RESEARCH ARTICLE

Green Synthesis of Gold Nanoparticles using *Melissa Officinalis L.* Aqueous Extract: Optimization, Antioxidant Activity and In Vitro Cell Viability

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ABSTRACT

Gold nanoparticles possess attractive properties that make them valuable in medicine. Recently, green synthesis of nanoparticles has been a target of research due to its simplicity, safety, eco-friendliness and cost-effectiveness. This study describes the green synthesis of gold nanoparticles via aqueous extract of Melissa officinalis L. (Mel). The various parameters affecting the synthesis of gold nanoparticles (pH, gold salt concentration, extract volume, temperature, and reaction time) were investigated and optimized. Ultraviolet–Visible spectrophotometry, Dynamic light scattering, Transmission electron microscopy, and Fourier-transform infrared spectroscopy were applied to characterize synthesized gold nanoparticles (GNP-Mel). The antioxidant activity of Mel extract and GNP-Mel was performed by 2,2-diphenyl-1-picrylhydrazyl free radical scavenging test. The effect of GNP-Mel on cell viability of H9C2 rat cardiomyocytes was evaluated using Alamar blue assay. The results indicated uniform spherical-shaped gold nanoparticles with an average particle size of 8 nm and a zeta potential value of -31.7 mV, which demonstrates the high stability of GNP-Mel. The Mel extract and GNP-Mel showed the highest antioxidant activity with percentages of 77.69 ± 1.18% and 59.64 ± 1.29% at a maximum concentration (of 100 µg/ml), respectively, which can be compared with Vitamin C (89.34 ± 1.34%). Moreover, the result of Alamar blue assay represented a higher than 80% cell viability in the presence of 20, and 50 µg/ml of GNP-Mel and likewise, a higher than 50% cell viability in the presence of 100 and 170 µg/ml of GNP-Mel. Overall, the findings show that the green synthesized gold nanoparticles are appropriate candidates for biomedical applications.

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INTRODUCTION

Nanotechnology has recently come to light as a promising tactic that can address issues with the current types of therapies [1, 2]. Gold nanoparticles (AuNPs) stand out among other nanoparticles for their special surface plasmon resonance (SPR) [3], ease of manufacture, and wide range of applications in the diagnosis and treatment of illnesses [4]. Furthermore, Gold nanoparticles are thought to be antimicrobial, antioxidant, anti-cancer, and anti-inflammatory activity [5-7]. AuNPs have found many applications in various biomedical areas due to their biocompatibility by increasing the adhesion and proliferation of cells by improving

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electrical communication between adjacent cells [8, 9]. Although the AuNPs are thought to be biocompatible, their preparation with toxic chemicals resulted in some harmful chemical species remaining on their surface, restricting their utility in medical applications. Finding a procedure that is safe, nontoxic, and biocompatible therefore appears to be essential. In comparison to chemical and physical techniques, the green synthesis of nanoparticles has benefits such as biocompatibility, high efficiency, eco-friendliness, and cost-effectiveness [10, 11]. Biological resources such as yeast, bacteria, fungi, actinomycetes, microorganisms, and various plant parts are now used to synthesize nanoparticles [12-14]. Plant extracts are particularly useful in the biological synthesis of nanoparticles due to their simplicity, stability, and faster synthesis rate when compared to other methods. In addition, plant bioactive compounds act as reducing agents, assisting in reducing metal ions to metal nanoparticles with distinct sizes, shapes, and significant antimicrobial efficiency [15]. *Melissa officinalis* L. (Mel) is a medicinal plant from the Lamiaceae family that contains flavonoids (quercetin, luteolin), polyphenolic compounds (rosemary acid, caffeic acid), triterpenes, and tannins [16, 17]. Also many studies in this field showed Mel has antioxidant [18], antibacterial, antidiabetic, anti-inflammatory [19, 20], anti-virus [21], and anticancer [22], and protects the brain and heart from ischemia [16, 23, 24]. Various studies have shown that Mel has very high antioxidant activity due to its chemical composition, containing large amounts of flavonoids, rosemary acid, gallic acid, and phenolic [16, 25]. Fierascu et al. (2017) synthesized gold and silver nanoparticles via the ethanolic extract of Mel and they studied their biological properties [26]. However, effective parameters for the synthesis of gold nanoparticles have not been examined. Dzimitrowicz et al. (2019) fabricated gold nanoparticles by aqueous extracts of Lamiaceae plants and studied the effect of follow-up treatment with atmospheric pressure glow microdischarge [27], but had not studied the antioxidant activity and cell viability of AuNPs. Consideration of reaction parameters is crucial to fabricate AuNPs with the desired characteristics [28]. Hence, the aim of the present work was to investigate a simple, stable and efficient process for the synthesis of gold nanoparticles using Mel leaves aqueous extract. Numerous parameters affecting synthesis of nanoparticles were studied. Analytical methods (UV-Vis, DLS, TEM, FTIR) were applied to characterize synthesized nanoparticles. The antioxidant activity of extract and Au nanoparticles were investigated. Further, the effect of prepared nanoparticles on the cell viability of H9C2 rat cardiomyoblast cells was assessed.

EXPERIMENTAL

Materials

Gold salt ($HAuCl_4.3H_2O$, 99%), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid (Vitamin C), DMSO (molecular biology grade), and Alamar blue were obtained from Sigma Aldrich, India. Sodium hydroxide and hydrochloric acid were prepared from Merck, Germany. Dulbecco's Modified Eagle Medium (DMEM) High Glucose, fetal bovine serum (FBS), Penicillin/streptomycin, Trypsin EDTA, Phosphate buffered saline (PBS) purchased from Gibco BRL, USA. All chemicals used in this study were prepared with the highest purity. H9C2 rat cardiomyoblasts cells were also obtained from the Cell Bank of Institute Pasteur of Iran.

Preparation of plant extract

Mel's leaves were harvested at full maturity from the ecological crops of Bagh Firuze (Tehran, Iran). Mel plant was identified in the herbarium of the School of Pharmacy, Tehran University of Medical Sciences, with the herbarium number PMP-1357. The plants were shade dried and ground to powder. The extraction was done by pouring 500 mg of the plant powders into 50 ml of distilled water and stirring for 60 min at 60 °C. The solution was filtered using the Whatman filter papers and stored at 4 °C for further green synthesis of gold nanoparticles from gold salt solution.

Green Synthesis of GNP-Mel

Initially, the aqueous plant extract (1ml, 1% w/v) was added to gold salt solution (5mL, 1 mM), and nanoparticles were obtained at 25 °C temperature after 120 min in stirring condition. The concentration of gold elements was determined by inductively coupled plasma emission spectrometry (ICP-OES) (730-ES, Varian). In order to obtain nanoparticles with appropriate morphology and size, factors affecting the synthesis of gold nanoparticles (pH of the reaction medium, extract volume, gold salt concentration, temperature, and reaction time) were investigated. In each step of optimization, one parameter was variable and the

Table 1 The different parameters in each synthesis of GNP-Mel.

S. No.	pH	Concentration of Au (mM)	Extract volume (ml)	Temperature (°C)	Time (min)
1	4	1	1	25	120
2	5	1	1	25	120
3	6	1	1	25	120
4	7	1	1	25	120
5	8	1	1	25	120
6	9	1	1	25	120
7	10	1	1	25	120
8	11	1	1	25	120
9	11	0.5	1	25	120
10	11	1	1	25	120
11	11	1.5	1	25	120
12	11	2	1	25	120
13	11	2.5	1	25	120
14	11	1	0.5	25	120
15	11	1	1	25	120
16	11	1	1.5	25	120
17	11	1	2	25	120
18	11	1	4	25	120
19	11	1	0.5	25	120
20	11	1	0.5	35	120
21	11	1	0.5	45	120
22	11	1	0.5	55	120
23	11	1	0.5	65	120
24	11	1	0.5	55	30
25	11	1	0.5	55	60
26	11	1	0.5	55	90
27	11	1	0.5	55	120

other parameters were fixed. The spectra absorbance of all solutions was taken separately by UV-Visible spectrophotometer to select the best synthesis. Firstly, to study the pH value of the reaction, 8 series of solutions, including 1 ml of extract and 5 ml of gold salt solution (1 mM) with pH of 4, 5, 6, 7, 8, 9, 10, 11 were made and stirred at 25 °C temperature for 120 min. The ideal pH was selected for the next step. Sodium hydroxide and hydrochloric acid were utilized with a concentration of 0.1 M to adjust the pH of the solution. Secondly, to optimize the effect of gold salt concentration on the synthesis, 1 ml of extract volume was added to 5 ml of different concentrations of gold salt solution (0.5, 1, 1.5, 2, 2.5 mM) with optimal pH, while other parameters were like the previous step. The best concentration of gold salt solution was chosen. Then, different volumes of extract (0.5, 1, 1.5, 2, 4 ml) were added to the optimal concentration of gold salt solution. At the same time other parameters were similar to the former step and the best extract volume was selected for the following action. Next, Solutions with optimal conditions were made separately at 25, 35, 45, 55, and 65 °C temperatures for 120 min and the ideal temperature was chosen. Finally, to evaluate the reaction time, a solution was made at all previously optimized conditions at different times, from 30 min to 120 min with a time interval of 30 min. The suitable reaction time was selected. All parameters in each synthesis of GNP-Mel are reported in Table 1. After preparing gold nanoparticles, the solution was washed in water to remove the residues of gold ions and non-reactive molecules of the extract using centrifugation at 4000 rpm for 15 minutes by Amicon 30 KDa MWCO filters. As a means to ensure complete removal of unreacted residues, the ultraviolet-visible absorbance spectrum of the supernatant was recorded and the washing steps were repeated until no specific absorbance was observed like the baseline (distilled water). In addition, the stability of synthesized gold nanoparticles was studied after 3, 30 and 90 days.

Characterization of GNP-Mel

The synthesized GNP-Mel and Mel extract were

analyzed by Ultraviolet–Visible spectrophotometry (UV-Vis) (Cecil CE 7250 Spectrophotometer) with a scan range of 200–900 nm. The particle size and surface charge of AuNPs were obtained with Dynamic Light scattering (DLS) and zeta potential study (HORIBA - SZ100, Nanoparticle Analyzer). Fourier-transform infrared spectroscopy (FTIR) (Thermo, Avatar) in the range of 400–4000 cm^{-1} was used to ensure the presence of the extract in the nanoparticles and the reduction of nanoparticles. The determination of particle size, distribution and morphology was carried out using transmission electron microscope (TEM) (Zeiss-EM10C) images at 100 kV acceleration voltages.

Antioxidant activity

The DPPH free radical scavenging activity of several concentrations (20, 40, 60, 80 and 100 µg/ml) of GNP-Mel and extract were studied by the modified method of Abbasi, et al. and also compared with antioxidant properties of ascorbic acid [28]. Briefly, 0.5 ml of Mel extract and GNP-Mel (20–100 µg/ml) were mixed with 1.5 ml DPPH solution. The mixtures were shaken and incubated for 30 minutes in a dark place. After incubation, the absorbance of the solutions was recorded at 517 nm. Vitamin C was used as the standard antioxidant with the same concentration range. The percentage of inhibition was calculated using this formula:

$$\text{Radical scavenging activity (\%)} = \frac{Absorbance\,of\,control - Absorbance\,of\,test}{Absorbance\,of\,control} \times 100$$

Cell viability study

The model cell line, H9C2 rat cardiomyoblasts cells, was used for cell viability assay. In brief, H9C2 cells were maintained in DMEM High Glucose supplemented with 10% FBS and 1% penicillin-streptomycin followed by incubation at 37 °C under 5% carbon dioxide and 95% humidified atmospheric condition. For fresh feeding, the medium was changed every second day. The effect of the synthesized GNP-Mel on H9C2 cell viability was assessed by Alamar blue test. For the assay, after 3 days, the cells reaching up to 80% confluency were treated with 0.25% Trypsin EDTA and seeded in a 96-well plate (5×10^3 cells/well). After 24 h of incubation phase, the cells were washed with PBS and treated with different concentrations of synthesized GNP-Mel (20, 50, 100, 170 and 250µg/ml) in complete culture and incubated for 48 h. Next, Alamar blue (resazurin 0.01% w/v) was added to each well and the plates were incubated for 4 h at 37 °C. Finally, The cell viability was calculated by reading the absorbance at 570 nm and 630 nm in an absorbance microplate reader (BioTek ELx808) [29].

Statistical analysis

All findings were given as mean ± standard deviation (SD) and studies were performed with 5 replicates. OriginPro software was utilized for the graphs.

RESULTS AND DISCUSSION

Optimization and synthesis of GNP-Mel

After mixing the gold salt with Mel aqueous extract, the initial formation of gold nanoparticles from the plant was optically verified by observing the color change of a solution. This change in color from light yellow to a purple-red colour, was due to the reduction of gold salt into gold nanoparticles, which indicates the successful synthesis of gold nanoparticles. The spectroscopy graph of the GNP-Mel solution demonstrated the highest absorbance in the range of 500-600 nm, related to the surface plasmon resonance (SPR) band of gold nanoparticles [30]. In addition, the graph of spectroscopy of the extract did not show any absorbance at the same range. Thus, the formation of GNP-Mel was validated by UV-Vis absorbance spectra (Fig. 1).

The effective parameters on the characteristics of gold nanoparticles, including pH of the reaction solution, concentration of gold solution, volume of extract, temperature, and reaction time, were studied to obtain suitable characteristics of Au nanoparticles. In each step of optimization one parameter was variable and the other parameters were fixed. The UV-Vis absorbance spectra of each synthesized AuNP solution are shown in Fig. 2. At first, eight series of gold nanoparticle solutions at different pH (4, 5, 6, 7, 8, 9, 10, 11) were synthesized. According to Fig. 2a, with increasing pH, the optical absorbance rate of gold nanoparticles increased and the maximum absorbance (0.99 a.u., 532 nm wavelength) was observed at pH = 11. Also, this pH showed a blue-shift of the SPR band indicating a decrease in particle size [31]. Thus, pH = 11 was chosen as the appropriate pH for following synthesis. Secondly, Au nanoparticles were synthesized at different concentrations of gold salt solution (0.5, 1, 1.5, 2, 2.5 mM). As shown

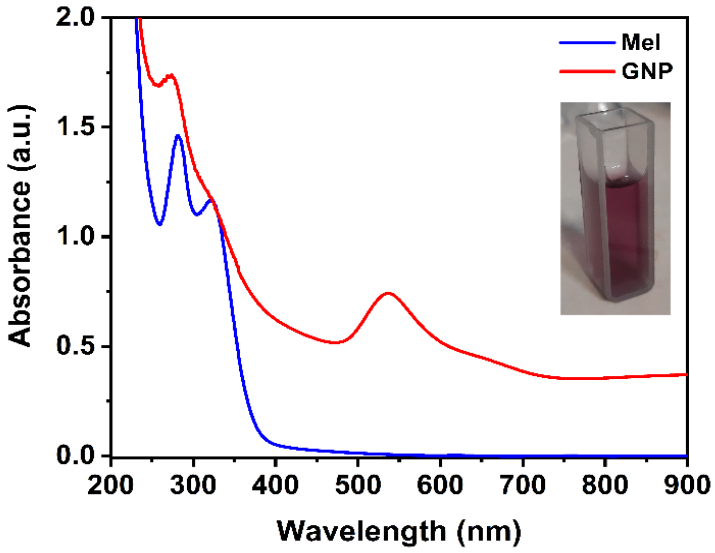

Fig. 1. UV-Vis spectra of Mel extract and GNP, GNP-Mel optical picture (Insert).

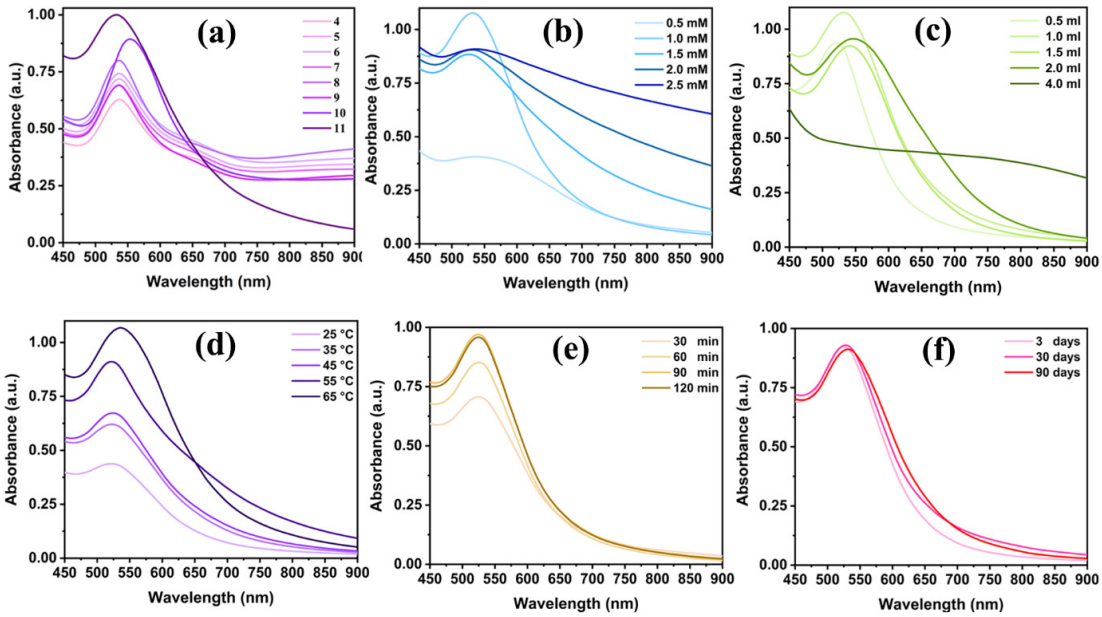

Fig. 2. The optimization of different factors (a) pH, (b) Gold salt concentration, (c) Extract volume, (d) Temperature, and (e) Time, on the UV-Vis absorbance spectra of synthesized GNP-Mel. (f) The UV-Vis spectra of stability investigation at different times.

in Fig. 2b., with increasing the concentration of Au salt to 1 mM, the absorbance related to gold nanoparticles increased to 1.07 a.u. at 532 nm wavelength. However, a significant decrease in absorbance was observed at higher concentrations. Therefore, a concentration of 1 mM of gold salt was considered suitable concentration for the next synthesis. Thirdly, gold nanoparticles were synthesized using a series of extract volumes (0.5, 1, 1.5, 2, 4 mL). The result showed the maximum absorbance (1.07 a.u. at 532 nm wavelength) was related to AuNPs with 1 ml of extract volume. But the volume of 0.5 ml of extract was selected due to its SPR with higher absorbance (0.93 a.u.) and lower width of wavelength (521 nm). The SPR of other extract volumes demonstrated a redshift

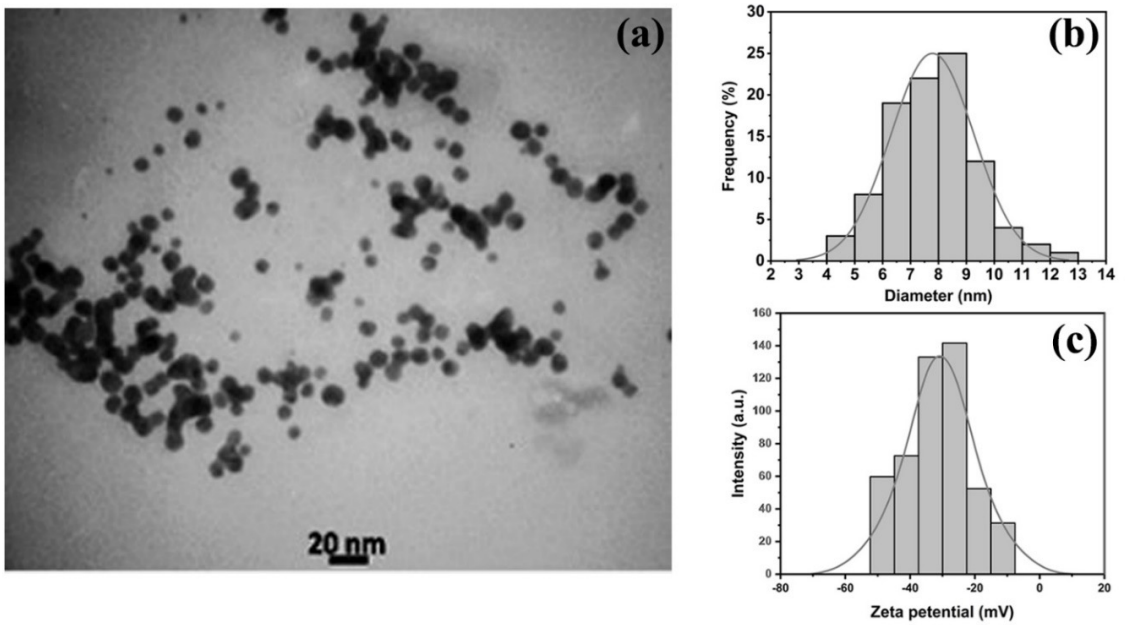

Fig. 3. (a) GNP-Mel electron microscope image. (b) Frequency distribution diagram of gold nanoparticle size. (c) The Zeta potential of GNP-Mel.

indicating an increase in particle size (Fig. 2c) [31]. Then, Au nanoparticles were synthesized at five different temperatures (25, 35, 45, 55, 65 °C). According to Fig. 2d, the amount of optical absorbance increased dramatically to 1.06 a.u. at 535 nm wavelength by increasing the temperature up to 65 °C. But a temperature of 55 °C was chosen as the ideal temperature due to its sharper and more symmetrical SPR band (absorption in 0.91 a.u. at 522 nm wavelength) compared to other bands, that could indicate a good uniformity in size distribution [30]. At last, a GNP-Mel was made at all previously optimized conditions. The UV-Vis absorbance spectra of the solution were considered from 30 min to 120 min of reaction forming at 30 min of intervals. The amounts of optical spectra of synthesized gold nanoparticles increased by exposure time of reaction up to 90 min (0.96 a.u. absorbance at 524 nm wavelength), but after 90 min, there was any change in the amount of absorbance, so the suitable time for the synthesis was considered at 90 min (Fig. 2e). The time variation had little effect on the UV-Vis absorbance spectra and temperature was the most effective parameter. The UV-Vis absorbance spectra of optimal solution were examined after 3, 30 and 90 days. Results showed a negligible decrease in absorbance after 90 days, which indicates high colloidal stability (98%) of synthesized AuNPs (Fig. 2f). Hence, optimal parameters (pH:11, extract volume: 5 ml, gold sault concentration: 1mM, temperature: 55°C, and reaction time: 90 min) for the fabrication of GNP-Mel were used for further characterization and study.

Analysis of TEM

The size and shape of GNP-Mel were identified using transmission electron microscopy (TEM). The TEM image indicated the formation of gold nanoparticles in a spherical and uniform shape (Fig. 3a). By measuring 200 nanoparticles of TEM image performed by ImageJ software, the average particle size was estimated at 8 ± 1.2 nm (Fig. 3b). Generally, the properties of GNPs are mainly affected by their size and shape. The earlier studies confirmed nanospheres show less cytotoxicity than nanostars and nanorods [32]. Additionally, in a study with different sizes of spherical gold nanoparticles, the smaller ones (10 nm) were found to be spread more widely in various organs of rats than larger particles [33]. The intracellular uptake or movment through the natural barrier in the body is important for delivering certain drugs, treatments, and diagnostics [34]. These results demonstrate that the synthesized spherical nanoparticles could be appropriate for biomedical applications.

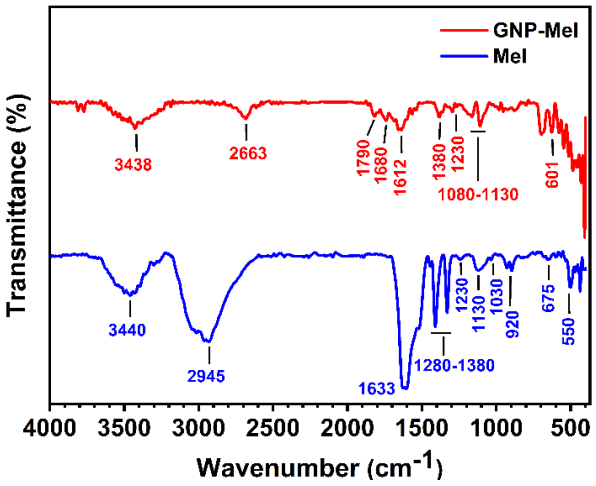

Fig. 4. FTIR spectra of aqueous extract of Mel and GNP-Mel.

Analysis of DLS

Dynamic light scattering (DLS) was used to determine the hydrodynamic diameter of GNP-Mel. The sizes of Au nanoparticles taken by DLS (20 nm) were larger than the TEM result, which could be attributed to the nanoparticle surface being charged and water molecules accumulating on the surface, increasing the hydrodynamic diameter. Zeta potential is the main factor in predicting the dispersion stability of nanoparticles over time. In previous studies, nanoparticles with a value greater than +25 mV or less than -25 mV of Zeta potential generally had high ratios of stability [35]. The zeta potential of GNP-Mel was about -31.7 mV indicating the high colloidal stability of these GNPs (Fig. 3c), as in the UV-Vis spectra of GNP-Mel after 90 days was observed (Fig. 2f).

Analysis of FTIR

FTIR analysis was carried out to determine the active groups in the samples as reducing and stabilizing factors of the synthesis [36]. The FTIR results of *Melissa officinalis L.* extract and GNP-Mel are shown in Fig. 4. As shown, the Mel extract has the broad strong band at 3440 cm^{-1} is allocated to the OH groups of alcohols or phenols compounds and NH stretching of amid A. The strong peaks at 2945 cm^{-1} indicate CH stretching vibrations of alkanes and secondary amines of compounds. The sharp peaks at 1633 cm^{-1} specify C=C group of aromatic compounds. The sharp peaks at 1280 -1380 cm^{-1} correspond to the CH3 groups stretching vibrations of carbohydrates in the extract. The weak peaks at 1230 indicate Amine III and amine I of proteins in the extract. The peaks at 1130 cm^{-1} indicate the C-O in-plane bending of alkanes, alcohols, carboxylic acid, esters and ethers. The weak bands at 920 cm^{-1} and 1030 cm^{-1} assigned different types of vibration of C-O-C groups. The peaks at 550 and 675 cm^{-1} designate =CH group of aromatic bicyclic monoterpenes [37, 38]. The many peaks presented in FTIR spectra of Mel extract, such as two bands related to phenolics, are shifted following the addition of the HAuCl4 solutions for GNP synthesis. The broad peak at 3438 cm^{-1} is indicated to the OH groups of alcohols or phenols compounds and NH stretching amid A. The peaks at 2663 cm^{-1} indicate CH stretching vibrations of alkanes or C=C group of aromatic compounds. The weak peak at 1790 cm^{-1} and vibration frequencies at about 1680 cm^{-1} are attributed to carbonyl stretch. The peaks at 1612 cm^{-1} specify C=C group of aromatic compounds. The sharp peaks at 1380 cm^{-1} correspond to the CH3 groups stretching vibrations of carbohydrates. The weak peaks at 1230 cm^{-1} indicate Amine III and amine I of proteins in extract. The peaks at 1080-1130 cm^{-1} indicate the C-O bending groups of organic compounds. Finally, the peaks at 626 cm^{-1} indicate the presence Au–O band of biosynthesized Au NP. FTIR spectra of biosynthesized Au NP showed shifts in the multiple bands were observed, resulting in the synthesis of Au NP. For the aqueous leaf extracts of the plants, the changes in the position of the bands were associated with the hydroxyl groups of the phenolic compounds, amines, and phenols. However, FTIR results indicated phenolics play an important role in the reduction and stabilization of

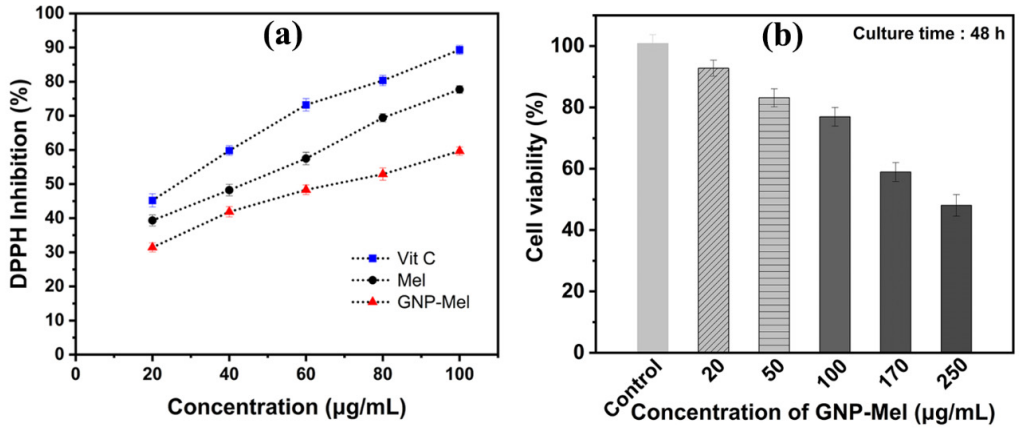

Fig. 5. (a) Antioxidant activity of Vitamin C and synthesized AuNPs. (b) Investigation the effect of GNP-Mel on H9C2 cell viability by Alamar blue method for 48h (mean SD, n = 5).

the Au (III) ions to Au0 during the biosynthesis of AuNPs by the natural plant extracts [27, 39].

Antioxidant activity of Mel and GNP-Mel

Antioxidants play a vital role against free radicals that cause the destruction and mutation of cells. Nowadays, the use of herbal products has increased due to high antioxidant activity [40]. In this study, DPPH (1,1-diphenyl- 2-picryl-hydrazyl) was used as a famous method to determine the antioxidant properties of Mel extract, GNP-Mel and ascorbic acid (standard antioxidant) (Fig. 5a) [41]. Several concentrations (20, 40, 60, 80, 100 µg/mL) of extract, GNP-Mel and Vitamin C were used for this assay. The test result showed a significant effect of free radical inhibition depending on increasing concentrations. The Mel extract showed the highest antioxidant activity at a concentration of 100 µg/mL, with 77.69 ± 1.18% inhibition, which was similar to the standard ascorbic acid that had 89.34 ± 1.34% inhibition. Pervious studies have shown that the Mel plant has a remarkable capacity for scavenging free radicals due to its high amount of polyphenolic compounds [42-44]. The synthesized GNP-Mel had a slightly lower antioxidant activity, with up to 59.64 ± 1.29% inhibition of DPPH radicals. Similar results have been reported for Au NPs synthesized by leaf extracts of Centaurea behen, Ziziphus nummularia, and Glaucium flavum, where the DPPH scavenging activity was concentration-dependent [6, 45, 46].

Cell viability study of GNP-Mel

The main factor for the usefulness of a compound in medicinal applications is a correct balance between curative ability and toxic side effects [47]. Therefore, for cell viability analysis, different concentrations of GNP-Mel were evaluated by Alamar blue test on H9C2 rat cardiomyoblasts cell line. Cell treatments by GNP-Mel (20, 50, 100, 170 and 250µg/mL) were performed on H9C2 cell lines for 48 hours. The result of Alamar blue assay showed cell viability depended on concentrations of gold nanoparticles. In addition, it showed a higher than 80% cell viability in the presence of 20, and 50 µg/mL of gold nanoparticles and likewise, a higher than 50% cell viability in the presence of 100 and 170 µg/mL of gold nanoparticles (Fig. 5b). Therefore, these results indicated that the low concentrations of GNP-Mel are cytocompatible.

CONCLUSION

The present study reports a simple and fast green synthesis method of gold nanoparticles that is reproducible and environmentally friendly without other reducing agents. We used variant physio-chemical characterization tests such as UV-Vis, ICP-OES, DLS, TEM, and FTIR to validate the fabrication of GNP-Mel. Optimization and stabilization of Au nanoparticles by using extract of Melissa officinalis L were done successfully. Mel extract presented excellent chemical-reducing, stabilizing and antioxidant characteristics. In addition, the results of DPPH and Alamar blue tests indicated that GNP-Mel had significant antioxidant and cell viability depending on concentrations of gold nanoparticles. In conclusion, the findings suggest that the green synthesized GNP-Mel has suitable potential to be employed in biomedical fields.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest in this project.

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