvents, the large amount of energy used, and dangerous waste, so the green synthesis method emerged [6].

The green synthesis method uses natural materials such as plant extracts [7]. The principle of green synthesis of gold nanoparticles is the synthesis of gold nanoparticles using natural material extracts, where natural material extracts will reduce Au^{3+} ions to Au^{0} [4]. Natural extracts must contain secondary metabolites, such as butterfly pea leaves (*Clitoria ternatea* L.). The butterfly pea plant has been widely studied and has various benefits because it contains flavonoids, flavanol glycosides, kaempferol glycosides, quercetin glycosides, and myricetin glycosides. The results of multiple studies show that *Clitoria ternatea* L. influences the pharmacological field as an antimicrobial, antiparasitic, anti-inflammatory, anticancer, antioxidant, and antidepressant [8]. Butterfly pea leaves contain flavonoids of 5.96 mg EK/g [9]

Gold nanoparticles resulting from this synthesis process can be used as a synthetic antioxidant that does not have a carcinogenic effect. Gold nanoparticles have strong, long-lasting antioxidant activity and are one of the most effective antioxidants in reducing free radicals [10]. Free radicals are molecules or atoms with unpaired electrons, so free radicals are very reactive, negatively impacting health [11]. Antioxidants can play a role in preventing damage to cellular components resulting from free radical chemical reactions [12]. Antioxidant activity testing is needed to determine the antioxidant activity of gold nanoparticles in reducing free radicals, which can be carried out using the DPPH method. The principle of the antioxidant activity test method is to measure the reduction of DPPH free radicals by an antioxidant compound with a UV-Vis Spectrophotometer to determine the percent reduction value, which indicates free radical reduction activity [13]. The antioxidant reaction with DPPH will decrease the amount of DPPH in the solution, thereby reducing the DPPH absorbance value.

Research Methods

Making Butterfly Pea Leaf Extract

The butterfly pea leaf extract was prepared using the infusion method. Seventy-five grams of butterfly pea leaves were ground using a blender/ copper, and 100 mL of aquades was added (the ratio of sample: aquades was 3:4).

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Next, prepare a water bath to heat the aquades with a 1000 mL beaker on a hot plate [14]. The Erlenmeyer containing the butterfly pea leaves is placed in a water bath, then heated for 15 minutes, stirring occasionally, and filtered using filter paper to separate the filtrate and residue from obtaining the butterfly pea leaf extract filtrate. This mechanism is shown in Figure 1.

Figure 1. Extraction of Butterfly Pea Leaf

Synthesis of Gold Nanoparticles

$HAuCl_4$ solution was measured in 2 mL using a measuring cup and then put into a 100 mL measuring flask, adding aquades and shaking until homogeneous [15]. Next, 2 mL of butterfly pea leaf extract was added. Then, it was heated at a temperature of 90˚C while stirring using a magnetic stirrer at a speed of 500 rpm until the color changed to ruby red and cooled to room temperature.

Characterization of Gold Nanoparticles

The synthesized gold nanoparticles (20 ppm) were measured using a UV-Vis spectrophotometer at 200-800 nm to calculate the maximum wavelength and analyzed using TEM to observe the particle size [16].

Antioxidant Activity

Making a 0.003% DPPH solution, 0.003 grams of DPPH was dissolved using 100 mL of ethanol, and the maximum wavelength of DPPH was measured with a UV-Vis spectrophotometer [10]. After that, DPPH was reacted with gold nanoparticles at 20, 10, 5, and 2.5 ppm concentrations and was incubated for 30 minutes in a dark environment. Then, it is measured using the DPPH wavelength with a UV-Vis spectrophotometer. The IC_{50} value is the strength value of the antioxidant activity of a sample, which is calculated using the following formula:

$$IC_{50} = \frac{(50-b)}{a}$$ [17]

Antioxidant streng based on IC_{50} value can be shown in Table 1 [18].

Table 1. Antioxidant Strength Based on IC_{50} Value

IC_{50}	Strength Antioxidant
<50 ppm	Very strong
50-100 ppm	Strong
101-250 ppm	Currently
250-500 ppm	Weak
>500	Very weak

Results and Discussion

Butterfly Pea Leaf Extract

Butterfly pea leaf extract (Clitoria ternatea L.) was obtained using the infusion method. The extraction process includes cleaning fresh red shoot leaves, grinding, and mixing with distilled water as a polar solvent [19]. Seventy-five grams of butterfly pea leaves were crushed using a Cooper, and 100 mL of aquades were added. In this infusion method, heating is carried out using a temperature of 90˚C for 15 minutes in a water bath to avoid changes in bioactive compounds and optimize the extraction process to produce an extract rich in bioactive compounds [20]. The solution used is aquades because they are polar, so it is possible to dissolve the flavonoid compounds in butterfly pea leaves. Aquades solvent can bind 5 bioactive compounds: flavonoids, tannins, triterpenoids, phenols, and saponins. This bioactive compound will reduce Au^{3+} and can be a capping agent for gold nanoparticles. The use of aquades as a solvent is used because some organic solvents have toxic (carcinogenic) and dangerous (flammable) properties, such as ethanol, methanol, CHCl3, ether, hexane, etc [10]. Aquades solvent is non-toxic, does not leave residue, and is safe when applied in antioxidant preparations. The resulting butterfly pea leaf extract is used as a bioreductant.

Synthesis of Gold Nanoparticles

The synthesis of gold nanoparticles is carried out to form gold into nanometer-sized particles. The synthesis of gold nanoparticles in this study used $HAuCl_4$. $HAuCl_4$ is made by dissolving 1 gram of gold metal in 8 mL of royal water (aquaregia) and then diluting it using a 1000 mL volumetric flask to obtain 1000 ppm $HAuCl_4$. Aquaregia solvent can dissolve gold perfectly because aquaregia is a strong acid that dissolves metal compounds [15]. In dissolving the gold metal, heating is carried out to evaporate the by-product gas in this reaction [15]. The reaction that occurs when making $HAuCl_4$ is a redox reaction. In this redox reaction, the uncharged Au ion (Au^0) is oxidized to the trivalent Au ion (Au^{3+}), resulting in the formation of the tetrachloroaurate (III) anion. The following is the reaction equation that occurs in making $HAuCl_4$:

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

To form gold in nano size (AuNPs), it is synthesized by reacting the $HAuCl_4$ solution with the bioreductant of butterfly pea leaf extract. The active compounds in butterfly pea leaves can reduce gold (Au^{3+}) to gold (Au^0). The active compounds in butterfly pea leaves contain hydroxyl groups with paired free electrons (PEB). These lone electron pairs can reduce Au^{3+} and act as a stabilizing agent for the gold nanoparticles formed [21]. The reactions that occur in plant bioactive compounds and gold (Au^{3+}) are shown in Figure 2.

Figure 2 explains that Au3+ ions undergo reduction in the reaction mixture, while Au^{+} is disproportionate back to Au^{3+} and Au^{0}. The newly generated Au^{3+} ion then oxidizes the additional 3,4-dihydroxyquercetin group obtained after the first 2 electrons of oxidation, giving the final reaction product. In the gold nanoparticle synthesis process, a disproportionation reaction occurs.

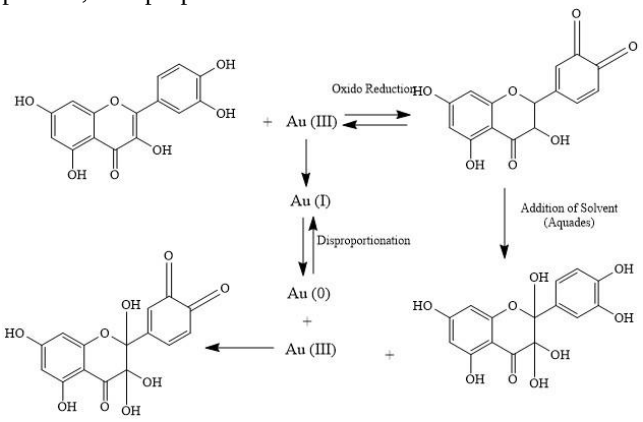

Figure 2. Reaction Mechanism for the Formation of Gold Nanoparticles Using Flavonoids (Quercetin)

The disproportionation reaction is a redox reaction where the oxidizing and reducing agents are the same substance. Gold particles, when reacted with active compounds, will produce AuNPS. Gold (Au^{3+}) reacts with electrons from active compounds to form Au^{2+}, then Au^{2+} will react with each other to produce Au^{3+} and $Au^{+,}$ where Au^{3+} will respond again with electrons, and Au^{+} will react with each other to form Au^{2+} and Au^{0}, then Au^{2+} will react with each other. The reaction is as follows :

$Au^{3+} + e^{-} \rightarrow Au^{2+}$

$Au^{2+} + Au^{2+} \rightarrow Au^{3+} + Au^{+}$

$Au^{3+} + Au^{+} \rightarrow Au^{2+} + Au^{0}$

The growth mechanism of the gold nanoparticle synthesis process using bioreactors generally consists of 3 phases. The first phase is activation; plants reduce Au3+ ions in the $HAuCl4$ solution to form Au^{0}. Then the second phase is the growth phase. This process is called the ripening process, where small nanoparticles spontaneously combine into larger particles. In the final phase, namely the termination phase, capping occurs on the gold nanoparticles to form AuNPs of a certain diameter. This mechanism is shown in Figure 3.

The gold nanoparticle synthesis in this research used 1000 ppm $HAuCl_4$ diluted to 20 ppm $HAuCl_4$. 2 mL of butterfly pea leaf extract was reacted with 2 mL of 20 ppm $HAuCl_4$ to produce 20 ppm gold nanoparticles. The extract volume of 2 mL was chosen to prevent the agglomeration process because many side compounds do not play a role in reducing Au^{3+}. The gold nanoparticle synthesis process was carried out by heating at a temperature of 90˚C and a magnetic stirrer with a speed of 500 rpm. This is done to speed up the synthesis process. The temperature of 90˚C is used because temperature is a determining parameter in the synthesis process. A significant increase in temperature will cause aggregation because the higher the heating temperature, the more reactions occur [22]. The formation of gold nanoparticles is characterized by a color change from golden yellow to ruby red.

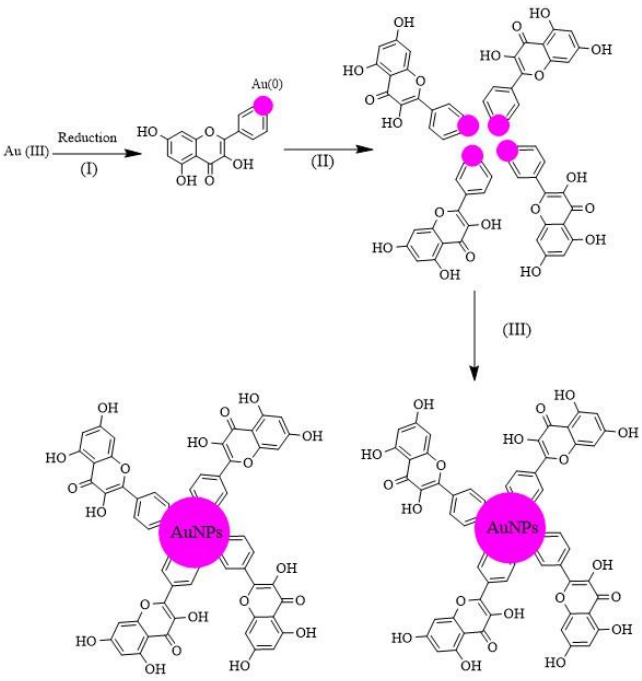

Figure 3. Growth Phase of Gold Nanoparticle Synthesis Process

Figure 4. Gold Nanoparticles 20 ppm

Characterization of Gold Nanoparticles UV-Vis Spectrophotometry

$HAuCl_4$ was measured using a Shimadzu UV-1900 UV-Vis Spectrophotometer with a 200-800 nm wavelength, resulting in a $HAuCl_4$ wavelength of 309 nm. The most stable gold nanoparticles are shown at a concentration of 20 ppm [15]. Apart from the shift in wavelength, the color change from pale yellow to ruby red indicates successful synthesis [23]. The increasingly intense color at higher concentrations suggests an increase in the density of gold nanoparticles, which can be explained by approaches based on cluster diameter and cluster concentration or density. Indicators of the stability of gold nanoparticles can be seen from aggregation and wavelength shifts. So, the gold nanoparticles that formed (20 ppm) were characterized using a Shimadzu UV-1900 UV-Vis Spectrophotometer. The measurement results show the maximum wavelength is 550 nm with an absorbance of 0.710. The spectrum from

the UV-Vis spectrophotometer results from 20 ppm gold nanoparticles is shown in Figure 5.

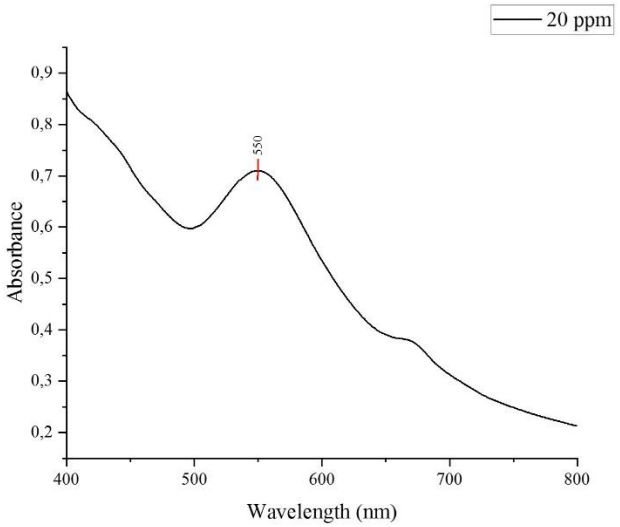

Figure 5. Graph of Maximum Wavelength of Gold Nanoparticles 20 ppm

TEM

The synthesized gold nanoparticles were then characterized using TEM (Transmission Electron Microscopy) to determine the size of the gold nanoparticles formed. The sample characterized was 20 ppm gold nanoparticles because gold nanoparticles were stable at this concentration and had a greater number of nanoparticles than other concentrations. Figure 6 shows the analysis of gold nanoparticles using TEM with a magnification of 150.000, which have various sizes. Then, the results were analyzed using ImageJ software to obtain the smallest nanoparticle size of 3.76 nm and the largest size of 8.56 nm with an average particle size of 6.58 nm.

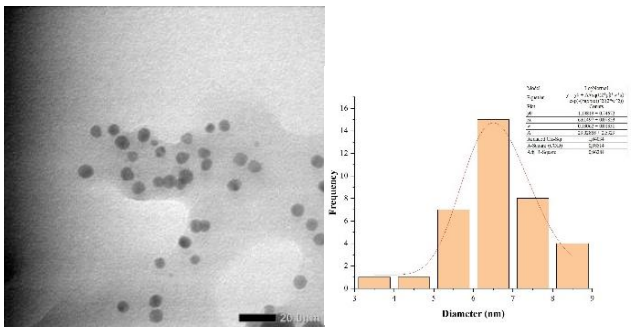

Figure 6. Characterization of 20 ppm Gold Nanoparticles using TEM (a) Magnification 10.000 (b) Results of Characterization of Gold Nanoparticle Cluster Diameters After Processing Using Origin Lab

Antioxidant Activity Testing

The DPPH reduction method is an antioxidant activity test with the working principle, namely changing the intensity of the purple color to yellow when gold nanoparticles (test sample) are reacted with DPPH. This color change causes the absorbance of the maximum DPPH wave, as measured by a UV-Vis spectrophotometer, to change so that free radical scavenging activity can be obtained, expressed using the IC_{50} value [24]. First, the maximum wavelength (λ) of 0,003% DPPH was measured, and the maximum length of DPPH was 517 nm with an absorbance of 0,600. The maximum wavelength shows the sample solution's maximum absorbance and the greatest sensitivity. Afterwards, the amount of antioxidant activity in the sample was measured using this maximum wavelength. The gold nanoparticles formed (20 ppm) were then diluted to various concentrations, namely 10, 5, and 2,5 ppm, aiming to determine the optimum concentration of gold nanoparticles in reducing free radicals.

Table 2. Results of Absorbance Measurements of Gold Nanoparticles and DPPH at λ 517 nm

Sample	Absorbance at length 517 nm wave			
	1	2	3	Average
DPPH + Nanogold 2.5 ppm	0.195	0.196	0.195	0.195
DPPH + Nanogold 5 ppm	0.289	0.291	0.288	0.289
DPPH + Nanogold 10 ppm	0.317	0.316	0.316	0.316
DPPH + Nanogold 20 ppm	0.456	0.456	0.458	0.457

Based on the data in Table 2, there was a decrease in DPPH absorbance after it was reacted with gold nanoparticles. This can happen because there is an interaction between DPPH and the contributing electrons, namely the Au atoms in the gold nanoparticles so that the DPPH will be reduced, causing the color of the solution mixture to fade. The absorbance data is then used to calculate the percent dampening, as shown in Table 3. The percent dampening is calculated using the formula:

$$\% \text{ inhibition} = \frac{(\text{A Blanko} - \text{A Sampel})}{\text{A Blanko}} \times 100\%$$

Information :
A blank = DPPH Absorbance (0.600)
A sample = Absorbance of sample (Absorbance of sample-absorbance of gold nanoparticles)

Based on table 2, data on the percent reduction of DPPH by antioxidant substances, namely gold nanoparticles, is obtained. In DPPH, there is a radical N atom that bonds with the Au atom. This is because there is a coordinating covalent bond between the Au and N atoms. The N atom, which has a lone pair of electrons, will be captured by the Au atom to form an Au-N bond. When a coordinating covalent bond is formed, it indicates that the radical N atom has become stable, so it can be seen that the Au atom has reduced (dampened) the DPPH free radical. The damping reaction is shown in Figure 8.

Table 2 presents the calculation of percent dampening, which shows that gold nanoparticles have greatly dampened DPPH. The greater the concentration of gold nanoparticles, the greater the percentage of DPPH radical scavenging so that free radical scavenging becomes more effective. The increasing absorbance refers to the resulting physical condition; that is, the greater the concentration of gold nanoparticles, the more intense the color of the solution becomes, but the solution is still ruby red (the color of gold nanoparticles), so the reduction is

carried out using the absorbance of gold nanoparticles before reacting with DPPH. However, gold nanoparticles at a concentration of 5 ppm experienced a decrease in absorbance, thus forming a non-linear valley. This can happen because the interaction of gold nanoparticles with DPPH radicals is not optimal.

Table 3. Percent Inhibition of DPPH by Gold Nanoparticles

Concentration Nano gold (ppm)	DPPH Absorbance	Absorbance Sample (Nano gold + DPPH) (A)	Absorbance Nano gold (B)	% Inhibition (%)
2.5	0.600	0.195	0.053	76.3
5		0.289	0.113	70.6
10		0.316	0.229	85.5
20		0.457	0.414	92.9

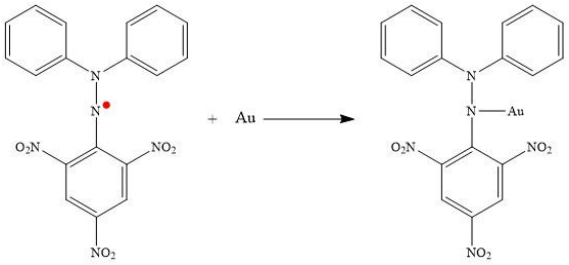

Figure 7. DPPH Attenuation Reaction by Gold Nanoparticles

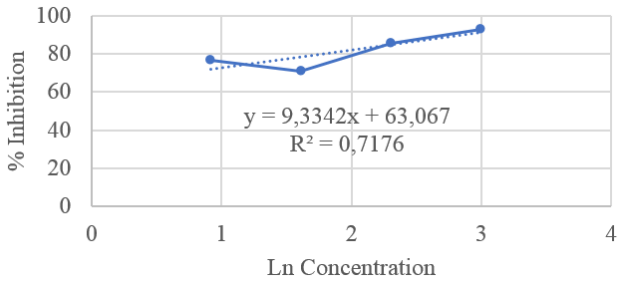

Figure 8. Percent Curve of DPPH Reduction by Gold Nanoparticles at Concentration 20, 10; 5, and 2.5 ppm

The percent damping data in Table 2 is then used to determine the IC_{50} value. The IC_{50} value is calculated by creating a curve of the relationship between the test sample concentration and the attenuation percentage. The research data in Figure 9 shows the relationship curve between the concentration of the test solution and the % inhibition. The regression equation y = 9.3342x + 63.067, with a value of R^2 = 0.7176. The IC_{50} value is a number that indicates the effective concentration of the gold nanoparticle test sample, which can inhibit the oxidation process by 50% of the total DPPH.

The research data based on Figure 9 shows that the IC_{50} value of gold nanoparticles synthesized using butterfly pea leaves is 0.25. The strength values of antioxidant activity are shown in Table 3 [18]. Based on table 3, gold nanoparticles synthesized using the bioreductant butterfly pea leaf extract are very strong antioxidants with an IC_{50} value of 0.25 ppm. This value shows that at a concentration of 0.25 ppm, gold nanoparticles can reduce the activity of free radicals (or other oxidant compounds) by 50% of the total number of free radicals present. Table 3 showed antioxidant streng based on IC_{50} value [18]. Based on table 3, gold nanoparticles synthesized using the bioreduction butterfly pea leaf extract are strong antioxidants with an IC_{50} value of 0.25 ppm.

Conclusion

The results of green synthesis of gold nanoparticles synthesized using a bioreductant from butterfly pea leaf extract (*Clitoria ternatea* L.) are rubby red, with a maximum wavelength of 550 nm and an average particle diameter of 6.58 nm. The antioxidant activity test of gold nanoparticles produced a reduction percentage of 76.3, 70.6, 85.5, and 92.9%; a concentration of 20 ppm shows the optimum percent reduction. The IC_{50} value is 0.25 ppm, which indicates a very strong antioxidant.

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