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Biosynthesized gold nanoparticles-doped hydroxyapatite as antibacterial and antioxidant nanocomposite

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Abstract

A composite of hydroxyapatite (HA) doped with green synthesized gold nanoparticles (Au NPs) was prepared. Au NPs were produced via the bioreduction of HAuCl₄ with Clitoria ternatea flower extract and utilized in HA synthesis, using Ca(OH)₂ and ammonium diphosphate as precursors. The aim of this research was to analyze the structure of the composite and conduct an antibacterial activity test involving Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, and Streptococcus pyogenes. In addition, antioxidant activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl radical scavenging. Au NP formation monitoring was conducted by UV–visible spectroscopy and particle size analysis, and the synthesized composite was studied using X-ray diffraction, scanning electron microscopy, and transmission electron microscopy. The results revealed homogeneously dispersed Au NPs (particle size ranging from 5 to 80 nm) in the HA structure. The nanocomposite demonstrated enhanced antibacterial activity against the tested bacteria compared to HA, with minimum inhibition concentrations of 3 μg ml⁻¹ for E.coli and S.aureus and 10 μg ml⁻¹ for K. pneumoniae and S. pyogenes. The nanocomposite expressed antioxidant activity, as shown by 2,2-diphenyl-1-picrylhydrazyl scavenging activities of 66% and 58% at concentrations of 100 μg ml⁻¹ and 50 μg ml⁻¹, respectively.

1. Introduction

Biomedical technology is developing together with the emerging nanotechnology researches. One exciting research which has received great attention in biomedical researches is artificial bone technology. Within this scheme, hydroxyapatite (HA) is attracted interests owing to its resemblance to the mineral part of human hard tissue consist of bones and teeth. The biocompatibility, bioreorbable, osteoconductive and thermally stable properties of the HA [Ca₁₀(PO₄)₆(OH)₂] are potential for many modifications for specific purpose, including the antibacterial properties [1–3]. The antibacterial and antioxidant characters of bone/tissue engineering are highlighted especially in correlation with the surgery and advanced artificial organ development. Functionalization of HA with some antibacterial agents notified the effectiveness of metal nanoparticles-substituted HA. Many researches explored the synthesis of Ag-substituted HA with good capability, however, some concerns with regards to the cytotoxicity of Ag made an intensive exploration for other dopants as alternatives [4, 5]. Gold is one of the metals allowed in the biomaterials, and in addition it exhibited good antibacterial properties. Beside of the antibacterial feature, the capability of biomimetic material in accelerated healing of fractured bones/implants via scavenging reactive oxygen species (ROS) and in-turn also provide a
protection against osteoporosis is also important. The incorporation of antioxidant such as acid, nanoparticles and other complex compounds were attempted [6]. The enhanced antibacterial activity by the formation of nanoparticles for some metals and metal oxides such as Au, and iron oxides were reported [7, 8]. For example, poly(γ-glutamic acid) capped gold nanoparticles exhibited higher activity compared to gentamicin as well-known antibacterial agent [7, 9]. As many other metals, the gold nanoparticles (Au NPs) were reported to determine the designed antibacterial and antioxidant properties of the nanocomposite [10–12].

Moreover, the existing synthetic routes are associated with extensive chemical modification and such intensification process such as microwave, ultrasound, furnace, hydrothermal, etc [13]. The green synthesis approach by using plant extracts as the bio-reductors for the synthesis were also reported intensively [10, 14, 15]. The non-toxic and abundantly resource are the advantageous beside of the capability of secondary metabolites as reducing or stabilizing agents with low cost and environmental-friendly. Clitoria ternatea or commonly known as butterfly pea has been reported to has capability become bio-reductor for the synthesis of some nanoparticles such as silver, gold, and tin oxide. Previous study reported the effectiveness of Clitoria ternatea flower extract (CTE)-mediated Au NPs as antibacterial agents due to the high content of secondary metabolite compound. The effective bioreduction produced the effective AuNPs with antibacterial activity against Eschericia coli, Staphylococcus aureus, Klebsiela pneumoniae, and Streptococcus pyogenes [16, 17]. However, investigation on the doping of CTE-mediated Au NPs to HA has not been reported yet.

Moreover, Based on these backgrounds, this research aimed to prepare Au NPs-doped hydroxyapatite (Au/HA) with The study on physicochemical properties of Au/HA and its antibacterial and antioxidant features are the focuses of this study.

2. Materials and methods

2.1. Materials

Clitoria ternatea flower was collected from Sleman district, Yogyakarta, Indonesia. The aqueous flower extract (furthermore called as CTE) was prepared by grinding 25 g of the flower followed by maceration in 100 ml of water for an hour followed by filtration. Chemicals in analytical grade consist of Ca(OH)2, (NH4)2HPO4, ethanol were purchased from Merk-Millipore (Germany). Other chemicals of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and HAuCl4 were produced by Sigma-Aldrich (Germany). The tested bacteria consist of E.coli ATCC 25922, K. pneumoniae 2968, S.aureus 0364, S. pyogenes 0130 were supplied from ATCC Company, USA. The cultures were stored and purified at the Microbiology Laboratory, Department of Pharmacy, Universitas Islam Indonesia.

2.2. Synthesis of Au/HA

The synthesis of Au/HA was firstly started by the synthesis of Au NPs using the method reported in previous work [17]. About 5 ml of CTE was mixed with 2 ml of 10 mM HAuCl4 followed reflux for 2 h. As-prepared Au NPs was utilized for the preparation of Au/HA together with Ca(OH)2 and (NH4)2HPO4, Ca(OH)2 and Au NPs were mixed in a double-distilled water by setting the atomic ratio of Au/[Ca + Au] at 0.1 under stirring at room temperature. Furthermore, (NH4)2HPO4 was slowly added into the mixture with the setting [Ca+Au]/P molar ratio of 1.67. The precipitate was obtained and it was kept in an autoclave at 110 °C overnight. The resulting slurry was then dried in before sintering at 700 °C for 1 h. Figure 1 represents the schematic diagram of the synthesis process.

2.3. Characterization of materials

The Au NPs formation was identified by UV-visible spectrophotometry and particle size analyzer. A HITACHI U2010 UV-visible spectrophotometer and a HORIBA particle size analyzer were employed for those analyses. The morphological and microstructural analysis of the Au/HA was executed by Scanning Electron Microscopy (SEM) performed on a Phenom X. The samples were sputter coated with gold prior to examination. XRD analysis was conducted for on Shimadzu X6000 instrument, which was operated at 20 kV voltage and using Ni-filetered Cu-Kα (λ = 1.5406 Å) the diffraction angle in the 20°–80° range. Form and size analysis was investigated using TEM (JEOL-JEM-2100, Tokyo, Japan) operated at 20 kV. X-ray fluorescence spectrophotometer of Philips EDAX DX-95 equipped with DX-4 data processing was employed for elemental analysis, while the XPS was performed on a V.G. Scientific ESKALAB MKII instrument. Prior analysis, about 0.2 mg of sample was slightly pressed into a pellet with the diameter of 15 mm and then mounted on the sample holder for degassing to achieve at the pressure below 10⁻⁸ Pa during 4 h. The monochromatic Al Kα radiation with a photon energy of 1486.6 ± 0.2 eV was employed for analyses.
2.4. Antibacterial and antioxidant activities test

The antibacterial activity of Au NPs, HA, and Au/HA was tested on E.coli, K. pneumoniae, S.aureus and S. pyogenes. The nutrient agar (NA) medium and all glassware were sterilized, and the experiments were performed in an aseptic environment. The dose of sample was 0.1 mg/5 ml, added to the disk, and the incubation was conducted for 24 h for furthermore, the inhibition zones of the tested samples were examined.

The antioxidant activity of Au/HA in comparison with Au NPs and HA was determined by DPPH assay method following the method introduced by Brand-Williams et al [18]. The capability of material to scavenge the DPPH radical was calculated using the colorimetric method measured at 517 nm using following equation (equation (1)):

\[
\text{DPPH scavenged(\%)} = \frac{A_c - A_s}{A_c} \times 100
\]

where \(A_c\) and \(A_s\) are the absorbance of the control and test sample, respectively, measured at 517 nm after DPPH addition.

3. Results and discussion

3.1. Material characterization

The preparation of Au/HA was initiated by the synthesis of AuNPs by using CTE. The principle of the reaction is bio-reduction of \(\text{Au}^{3+}\) to \(\text{Au}^0\) employed by the secondary metabolite in the CTE. As previously described by some papers, CTE is composed of mainly some anthocyanin compounds such as tretatin and delphinidin, and flavon compounds such as quercetin, myricetin and kaemferol [12]. There are possible oxidation-reductions from the functional group site, and one of these is the oxidized hydroxyl to carbonyl which corresponding to the \(\text{Au}^{3+}\) reduction into \(\text{Au}^0\).

The proof of the reduction is identified by UV-visible spectrophotometric and particle size analyses. As can be seen in figure 2(a), it is seen that CTE exhibits the peaks at 230–300 nm and 500–550 nm which are corresponding to the existence of flavonoid and anthocyanine content. The formation of Au NPs is observed by the shoulder peak at the about 362 nm along with the blue shifting spectrum at lower wavelength range. The presence of shoulder peak is the indication of the mixed spectra of the surface plasmon resonance and the aromatic rings of the secondary metabolite content, which act as the capping agent to metallic nanoparticles. In addition, the blue shifting is the representation of oxidized from of the related chromophore [19].

The absorption around 542 nm is identified due to the surface plasmon resonance (SPR) of the metal nanoparticles as the collective oscillations of the free electrons in of the metal. This peak is similar with were reported in the biosynthesized Au NPs for example by using Myristica fragran [20], Brazilian red propolis [21], and Tribulus terrestris [22].

The low intensity of the Au NPs peak at around 400 nm suggest the formation of the Au NPs within the nanometer range. This is also in line with the particle size distribution data (figure 2(b)) represented the particles size at the range of 8–120 nm with the mean particle size of 58 nm. This size is coincided with previously reported [17], and furthermore utilized to be dopant in Au/HA synthesis. The distribution of Au NPs in the Au/HA structure was performed by the use of AuNPs in the precipitation reaction:
Figure 2. (a) UV-visible spectra of CTE and Au NPs (b) Particle size distribution of Au NPs.

Figure 3. XRD patterns of Au/HA and HA.
The mechanism is widely used since it is the simple procedure \([23–25]\). Referred to the previous experiment for Ag/HAp synthesis \([5]\), the hydrothermal treatment was performed by autoclave set at 150 °C.

### Table 1. Elemental analysis using XRF.

| Component | Percetange (% wt.) | Au/HAp |
|-----------|-------------------|--------|
|           | HA                |        |
| O         | 39.03             | 30.54  |
| Ca        | 46.49             | 48.02  |
| P         | 14.46             | 16.40  |
| Au        | —                 | 4.94   |

\[\text{10CaO} + 6(\text{NH}_4)_2\text{HPO}_4 + 4\text{H}_2\text{O} \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 12\text{NH}_3\text{OH}\]
The change on structural form of the Au/HA is identified by the XRD analysis presented in figure 3. Both HA and Au/HA depict reflections which are associated with HA structure. The peaks at 26.2°, 33.8°, 34.2°, 39.9°, 47.6°, 49.6 and 54.0° (2θ) correspond to the (002), (211), (300), (130), (222), (213) and (004) reflection planes, respectively [26, 27]. In addition, the low intense peak at 38.2° is identified as proof of the existence of (111) plane of Au NPs refer to the JCPDS standard No. 04-0784 [20, 28]. Similar pattern was also identified by the synthesis of Au-dotted hydroxyapatite [29]. The small and less intensity suggest the nanoparticle form of the metal. The presence of nanoparticles form is also proven by the SEM analysis. As can be seen in the SEM inspection result in figure 4, the insignificant change of the HA and Au/HA surface morphology is showed, suggest that there is no such structural change on the AuNPs doping. The XRF spectra revealed the amount of 4.94% wt. Au identified a peak at around 2.16 eV, which is slightly less than the theoretical value which laid at around 6.5% wt (table 1). In addition, based on the elemental analysis results the molar ratios of Ca/P are 1.68 and 1.58 for HA and Au/HA, respectively. The value from HA is slightly higher compared to the theoretical value of 1.67, which probably caused by the loss composition during process, in addition, the less ratio in Au/HA is due to the Au NPs incorporation.

Furthermore, the doped Au NPs can also see from TEM image (figure 5). The prismatic forms of HA in relative homogeneous size are imaged at the range size of 100–150 nm, suggesting the formation of crystalline and nanosize HA (figure 5(a)). The HRTEM image (figure 5(b) and inset figure) is furthermore revealed the HA structure as the fringes space of 0.34 nm which associated with (221) reflection of HA. Refer to the TEM images, the form of HA prepared this research is similar with the crystalline nanosized HA [29–31]. There are nanosized dots dispersed on HA facets appeared in Au/HA sample with the range size of 5–80 nm in size, consisting

Figure 6. (a) Survey scan spectrum of Au/HA (b) Deconvoluted Au_{4f} spectrum.
random spherical shapes (figure 5(c)). The lattice fringe spacing from HRTEM image (figure 5(d) and the inset figure) indicated in the image is 0.24 nm, which corresponds to the (111) facet of the gold cubic phase [31, 32].

The analysis using XPS was employed for qualitatively and quantitative determination of surface components of Au/HA. The survey scan spectrum of Au/HA in figure 6(a) exhibits the presence of Ca, P, O, and Au which represented as Ca 2p (347.3 eV), P 2p (133.09 eV), O 1s (523.1 eV), and Au 4f (83.7 eV), respectively [33, 34]. The deconvolution to the Au 4f spectrum revealed the doublet peaks of Au 4f 7/2 and Au 4f 5/2, at 83.7 and 87.4 eV ascribed to Au 0. The spectra imply the presence of Au metal and non-ionic form.

The quantitative measurement for Au content in the Au/HA was calculated based on the ratio of Au/Ca peak intensity which revealed the amount of 0.094 [35]. This ratio means that the atomic percentage of Au is less than the theoretic Au/Ca ratio of about 0.14. Thus, the ratio of Au/Ca obtained from the XPS results is similar with that of the chemical analysis results using XRF.

### 3.2. The antibacterial test

The antibacterial effect of Au/HA in the culture media was studied by an adapted disc diffusion method on four different-strains (E. coli ATCC 25922, K. pneumoniae 2968, S. aureus 0364, S. pyogenes 0130). The antibacterial activity data of the tested sample at varied concentration of 2 μg ml⁻¹–50 μg ml⁻¹ are well demonstrated by the considerable inhibition zones obtained against the bacteria.

![Figure 7. DPPH scavenging activity of samples.](image-url)

#### Table 2. Inhibition zone data in antibacterial activity test of samples.

| Tested bacteria | Inhibition zone (mm) |
|----------------|----------------------|
|                | 2 μg ml⁻¹ | 3 μg ml⁻¹ | 10 μg ml⁻¹ | 20 μg ml⁻¹ | 30 μg ml⁻¹ | 40 μg ml⁻¹ | 50 μg ml⁻¹ |
| E. coli        | —       | 7.8      | 9.5      | 10.9      | 12.0      | 16.0      | 16.2      |
| S. aureus     | —       | 10.5     | 11.2     | 13.5      | 16.0      | 16.0      | 16.2      |
| K. pneumoniae | —       | —        | —        | 9.0       | 10.0      | 11.2      | 13.5      |
| S. pyogenes   | —       | —        | 9.0      | 9.5       | 10.5      | 13.0      | 15.0      |

| Au NPs |
|--------|
| E. coli | — | — | 19.6 | 19.9 | 22.0 | 24.0 | 24.2 |
| S. aureus | 28.5 | 33.0 | 33.1 | 34.1 | 34.1 | 34.1 |
| K. pneumoniae | 8.5 | 9.0 | 10.9 | 11.5 | 11.7 | 12.0 | 14.0 |
| S. pyogenes | — | 7.5 | 12.0 | 12.6 | 16.6 | 18.0 | 18.0 |

| HA |
|----|
| E. coli | — | — | — | 8.7 | 9.0 | 9.5 | 13.0 |
| S. aureus | — | — | 9.0 | 9.0 | 9.8 | 10.4 | 13.5 |
| K. pneumoniae | — | — | — | — | 9.0 | 10.9 | 11.5 |
| S. pyogenes | — | — | — | — | 7.6 | 8.5 | 9.5 |

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The data of inhibition zone presented in table 2 suggest that Au NPs exhibits the highest antibacterial activity as it showed the highest inhibition zone and lowest tested concentration. In addition, it was found that the minimum inhibition concentration (MIC) was $0.25 \mu g \text{ ml}^{-1}$. Due to the inhibition zones data, it can be concluded that Au/HA antibacterial activity is within the range activity of ‘strong’. The consistence data with Au NPs reflected that the antibacterial activity of Au/HA is due to the capability of surface plasmon resonance of the AuNPs for destroy the bacterial membrane [36]. Similar effect was also reported by previous works [37–39].

3.3. Antioxidant activity

The DPPH radical scavenging assays was employed for antioxidant activity identification, and the obtained DPPH scavenging activity are presented in figure 7. It is noticed that Au NPs represents the scavenging activity of 89.2%, meanwhile HA shows the activity of 24.34%. Those values were achieved by the concentration of 100 $\mu g \text{ ml}^{-1}$, and the activity of Au NPs is higher compared to the antioxidant activity of Au NPs synthesized using Mangifera indica [40] and Acalypha indica [41] which the activity laid around 70%–82% on similar concentration. The antioxidant activity of ~66% and 58% are achieved by Au/HA at the concentration of 100 $\mu g \text{ ml}^{-1}$ and 50 $\mu g \text{ ml}^{-1}$, respectively. Refer to the highest and potential antioxidant activity of Au NPs, the significant increase is correlated with the surface activity of dispersed nanoparticles. The reduced activity of about 30% from the Au NPs is related with the smaller amount, but it is comparable in similar value with Ag-doped hydroxyapatite (Ag/HA) [6].

Finally, from the psychosocial character, antibacterial, and antioxidant activity data, Au/HA represent potential for further development in biomedical technology.

4. Conclusion

The composite of Au/HA was successfully synthesized using Clitoria ternatea flower extract for the green synthesis of Au NPs. The composite showed the nanoparticles dispersed in the composite giving influence for the homogeneous performance as well as the antibacterial activity. The nanocomposites exhibit remarkable antibacterial activity against Eschericia coli, Staphylococcus aureus, Klebsiela pneumoniae, and Streptococcus pyogenes. In addition, the nanocomposite exhibit antioxidant activity which provides a reference for designing and developing novel antibacterial materials for various applications, especially as antibacterial component in biomedical technology.

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Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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