Research Article

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Gold nanoparticles from *Celastrus hindsii* and HAuCl_4 : Green synthesis, characteristics, and their cytotoxic effects on HeLa cells

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Abstract: The extract from *Celastrus hindsii* (*C. hindsii*), a plant that naturally grows in the forests of several provinces of Vietnam, has been traditionally used as an alternative medicine for the treatment of inflammation because of its anticancer and antitumor properties. This study reported the green synthesis of stable gold nanoparticles (Au-NPs) derived from HAuCl_4 using the extract of *C. hindsii* as reducing and capping agents. Their particle size could be controlled by adjusting the ratio of the extract to HAuCl_4 solution used (1.25%, 2.5%, 3.75%, 5.0%, and 6.25%). The optimal ratio of the extract was 3.75% (Au-NPs-3.75%). The X-ray powder diffraction analysis demonstrated that the Au-NPs was successfully synthesized. Fourier-transform infrared spectroscopy result indicated the possible presence of phenolic acids and flavonoids (acting as reducing agents and potential

natural antioxidants). Transmission electron microscopy images showed that the particle diameter of Au-NPs-3.75% varied between 13 and 53 nm (average: ~ 30 nm) in its spherical shape. The biosynthesized Au-NPs-3.75% exhibited dose-dependent cytotoxicity against HeLa cells, and the inhibitory concentration (IC_{50}) was $12.5 \mu\text{g/mL}$ at 48 h. Therefore, Au-NPs that were synthesized from environmentally friendly method without the presence of potentially toxic chemicals were highly possible in biomedical applications.

Keywords: biosynthesis, *Celastrus hindsii*, cytotoxicity, gold nanoparticles, HeLa cells

1 Introduction

Nanoscience and technology has attracted more concerns from scientific communities because of its wide application. Among current nanomaterials, gold nanoparticles (Au-NPs) have been widely applied (especially in biomedication [1,2]) because of their unique and advantageous properties. The multiple surface functionality of Au-NPs makes it easier to attach with drugs [3], oligonucleotides [4], and antibodies [5]. The unique surface plasmon resonance (SPR) of Au-NPs triggers their photostability and extremely efficient absorptivity of light [6]. Therefore, Au-NPs offer multiple modalities for biological and medical applications [7] such as cancer nanotechnology [7,8], tissue engineering, and drug and gene delivery [6].

Green nanotechnology is a thrust area of research with a significant focus on developing eco-friendly methods for the production of high-quality nanostructures with sustainable commercial viability. Among existing Au-NPs synthesis, the method based on biological systems (i.e., microbes, fungi, and extracts) is the most common and appropriate because of its simplicity and low preparation cost. Besides, this method is eco-friendly and green,

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resulting from the absence of toxic chemicals during the synthesis process. In general, various plant species (the extracts from them) can serve as potential reductant and capping agents for synthesizing Au-NPs. In the literature, the extracts of many plant species have been applied for the successful biosynthesis of Au-NPs. They included *Chenopodium album* [9], *Sorbus aucuparia* [10], *Sugar beet pulp* [11], *Terminalia catappa* [12], *Hibiscus rosa sinensis* [13], *Mangifera indica* [14], *Syzygium aromaticum* [15], *Anacardium occidentale* [16], *Macrotyloma uniflorum* [17], and *Murraya koenigii* [18]. The gold nano-materials biosynthesized from those extracts often exhibit their excellent compatibility with biomolecules [19]. Different methods that were used for the plant extract might lead to different properties of resultant Au-NPs. In addition, the application of the extract method is strongly dependent on the unique nature and property of each plant. Therefore, the extract method often plays an important role in controlling the properties and sizes of resultant Au-NPs [9].

Celastrus hindsii (*C. hindsii*; its family: Celastraceae) that is abundantly found in America, China, and Vietnam [20,21] can be used as herbal and traditional medicines for treating cancers [20,22]. In Vietnam, *C. hindsii* plants naturally thrive in forests and have been chemically studied because of their biological activities. For example, Duyen et al. [23] used ethanol and then fractionated with different solvents (*n*-hexane, *n*-butanol, and ethyl acetate) to extract certain chemicals (i.e., vitamin C, vitamin E, and polyphenols) from the leaves of *C. hindsii*. They found that the highest cytotoxicity effects on the cells of lung cancer and liver cancer were obtained by using the ethyl acetate fraction, with an inhibitory concentration (IC_{50}) value being 13 and 33.7 mg/mL, respectively. They concluded that the extract of the leaves of *C. hindsii* has strong antioxidant and cytotoxicity activities. An identical conclusion was reported by Pham et al. [21]. The authors used ethanol assisted with ultrasound to extract flavonoids from the leaves of *C. hindsii*. The IC_{50} values for cancer cell lines were 88 μ g/mL (MCF7), 120 μ g/mL (A549), and 118 μ g/mL (HeLa).

In this study, the extract of *C. hindsii* leaves and stems was used as a natural reducing and capping agent for biosynthesizing well-dispersed Au-NPs. A simple method of extraction without any chemicals or solvents was developed. The extract and Au-NPs were well-characterized by various techniques. Au-NPs were then applied for exploring their cytotoxic effects on HeLa cells.

2 Materials and methods

2.1 Materials and reagents

The dried leaves and stems of *C. hindsii* were procured from local herbal markets. Gold(III) chloride trihydrate (99.9%; $HAuCl_4 \cdot 3H_2O$) is acquired from Sigma Aldrich. Other chemicals were of analytical grade, such as Ammoniac, Dulbecco's modified Eagles medium, phosphate-buffered saline, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), and dimethyl sulfoxide. Those chemicals were purchased from Sigma-Aldrich and ACROS Organics and directly used.

2.2 Preparation of the plant extract from *C. hindsii*

The leaves and stems of *C. hindsii* were thoroughly washed with de-ionized water, dried in the air, and then ground into powder particles. A mixture of *C. hindsii* powder (50 g) and de-ionized water (500 mL) was heated up to 60°C for 30 min, and cellulose was then removed by filtrations to obtain the aqueous black extract. After 100 mL of the extract had been evaporated under reduced pressures, 1.55 g of *C. hindsii* powder was obtained. Finally, the extract of *C. hindsii* powder was dispersed in 100 mL of de-ionized water.

2.3 Green synthesis of Au-NPs

The synthesis method of Au-NPs was adapted from the literature [24,25]. Briefly, the extract of *C. hindsii* (at different volumes: 0.25, 0.5, 0.75, 1.0, and 1.25 mL) was added to a $HAuCl_4 \cdot 3H_2O$ solution (1.0 mM and pH 2.75) in a flask (under stirring 250 rpm) at 30°C for 1.0 min. The total volume of the mixture of the extract and the $HAuCl_4$ was 20 mL. This means that the ratio of the extract (v/v) used was 1.25%, 2.5%, 3.75%, 5.0%, and 6.25%. For convenience, the final samples were named according to the ratio of the extract used, such as Au-NPs-1.25%, Au-NPs-2.5%, Au-NPs-3.75%, Au-NPs-5.0%, Au-NPs-6.25%, respectively. After the above reaction time (~1.0 min), the mixture containing Au-NPs was centrifuged at 10,000 rpm for 30 min, repeatedly washed with de-ionized water,

and then stored in de-ionized water (20 mL) at 4°C for further use.

2.4 Characterization of the prepared Au-NPs sample

The prepared Au-NPs samples were distinguished by UV-Visible spectrometer (Shimadzu UV-1800) for evaluating their fascination potentials. The UV-Visible spectra of the Au-NPs samples were observed at a wavelength range between 450 and 650 nm. In addition, their morphological properties (including their size and shape) were analyzed by transmission electron microscopy (TEM) using a JEM1010-JEOL microscope. The aqueous re-dispersed suspension of a drop of Au-NPs was located on a carbon-coated copper grid and air-dried before the TEM assessment. Moreover, the crystallographic structure of the synthesized Au-NPs was detected by X-ray diffraction (XRD; EQUINOX 5000 diffractometer) using Cu K radiation ($\lambda = 0.1542$ nm). Meanwhile, the Fourier-transform infrared spectroscopy (FTIR) spectra of *C. hindsii* and Au-NPs were used to determine the presence of main functional groups on the materials' surface. The FTIR absorption spectra were conducted at room temperature at the level of 4,000–500 cm^{-1} using the IR Prestige-21 Shimadzu spectrometer. Finally, the concentration of Au in Au-NPs and solution was determined by inductively coupled plasma mass spectrometry (ICP-MS; PerkinElmer NexION 2000).

2.5 Anticancer activity of Au-NPs

The prepared Au-NPs sample anticancer activity was analyzed by calculating the inhibitory effect (IC_{50}) in HeLa cell line using MTT assay [26]. HeLa cells were cultured in a composite of DMEM/F12 with 10% FBS and 1% penicillin/streptomycin. The cells were maintained at 37°C with a 5% CO_2 atmosphere and detached by trypsin/ethylenediaminetetraacetic acid (0.25%; Gibco) when confluence reached around 80–90%. The cell suspension was seeded in a 96-well plate at density 5×10^3 cells in 100 μL per well. Subsequently, they were cultured stably in media (DMEM/F12, 10% FBS, and 1% PS) in 24 h. After removing media, the cells were treated with various concentrations of Au-NPs (i.e., 1.5, 3, 5, 12.5, 25, 50, and 73 $\mu\text{g/mL}$) to each well for 48 h. The whole cultured cells were incubated at 37°C in a 5% CO_2 atmosphere. After

48 h of incubation, the images of cells cultured were captured using inverted microscopy (Eclipse Ti-U, Nikon) before adding dye steps.

After the incubation period, the medium was removed, and 100 μL of 7-hydroxy-3H-phenoxazin-3-one 10-oxide dye (resazurin; 0.02 mg/mL) was added to each well. Those mixtures were incubated within avoiding-light requirements for 3 h at 37°C and 5% CO_2 . The samples were read by a microplate reader (Varioskan™, ThermoScientific, USA) at a wavelength of 530 nm, and fluorescent signals were measured at 590 nm. Notably, the data were presented and reported as average $\pm$ standard deviation (SD).

3 Results and discussion

3.1 Characterization of Au-NPs

In essence, UV-visible absorption spectroscopy (an efficient technique) is often used to monitor the optical properties of quantum-sized particles [27]. Figure 1a shows the maximum band corresponding to the unique SPR at around 540 nm. The results confirmed the formation of Au-NPs because the presence of a maxima band of absorbance at around 540 nm is a specific signal to identify gold nanoparticles [28,29]. In addition, the color change of solution (Figure 1c) after reaction from yellow (pristine HAuCl_4) to ruby-red suggested the successful formation of Au-NPs [30]. Moreover, the SPR band indicated a slight shift towards a higher maximal wavelength number with an increase in the extract ratios from *C. hindsii* used (Figure 1a), consistent with the change of color observed in Figure 1c. The result suggested an increase in particle sizes of the resultant Au-NPs when the ratios of the extract used increased from 2.5% to 6.25% (Figure A1), except that of 1.25%. In fact, the sample of Au-NPs-1.25% was not reliable because some Au-NPs were not observed by TEM images (data not shown). This is consistent with its visible color in Figure 1c, so this sample was not used for further discussion. The average particle diameters of each gold nanoparticle increased as the following order: 14 nm (Au-NPs-2.5%) < 30 nm (Au-NPs-3.75%) < 36 nm (Au-NPs-5%) < 54 nm (Au-NPs-6.25%). A similar phenomenon was reported by Singh and Srivastava [31]. Notably, the SPR band became broader when the ratio of the extract was higher than 3.75% (i.e., 5% and 6.25%) because of the formation of large anisotropic particles [30]. Therefore, the sample (Au-NPs-3.75%) was used for further studies of its characteristics and anticancer activity.

Figure 1: (a) UV-Visible spectra of the synthesized gold colloids, (b) its stability over times, and (c) manifestation of different colors of Au-NPs samples prepared from different ratios of the *C. hindsii* extract.

The analysis of UV-Visible absorption spectroscopy was repeated for a period from 0, 5, 10, 20, and 30 days to evaluate the stability of Au-NPs-3.75%. Figure 1b indicates that the band of the water solution containing Au-NPs-3.75% was almost stable after 30 days. The result confirmed that Au-NPs-3.75% exhibited too high stability and good dispersion in the water solution without any aggregation. A similar conclusion was reported by other researchers who synthesized Au-NPs from the extract of *Anacardium occidentale* leaves [16] or marine algae *Padina gymnospora* [30].

Secondly, FTIR was performed to investigate the biomolecules of *C. hindsii* extract involved in the Au-NPs formation and identify the molecules responsible for capping and efficient stabilization of synthesized Au-NPs. The FTIR spectrum of *C. hindsii* extract (Figure 2a) was compared with that of synthesized Au-NPs (Figure 2b). As shown in Figure 2a, an intensive band at around $1,067\text{ cm}^{-1}$ can be assigned to the stretching vibration of the C–O bond derived from proteins in the plant extract [30]. Distinct bands in the regions of $1,238$ and $1,385\text{ cm}^{-1}$ may be attributed to the presence of stretching vibrations of alcohols, ethers, esters, carboxylic acids, and amino groups. A band at the $1,385\text{ cm}^{-1}$ is related to the carbon

double bonds (C=C) in aromatic of common flavonoids in *C. hindsii* [16]. Notably, the presence of the amides (–C(=O)N=) overlapping the carbonyl group (C=O) in proteins is identified at approximately $1,610\text{ cm}^{-1}$. Meanwhile, a band at $1,730\text{ cm}^{-1}$ is also related to carboxylic

Figure 2: FTIR spectra of (a) *C. hindsii* extract and (b) Au-NPs-3.75%.

groups. The stretching vibrations of the secondary amines in the N–H and O–H bonds are observed at $3,420\text{ cm}^{-1}$ [25].

The FTIR spectrum of Au-NPs-3.75% (Figure 2b) is similar to that of the *C. hindsii* extract (Figure 2a). However, the intensity of some important bands remarkably decreased, suggesting certain reactions exist between some compounds in the *C. hindsii* extract and HAuCl_4 to form Au-NPs. For example, the intensity of two bands located at around $1,385\text{ cm}^{-1}$ (identified the presence of the flavonoids) and $3,420\text{ cm}^{-1}$ (phenols) in the spectrum of Au-NPs-3.75% decreased compared to that in the *C. hindsii* extract. The result suggested that the phytochemical compounds (i.e., flavonoids and phenols) available in the *C. hindsii* extract simulated generating Au-NPs through the bio-reduction of Au^{3+} ions [16,31]. Meanwhile, a remarkable decrease in the intensity of a band at $1,610\text{ cm}^{-1}$ (amides) confirmed that the amide groups had a strong capability to capture Au-NPs.

Thirdly, the XRD result suggested that Au-NPs-3.75% was well-crystalline (Figure 3). The XRD peaks located at $2\theta = 38.18^\circ$, 44.38° , 64.57° , and 77.56° correspond to the (111), (200), (220), and (311) planes of the Au-NPs, respectively [12,17,32]. The standard diffraction bands showed the face-centered cubic phase of Au based on JCPDS 65-2870 and COD LINK 9008463 [33]. The result suggested that the Au-NPs were successfully synthesized by the *C. hindsii* extract and HAuCl_4 .

Furthermore, the properties of morphology, size, and shape of the prepared Au-NPs are explored by the TEM technique. Figure 4a shows that Au-NPs-3.75% exhibited two distinguishing morphologies, such as spherical (accounting for >95%) and triangular. An analogous observation was obtained by Singh and Srivastava [31]

Figure 3: XRD pattern of the biogenic Au-NPs-3.75%.

Figure 4: (a and b) TEM images of Au-NPs-3.75% and (c) distribution of its particle diameter.

for synthesizing Au-NPs using *black cardamom*. As expected, each sphere (or triangle) indicated a high mono-dispersed particle and non-agglomerated phenomenon (Figure 4b). This might result from the effective

stabilization of several bio-organic components or proteins (Figure 2a) in the *C. hindsii* extract [30]. Those compounds can act as an important ligand to encapsulate Au-NPs (Figure 4a). Notably, some previous studies demonstrated that the spherical shape of common nanoparticles could be simulating to increasing cytotoxic antibacterial effects such as silver nanoparticles [34] and Au-NPs [16]. Furthermore, the TEM images of the other samples (Au-NPs-1.25%, Au-NPs-2.5%, Au-NPs-5.0%, and Au-NPs-6.25%) are provided in Figure A2 for comparison.

The particle size of Au-NPs-3.75% (obtained from the TEM data) ranged from 13 to 53 nm in diameter, with the most diameter being in the range of 23–37 nm (centered at 30 nm) (Figure 4c). Some scholars also reported the similar diameters of Au-NPs such as 15–25 nm for Au-NPs performed from *Cordyceps militaris* [25], 25–35 nm from *black cardamom* [31], and 30–50 nm from *Marsdenia tenacissima* [24].

Finally, the total concentration of Au in Au-NPs-3.75% in 20 mL was determined by the ICP-MS technique. The result of three repeated experiments indicated that the Au concentration was $72.9 \pm 0.3 \mu\text{g/mL}$ (average $\pm$ SD). This concentration was used as a stock solution to prepare running solutions in the investigation of anti-cancer activity.

3.2 Anticancer activity of Au-NPs

Figure 5 shows the cytotoxic effect of *C. hindsii* Au-NPs on HeLa cells. The cell shape changed and became unstable when increasing the concentrations of Au-NPs ($\mu\text{g/mL}$). In the control group (0 $\mu\text{g/mL}$), the population of HeLa cells proliferate, have lance-shaped, and grow in clusters (Figure A3). In the presence of Au-NPs, the change of the shape and stretching due to chemical stress indicated the increase in membrane rupture cells when increasing the concentrations of Au-NPs [35,36]. Clearly, when high concentrations of Au-NPs (i.e., 25, 50, and 73 $\mu\text{g/mL}$) were used, the state of HeLa cells was unstable: they lose lance-shaped and their membranes are entirely ruptured. In addition, there was no “connecting” in adjacent HeLa cells because of the sparsed population. Those were some manifestations of cellular stress.

Au-NPs, as novel agents for cancer therapy, are gaining substantial demand in medical applications. However, studies on the cytotoxic activity of biosynthesized Au-NPs against cancer cell lines are limited. The MTT assay was used to assess the effect of Au-NPs on

Figure 5: (a) Effect of the concentrations of Au-NPs-3.75% on the viability of HeLa cells (average $\pm$ SD; raw data presented in Table A1); microscopic images of HeLa cells without treated with Au-NPs-3.75% (b) and after treated with Au-NPs-3.75% (12.5 $\mu\text{g/mL}$) for 48 h (c).

the proliferation of HeLa cells. The present study found that the induction of cell death modalities could be the possible mechanism for the antiproliferative activity of biosynthesized Au-NPs.

The dose-dependent cytotoxicity was also observed in Au-NP-treated HeLa cells. The results demonstrated that living cells decreased when increasing the concentration of Au-NPs, especially in the 73 $\mu\text{g/mL}$ group, nearly all death cells (only approximately 10% cell viability). Considering 50% of HeLa cell death, the inhibitory concentration (IC_{50}) value of biosynthesized Au-NPs is 12.5 $\mu\text{g/mL}$ in 48 h (Figure 5) [24]. The previous research showed that the amphiphilic nature of the Au-NPs helps them easily penetrate the cell membrane and increases metabolic activity; these factors increase reactive oxygen species (ROS), as a result creating hypoxia condition in the cancerous cells, which has an inhibitory effect on metabolic activities and endothelial balance, resulting in inhibiting these cells and leading to a decrease or a stop in growth at different concentrations [19,37]. In addition, the ingredients of *C. hindsii* extract are also believed to contribute to cancer prevention [20,21,23], and the surface of Au-NPs, after covered with biomolecules, can be delivered drug [38]. Briefly, the experimental results

proved the excellent anticancer activity of Au-NPs against the HeLa cell line [35,36].

4 Conclusion

The green synthesis of stable and spherical-shaped Au-NPs using the extract of *C. hindsii* as a simple, economical, nontoxic, and efficient green approach was successfully developed. The ratio of the extract used played a critical role in the size dispersity of NPs. The reduction of Au³⁺ occurred very fast at room temperature, and the Au-NPs remained stable over time. The crystalline nature of NPs was evident from clear lattice fringes in the TEM images and bands in the XRD pattern. FTIR spectra revealed that biomolecules responsible for capping and stabilizing Au-NPs were phenolic and polyphenolic compounds found in the extract. The high phenolic content in *C. hindsii* with a robust antioxidant activity reduced Au cations to Au-NPs.

Moreover, the cytotoxicity effects of Au-NPs on the HeLa cell line were assessed using cell viability and staining techniques, thus proving that Au-NPs exert cytotoxicity effects on HeLa cell lines. Further studies are required to elucidate the precise molecular mechanism involved in cell growth inhibition, thereby permitting the biosynthesized Au-NPs as chemo-preventive and therapeutic cancer agents.

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Conflict of interest: One of the authors (Hai Nguyen Tran) is a member of the Editorial Board of Green Processing and Synthesis.

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Appendix

Figure A1: Particles size distribution of Au-NPs prepared at different ratios of the extract used.

Figure A2: TEM images of Au-NPs prepared at different ratios of the extract used.

Figure A3: Cell growth inhibition assays of *C. Hindsii*-AuNPs on HeLa cells.

Table A1: The inhibition of cell growth assays of *C. Hindsii*-AuNPs on HeLa cells

Control		Concentration of Au-NPs (ppm)						
		1.5 ppm	3 ppm	6.25 ppm	12.5 ppm	25 ppm	50 ppm	73 ppm
Repeat 1	577	550	501	484	312	241	89	57
Repeat 2	656	500	479	457	311	134	71	44
Repeat 3	634	491	418	368	365	169	70	38
Repeat 3	693	548	505	452	331	180	92	69
Average	640	522	476	440	330	181	81	52
SD	48.5	31.1	40.2	50.2	25.2	44.5	11.6	13.8
SE	24.3	15.6	20.1	25.1	12.6	22.3	5.8	6.9