RESEARCH ARTICLE

Synthesis of Gold Nanoparticles using Bioreductants from the Aqueous Leaves Extract of *Dillenia philippinensis* Rolfe. (Katmon)

Marijen Y. Soto[1]* and Rey Alfred Ranola[2]

*Corresponding author's email address: marijensoto@rocketmail.com

[1]*Graduate School, Centro Escolar University, Manila, Philippines*
[2]*Physical Science Department, Centro Escolar University, Manila, Philippines*

ABSTRACT

Background and Objectives: Endemic plants are integral part of the ecosystem that provide innumerable benefits. Thus, emerging studies and applications using these endemic plants continue to increase globally. In this study, the preparation of gold nanoparticles (AuNPs) has been carried out by using the aqueous leaves extract of *Dillenia philippinensis* Rolfe. (Katmon) as the bioreductants.

Methodology: After the synthesis of AuNPs, the process was further subjected to optimization to assure the production of AuNPs with the best parameters. Synthesized and optimized AuNPs were characterized using FTIR, XRD, DLS, Zeta Potential, and TEM.

Results: Optimization results that provide the desired properties for AuNPs were a volume ratio of 1:2 ($HAuCl_4$: leaves extract), no significant difference ($p>0.05$) with the sequence of addition and reaction time, and acidic pH. The synthesized AuNPs' particles were found to contain hydroxyl and amine groups, had broad, amorphous, and spherical particles that have a mean diameter of 60.6nm, a PDI of 0.563, and a repulsion force of -13.720mV. The optimized and characterized AuNPs were then further used as a colorimetric sensing material, showing the potential applicability of AuNPs in the heavy metal analysis.

Conclusion: Gold nanoparticles were able to be synthesized with the use of bioreductants that are present in the aqueous leaves extract of Katmon. This concludes that endemic plants in the Philippines can be used for the synthesis of AuNPs and can be applied in the field of phytonanotechnology and other applied sciences.

Keywords: *gold nanoparticles, green synthesis, bioreduction, surface plasmon resonance, dillenia philippinensis, colorimetric sensing*

Introduction

Metallic nanoparticles are nanometer-sized materials that exhibit unique optoelectronic properties and catalytic activity [1-4]. These unique properties pave the way for their applications in various fields such as biomedical, health care, environmental, drug delivery, nonlinear optics, and space industry [2,3,5-9]. Gold Nanoparticles (AuNPs) have been a recent interest for detecting heavy metal ions by colorimetric sensing because of their unique optical properties, stability, chemical inertness, and a sensitive shift on the localized surface plasmon resonance (LSPR) peak can be detected by the naked eye or with the use of a colorimeter [3,7,9-11].

The synthesis of Gold Nanoparticles (AuNPs) can be carried out by chemical method, physical method [5,12], and microbiological method. However, the use of such methods is harmful to the environment due to the strong reductants, demands the use of high energy, and requires maintenance of microbial cultures which is time-consuming, respectively [3,5,7,9,10,12,13]. Hence, the emergence of green synthesis of nanoparticles from plant extracts has received increasing attention due to its advantages including the short synthesis reaction time, environmentally-friendly, and eliminate the need of maintaining microbial cultures.

Katmon is an endemic plant found in the Philippines [14,15]. Studies were conducted on the phytochemical secondary metabolites in both leaf and fruit parts and pharmacological assays such as antioxidant, antimicrobial, acute toxicity testing, and as an alternative to the colorants

used for acetaminophen syrups [14-18]. In this study, the preparation of AuNPs was carried out by using the Katmon aqueous leaves extract as the reducing agent.

Methodology

All chemicals and reagents used for the entire experiment were of analytical grade and were purchased from different local laboratory suppliers in the Philippines. All glassware were acid-washed with aqua regia, rinsed with deionized water, and oven-dried before use.

Preparation of Katmon Plant Extract

The leaves of Katmon that were collected from Polilio Island, Quezon Province were washed with deionized water and shade-dried for 7 days. It was then powdered using an Oster 4172 10SPD. Exactly 1.5g of powdered Katmon leaves were mixed with 100mL deionized water to yield a 1.5% aqueous extract (%w/v) concentration. The mixture was heated at 60ºC for 30 minutes. The aqueous extract was then filtered using Whatman 0.4micron filter paper and the filtrate was directly used for the synthesis of AuNPs [19].

Total Flavonoid Content (TFC) Analysis, Total Phenolic Content (TPC) Analysis, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay

The amount of flavonoids and phenolics in the Katmon extracts were also analyzed in this study. According to Silva-De Hoyos, *et al*. (2017) [20], the amount of total phenolics in the extract and the degree of its antioxidant property are similar to the amount of reducing agent in the aqueous extract.

Green Synthesis of Gold Nanoparticles (AuNPs)

The synthesis of gold nanoparticles (AuNPs) was based on the methods of Sett *et al*. (2016) with slight modifications. Exactly 1200uL or 1.2mL of 1.5% Katmon aqueous extract was mixed with 800uL or 0.8mL of 1mM chloroauric acid ($HAuCl_4 \bullet 3H_20$) solution to produce a total volume of 2000µL or 2mL. Formation of the wine-red color of the colloidal solution and with no visible aggregation indicates the presence of AuNPs [19]. Color change and the absorbance of the colloidal solution were closely monitored during the entire procedure.

Optimization Procedures

The properties of the synthesized AuNPs greatly rely on their shape, size, and morphology. Hence, modulating the different parameters (volume ratio of extract and chloroauric acid solution, the effect of pH, the effect of reaction time, and the sequence of addition) is an important procedure in this study. Beckman Coulter DU 730® Life Science UV-Vis Spectrophotometer was used for the entire experimentation.

Different volumes of Katmon aqueous extract and chloroauric acid were mixed to obtain the optimum absorbance. Table 1 shows the volume of $HAuCl_4$ and Katmon aqueous extract and the respective extract percentage of each 2mL microcentrifuge tubes.

The synthesized AuNPs were optimized according to their reaction to time (30 seconds to 60 minutes). The synthesized AuNPs were identified by a noticeable change in visual appearance in terms of color hue or by checking its absorbance in the UV-Vis Spectrophotometer at 541nm wavelength.

Table 1. *Volume ratio of HAuCl4 and Katmon aqueous extracts*

K	$HmAuCl_4$ (µL)	Plant Extract (µL)	Extract %
1	0	2000	1.5
2	200	1800	1.35
3	400	1600	1.2
4	600	1400	1.05
5	800	1200	0.9
6	1000	1000	0.75
7	1200	800	0.6
8	1400	600	0.45
9	1600	400	0.3
10	1800	200	0.15
11	2000	0	0

The effect of pH was optimized using two methods: 1) via the addition of NaOH/HCl (pH range of 1-12) to the synthesized AuNPs and 2) the addition of different volumes of McIlvaine's Buffer System (pH range of 2-8) to the synthesized AuNPs. A minimum shift in the wavelength would be considered as the optimum pH for the synthesis of AuNPs.

Characterization of Synthesized AuNPs

The formation of AuNPs was confirmed and characterized via the use of Fourier Transform Infrared (FTIR) Spectroscopy, X-Ray Diffraction Analysis (XRD), Dynamic Light Scattering – Particle Size Analyzer (DLS-PSA), Zeta Potential Analyzer, and Transmission Electron Microscope (TEM).

Fourier Transform Infrared Spectroscopy was used to identify the functional groups responsible for reducing the gold ions in chloroauric acid [13]. Cary 630 FTIR was used for this characterization process.

X-Ray Diffraction was used to obtain structural information on the AuNPs' crystallinity. The synthesized AuNPs were dropped cast in 1"x1" glass slides and dried at room temperature. It was then analyzed using the Maxima XRD-7000 Shimadzu.

Dynamic Light Scattering – Particle Size Analyzer (DLS-PSA) was able to measure the average particle size and distribution of AuNPs [21]. This was conducted using the Micromeritics Nanoplus-1.

Zeta Potential Analyzer measured the surface charge of the AuNPs [22]. This was conducted using Zeta-Meter 4.0.

Transmission Electron Microscope (TEM) was used to determine the morphology and shape of the synthesized AuNPs. The synthesized and optimized AuNPs were analyzed using the JEOL JEM 2100-F Field Emission Transmission Electron Microscope.

Colorimetric Sensing Analysis

The synthesized AuNPs were introduced to different heavy metals such as lead, mercury, and cadmium. Different concentrations (100ppm, 80ppm, 60ppm, 40ppm, and 20ppm) of the heavy metal ions were prepared. The sensitivity testing was done by mixing 1mL of the synthesized AuNPs with 1mL of different concentrations of heavy metal ions (3 trials per concentration). Their absorbances at 541nm were then measured using the UV-Vis Spectrophotometer.

Results & Discussion

The decoction of the Katmon leaves resulted in a brownish- colored solution.

TFC and TPC Assay

The amount of flavonoids and phenolics in the Katmon powdered extracts were also analyzed in this study as it was believed that these compounds elicit antioxidant properties [23]. Additionally, the amount of total phenolics in the extract is similar to the amount of reducing agent in the aqueous extract [20].

In this study, Katmon powdered extracts were observed to have 58.981mg/g ± $3.39E^{-17}$ (gallic acid equivalent) and 72mg/g ± 0 (quercetin equivalent) (n = 3). In the study conducted by Barcelo (2015), the fruits of Katmon showed 4.85 mg/100g (quercetin equivalent) while Recuenco, *et al.* (2016) observed that the fruit of Katmon contains 449 ± 29 mg/100g (gallic acid equivalent) and 190 ± 4 mg/100g (catechin equivalent). Hence, this suggests that a sufficient amount of phenolics on Katmon aqueous leaves extract would have the capability of reducing the gold ions into gold nanoparticles.

DPPH Assay

The main reason for utilizing plant samples in the synthesis of AuNPs is their phytochemical constituents that are capable of reducing gold ions to gold nanoparticles. To determine if the plant sample can be used for the synthesis of AuNPs, an antioxidant assay (such as DPPH Assay) was conducted.

The change of color in the DPPH solution indicates the extracts' ability to reduce DPPH radical. There is no significant difference ($p > 0.05$) between the percentage scavenging activities of a 3% decoction of the Katmon aqueous leaves extract (83.09% ± 0.1640) and the 3% Ascorbic Acid (89.59% ± 0.1639). According to Barcelo (2015), Katmon fruits were found to have the highest percentage of radical scavenging activity (91.13%) indicating their high antioxidant property. In other studies, *Dillenia indica* methanolic fruit extract was also found to have high antioxidant properties. These studies thereby indicate that the members of the Dilleniaceae family have high antioxidant properties.

By definition, IC_{50} or the half-maximal inhibitory concentration is the concentration of drug at which 50% of the target is inhibited [23]. Therefore, a higher IC_{50} level depicts a lower antioxidant effect of the plant sample while a lower IC_{50} means a more potent molecule. Katmon received an IC_{50} of 0.27g/100mL compared to ascorbic acid with 0.67g/100mL. Hence, this supports the notion that the Katmon aqueous leaves extracts are rich in antioxidant compounds.

Synthesis of Gold Nanoparticles

The presence of phytochemical constituents that are capable of antioxidant property as presented by the results of the Antioxidant Assay thereby supports the proposition that the Katmon aqueous leaves extract can be used for the synthesis of AuNPs.

From the numerous experiments conducted for the volume ratio, the 1.5% w/v Katmon aqueous leaves extract was chosen as the optimum concentration as other concentrations higher than 1.5% were observed to produce aggregated gold nanoparticles while concentrations lower than 1.5% were not enough to produce the desired properties of AuNPs (i.e. wine-red coloration). Additionally, the 1.5% aqueous leaves extract obtained a high antioxidant property

as shown in the DPPH results, thus justifying its utilization as the optimum w/v concentration.

Optimization of Synthesized AuNPs

It was stated from previous studies that the yield of AuNPs was small. Hence, the optimization of different parameters was done to increase their yield.

Effect of Volume Ratio

The presence of the localized surface plasmon resonance (LSPR) band was indicated by the absorption of light at wavelength 520-560nm. At this specific wavelength, the purplish-red color was transmitted; hence, explaining the optimum wine-red color of the AuNPs [24]. In a similar study by Rastogi, *et al*. (2013), increasing the amount of plant extract also increases the nucleation site for gold ion complexation thereby producing smaller gold nanoparticles. Whereas, decreasing the amount of plant extract produces fewer nucleation sites thereby forcing the complexation of gold ion to occur in only one nucleus leading to the formation of bigger particles. Hence, these statements imply that the optimization of the volume ratio between the Katmon aqueous leaves extract and $HAuCl_4$ is very important as it affects the AuNPs' size, color variation, and maximum absorption.

As shown in the inset of Figure 1, K2-K5 produced a more reddish color of AuNPs solution because of the red-shifting of the absorption band that is due to the large volume of Katmon aqueous leaves extract as compared to the low volume of $HAuCl_4$. Furthermore, the AuNPs solutions assigned as K6-K9 were observed to have a more purplish color due to the low volume of aqueous leaves extract and a high volume of $HAuCl_4$. It can be observed that although K8 has the highest lambda peak, it was not chosen for the study due to its purplish color that elucidates the blue (hypsochromic) shifting of the absorption band as shown in the inset in Figure 1.

The AuNPs solution with $HAuCl_4$ to Katmon aqueous leaves extract ratio of 1:2 (denoted as K5) is deemed to achieve the optimum parameters (i.e. wine-red coloration and with no visible aggregation). Hence, K5 was concluded as the optimum AuNPs for the study.

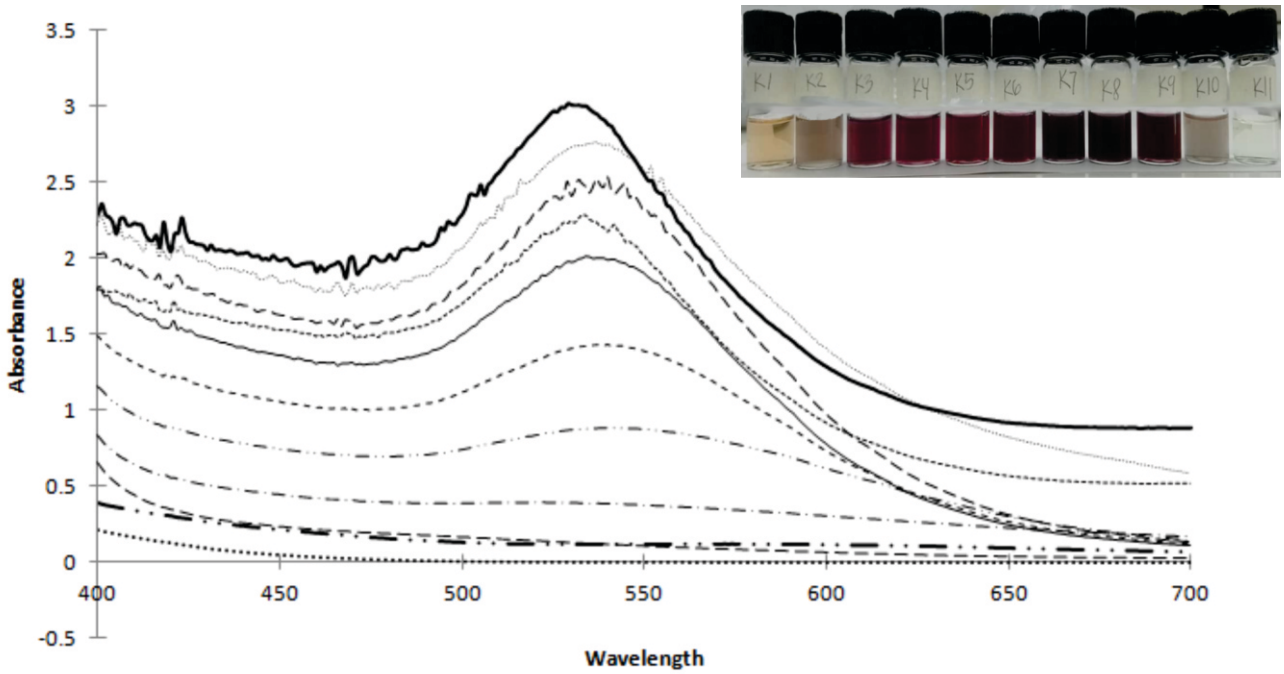

Figure 1. *Absorption spectra of AuNPs at different wavelengths (Inset: synthesized AuNPs)*

Effect of Reaction Time and Sequence of Addition

It was observed in related studies that the complexation of gold ions to the nucleation site present in the aqueous leaves extract was time-dependent [24,25]. Faster gold nanoparticle formation produces more isotropic and smaller gold nanoparticles. This is one of the advantages of gold nanoparticles over other metal nanoparticles as the quick color change of the mixture infers the rapid reduction of gold ions to gold nanoparticles. Hence, the formation of AuNPs in response to time was also optimized for this study.

It was found that the AuNPs' absorbance increased with time wherein it took 2 minutes to achieve the desired wine-red coloration and approximately 10 minutes for the AuNPs to reach the lambda peak. Thus, the AuNPs synthesized from the Katmon aqueous leaves extract can already be used for colorimetric assay after 10 minutes. This was also supported by the result of kinetics between $HAuCl_4$ and Katmon aqueous leaves extract, where it was observed that there were no changes in the absorbance peak at 8 minutes. Hence, this stabilization suggests the point of equilibrium in the chemical reaction at 10 minutes.

Furthermore, the lambda peak started higher in E2A (i.e. Extract to $HAuCl_4$) as compared to A2E (i.e. $HAuCl_4$ to Extract). The hypothetical reason for this phenomenon might be related to the mechanism of nanoparticle synthesis. The Katmon aqueous leaves extract contains polyphenols, particularly flavonoids, which are responsible for the reduction of gold ions to gold nanoparticles. Moreover, adding the extract to the chloroauric acid might intensify the interaction between the reducing agent and the gold ions. Nevertheless, it was observed that there is no significant difference ($p > 0.05$) with the sequence of addition and reaction time when grouped according to the lambda peak (Table 2).

Table 2. *Summary of the Lambda Peak in terms of Reaction Time and Effect of Addition*

Time	(a) Reaction Time ($HAuCl_4$ to Extract) Lambda Peak	(b) Reaction Time (Extract to $HAuCl_4$) Lambda Peak
30 sec	536	535
1 min	537	536
2 mins	537	536
5 mins	537	537
10 mins	539	540
15 mins	541	541
30 mins	543	543
60 mins	545	545

Conditions: 0.8mL $HAuCl_4$ and 1.2mL Katmon Leaf Extract (at pH4)

Effect of pH

Any changes in the environment of the gold nanoparticles will cause shifting of the LSPR band which was visually observed as color change [6]. As shown in the inset of Figure 2, it was found that at a very low pH (i.e. pH 1), the AuNPs show no color change on the solution. But at higher pH, the AuNPs' color starts to change from wine-red to a brownish solution which was observed as a slight bathochromic shifting of the wavelength (as shown in Figure 2) and also as a physical manifestation of aggregations.

Additionally, Figure 2 shows the spectra of the synthesized AuNPs after the addition of different volumes of McIlvaine's Buffer at different pH where it can be observed that there is a prominent hypochromic shifting of the wavelength. Changing the pH of the extract results in a change in charge of the phytochemical secondary metabolites, which affects their ability to bind and reduce gold ions [24]. This was also supported by the probability that there was an increased amount of functional groups that became available for nucleation when gold ions are at pH 3-4 [24].

Hence, the optimum pH of the synthesized AuNPs was at pH 4.0 as there were no observed bathochromic or hypsochromic shifting of the wavelength. Furthermore, pH at a range of 2.2-4.0 shows no significant difference ($p > 0.05$).

Characterization of Synthesized AuNPs

For the researcher to further understand why the AuNPs behave in such a manner, characterization was conducted. This was also vital for studying the physical and chemical properties of nanoparticles and their effect on the AuNPs' sensitivity.

Fourier Transform Infrared Spectroscopy (FTIR)

Evaluation of the functional groups responsible for the reduction of gold ions to gold nanoparticles was conducted via FTIR analysis. These functional groups could also serve as capping ligands whose main role is the stabilization of the nanoparticles to avoid further agglomeration [26].

The infrared (IR) spectrum of the synthesized AuNPs was compared with the Katmon aqueous leaves extract. Hydroxyl (O-H) group acquires sharp and broad stretching at 3336cm^{-1} which is due to the existence of flavonoids and phenolic

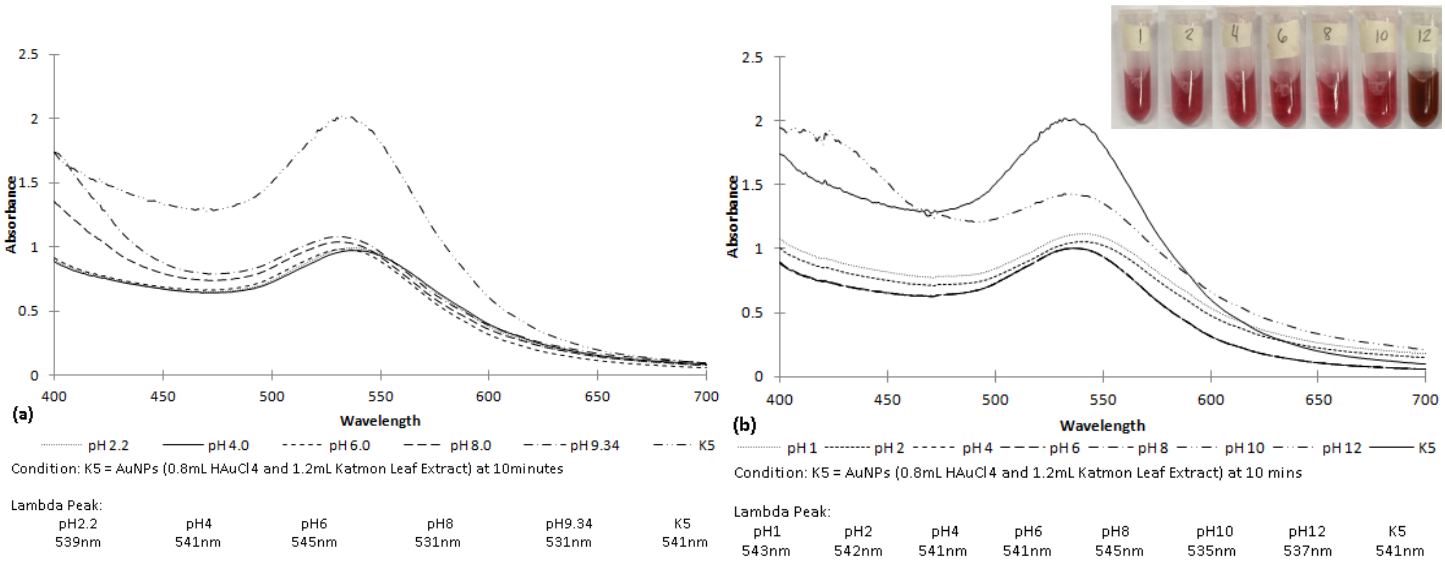

Figure 2. Absorption spectra of AuNPs at different pH using McIlvaine's Buffer System (a) and NaOH & HCl (b)

compounds in the extract. Both the spectra of Katmon aqueous leaves extract and the synthesized AuNPs showed medium N-H stretching at 1636cm^{-1} which is attributed to the presence of 1° amine while sharp and broad C-H stretching at 652cm^{-1} indicated the presence of alkynes.

It was found in similar studies that the most important reducing and capping agents in plant extracts were proteins, polysaccharides, flavonoids, and terpenoids [13,24,27]. Consequently, all those observations strongly inferred that the polyphenols present in Katmon aqueous leaves extract are functionally significant for reducing and capping gold ions to gold nanoparticles.

X-Ray Diffraction (XRD)

Identifying the crystalline pattern of the synthesized AuNPs was part of the characterization process as these patterns contribute to the properties related to AuNPs. Only crystalline parts yield sharp peaks specifically at 111°, 220°, and 222°. Amorphous materials did not possess periodically arranged atoms but they were randomly distributed in 3D space. X-rays would be scattered in many directions leading to a large bump distributed in a wide range (2θ) [26]. Hence, this explains the amorphous property of the synthesized AuNPs which is interpreted by the broad diffraction pattern of the synthesized AuNPs with an equivalent Bragg angle at 2θ = 22°.

Particle Analysis

In terms of particle size analysis, Dynamic Light Scattering (DLS) provides a mean diameter that was larger than those provided by the Transmission Electron Microscope (TEM). This was because DLS measured the hydrodynamic radius which includes the nanoparticle plus ligands attached to its surface [22]. Nevertheless, DLS could also provide information regarding the polydispersity of the nanoparticles aside from the measurement of mean diameter, hence, confirming it as an essential part of the characterization process.

Particle size analysis shows the exhibited average diameter of the AuNPs on the first day of synthesis to be 60.6 nm and the polydispersity index (PDI) to be 0.563 suggesting a medium polydisperse state, signifying that the AuNPs particles throughout the solution do not have the same sizes. The particle size of one-week-old AuNPs was also analyzed and it was found to have a mean diameter of 64.2 nm with a PDI value of 0.601, which was also interpreted as having a medium polydisperse state. These results suggest that the synthesized AuNPs from the aqueous leaves extract of Katmon were particularly small in size and were still stable after one week. Its polydispersity was also observed to increase slightly but still suggests moderate uniform sizes throughout the solution [28].

Zeta potential was an indication of the surface potential which could determine the magnitude of the nanoparticles' repulsive layer. Furthermore, zeta potential could also predict the stability of the colloidal system [22]. The measured zeta potential for the synthesized AuNPs was found at -13.720 mV ± 0.710 (n = 20); hence, proposing its minimal repulsion force which can lead to agglomeration of the particles.

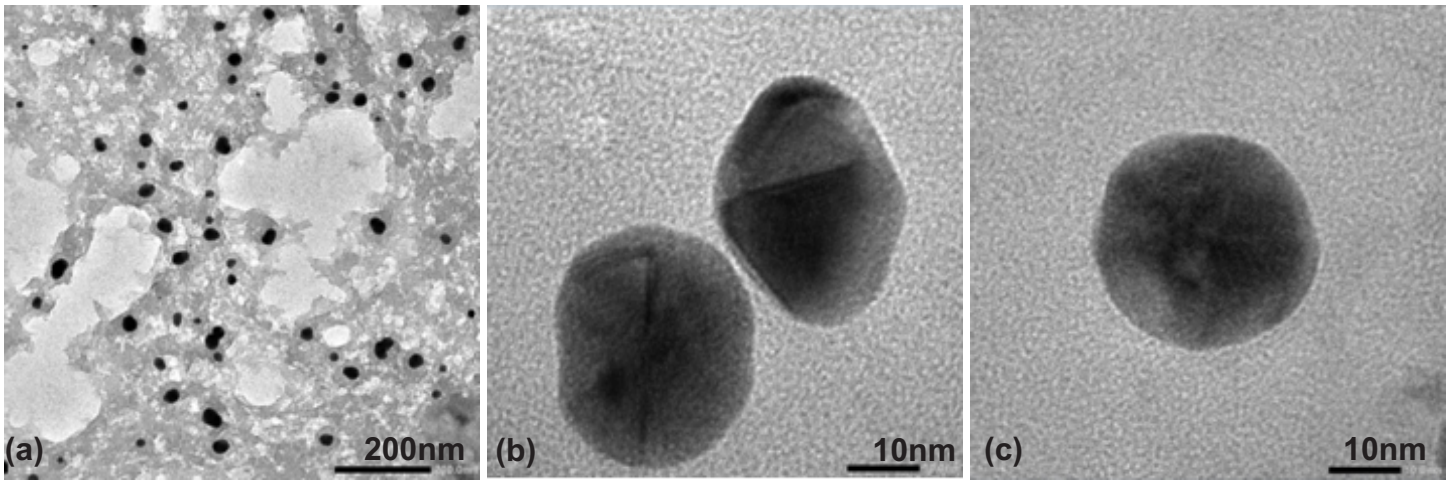

Figure 3. *TEM Micrographs depicting the polydispersity and morphology of the synthesized AuNPs*

Transmission Electron Microscope (TEM)

The LSPR band was dependent on the size and morphology of the synthesized AuNPs. This was explained by the red (bathochromic) and blue (hypsochromic) shifting of the LSPR band whenever there was a color change in the AuNPs solution. TEM was used to check the morphology of the AuNPs. Figure 3(a) shows the medium polydispersed particles in the 200nm magnification. Morphology of the synthesized AuNPs was found to be both spherical and non-spherical as shown in Figure 3 (b and c). The mean diameter for spherical-shaped AuNPs was found to be 47.734nm ± 4.359. In comparison, the synthesized AuNPs of the conventional method (Turkevich Method) were observed to have more aggregated particles [29]. Hence, this may infer that the plant-based synthesis method produces smaller AuNPs.

Colorimetric Sensing Property of the AuNPs

Selecting the wavelength in the spectrum with the highest absorbance (i.e. λPeak) was important for this study as the synthesized AuNPs would have their highest sensitivity at this wavelength. Additionally, the result for lead metal ion was shown in the table as it shows the best sensitivity and linearity.

As summarized in Table 3, the AuNPs' sensitivity at different λPeak was found to attain its highest sensitivity (1.1083 x 10-3) and the highest linearity (0.949) at 541nm (n=3). Hence, this wavelength was used for the colorimetric sensing assay.

The synthesized AuNPs were introduced to different heavy metal ions such as Pb, Hg, and Cd to determine which heavy metal ion would have the highest sensitivity towards AuNPs. Figure 4 shows the absorbance of the gold nanoparticles and the heavy metals at 541nm. As shown in Table 4, lead (m = 1.1083×10^{-3}) was observed to have the highest sensitivity and the highest linearity (0.949). It was also favorable to mention the selectivity of the AuNPs to mercury as this heavy metal ion's absorbance tends to have a unique sensing property (decreasing slope) compared to other heavy metal ions (Fig. 4). The AuNPs' different sensitivities (as shown in Table 4) may imply the diverse responsiveness of the ligands present in the AuNPs' surface towards the heavy metal ions present in the environment. Hence, this suggests future studies on the optimization and characterization of the AuNPs for the colorimetric detection of other heavy metal ions.

Table 3. *Sensitivity and Linearity of AuNPs and Pb ion at Different Wavelength*

λPeak	Sensitivity	Linearity
541nm	1.1083×10^{-3}	0.949
539nm	7.95×10^{-4}	0.758
537nm	7.6×10^{-4}	0.738
536nm	7.45×10^{-4}	0.723
534nm	6.8×10^{-4}	0.681

Conditions: 1mL AuNPs + 1mL different concentration of Pb ion (at 10 minutes, pH4)

Table 4. *Sensitivity and Linearity of AuNPs towards different Heavy Metal Ions*

Heavy Metal Ion	Sensitivity	Linearity
Pb	1.1083×10^{-3}	0.949
Hg	-1.47×10^{-3}	0.853
Cd	3.1×10^{-4}	0.659

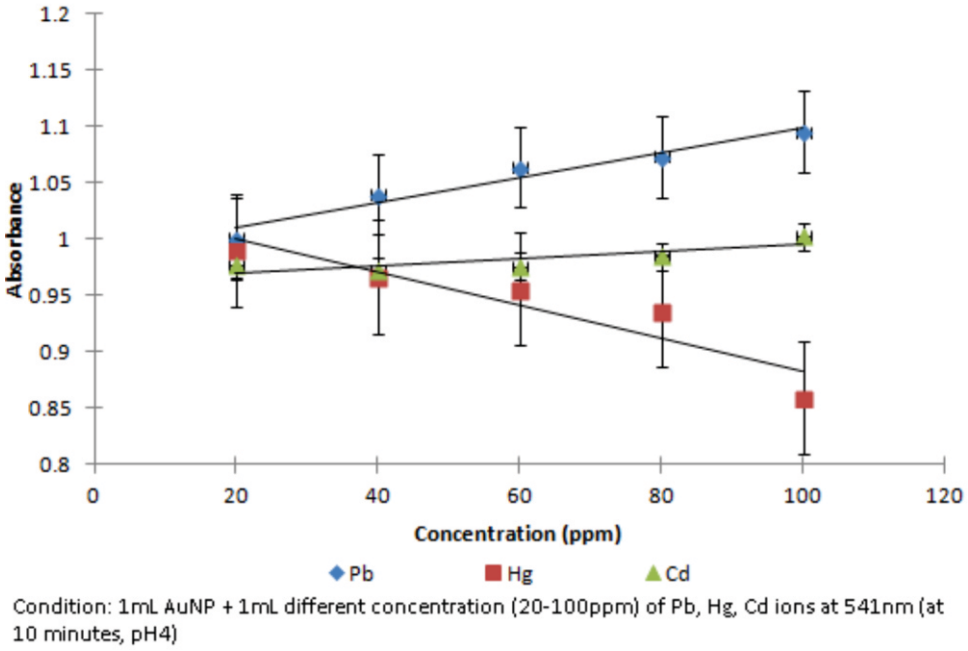

Figure 4. *Calibration Curve of AuNPs against different concentrations of lead, mercury, and cadmium at λPeak*

Conclusion

Synthesis of AuNPs with the use of bioreductants from plant samples has been drawing increasing interest due to their promising properties and different application in the field of phytonanotechnology and other applied science. Optimization results were found to be at a volume ratio of 1:2 ($HAuCl_4$ to Katmon aqueous leaves extract), and acidic pH displays the desired properties. Furthermore, there is no significant difference in the sequence of addition and reaction time. The synthesized AuNPs' particles were found to contain hydroxyl and amine groups; have broad, amorphous, and spherical particles that have a mean diameter of 60.6nm; 0.563 PDI; and a repulsion force of -13.720mV. Lead was chosen as the most sensitive ion as this heavy metal ion attained the highest sensitivity at 541nm. Despite the low sensitivity of AuNPs towards heavy metal ions, it is notable to conclude that the AuNP's colorimetric sensing ability showed to be very promising and this can be improved by further optimization of its parameters.

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