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A green approach for the synthesis of silver nanoparticles using *Lithospermum officinale* root extract and evaluation of their antioxidant activity

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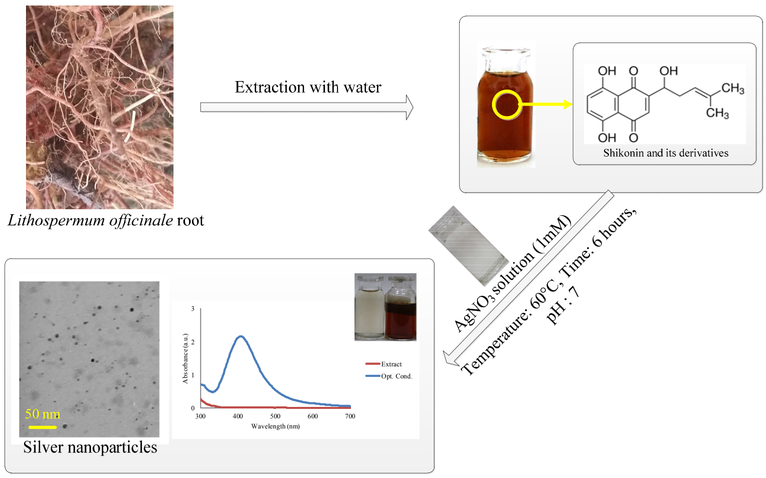
HIGHLIGHTS

- Green method for synthesis of the silver nanoparticles using *Lithospermum officinale*.
- Evaluation of antioxidant activity of synthesized silver nanoparticles.
- Therapeutic potential of the synthesized nanoparticles due to their antioxidant activity.

GRAPHICAL ABSTRACT



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ABSTRACT

Recently, the synthesis of silver nanoparticles has become an important subject in the bionanotechnology field. Many different chemical and physical methods could be used for silver nanoparticles synthesis, but they are limited due to the usage of toxic chemicals and the production of dangerous by-products. However, the usage of plant extract for silver nanoparticles synthesis is a green single-step method without using toxic chemicals. Herein, silver nanoparticles were synthesized using *Lithospermum officinale* root aqueous extract and their antioxidant activity was evaluated in vitro. The results showed that 1 ml of the extract could reduce 9 ml of silver ions (1 mM) to silver nanoparticles by heating the reaction mixture (60 ºC) for 6 hours at pH 7.0. The synthesized silver nanoparticles were detected by UV–Vis spectroscopy, TEM, FT-IR, DLS, and XRD. The synthesized silver nanoparticles spectrum had a maximum peak at 390nm, and TEM analysis indicated spherical particles, higher stability (zeta potential: -15.3 mV) and an average size of 7 nm. The antioxidant activity of the synthesized silver nanoparticles was 0.07 mg/ml compared to *L. officinale* root aqueous extract (0.142 mg/ml) which indicated higher antioxidant activity. So, it is concluded that the synthesized silver nanoparticles could be considered a clinical therapeutic potential due to its antioxidant property.

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1. Introduction

The field of nanotechnology, which is related to manufacturing materials at the range of nanometers, is growing rapidly because of its application in technology and science. Metal nanoparticles, especially silver nanoparticles, are widely used in many fields such as catalysis [1], plasmonics [2], optoelectronics [3], biological sensor [4,5], agricultural [6,7] and the pharmaceutical industry [8-10].

Many physical and chemical methods such as thermal decomposition [11], electrochemical [12], laser ablation [13], microwave, [14] etc. [15,16] have been applied for silver nanoparticles synthesis, but these are various limitations for these mentioned methods to such as high cost, harmful solvent and chemical systems, environmental contamination, hazardous by-products, higher temperature and higher pressure.

In comparison to physicochemical methods, green chemistry methods are cost effective, easily available, eco-friendly, and nontoxic [17,18]. In this method naturally occurring biomaterials, such as the extract of plants, were used for the metal nanoparticles synthesis applied in medicine and pharmaceutics [17,18]. It has been confirmed that the plant metabolites, such as polyphenols, phenolic acids, terpenoids, alkaloids, sugars, and proteins, play an important role as reducing, capping and stabilizing agents [19,20]. Many articles are available for silver nanoparticles synthesis using plants such as *Citrullus lanatus* [21], *Avena sativa* [22], *Prangos ferulaceae* [23], *Gmelina arborea* [24], *Parkia speciosa* [25], and *Azadirachta indica* [26]. Also in many cases, the biological activity of silver nanoparticles synthesized by plant extract has been researched. Elemike et al. [10] synthesized biological active silver nanoparticles using *Costus afer* extract, they found the nanoparticles indicated better antibacterial and antioxidant activity compared to the leaf extract. Jemal et al. [27] demonstrated a green and cost-effective method for silver nanoparticles synthesis from leaf extracts of *Allophylus serratus* and evaluated their antibacterial activity. The silver nanoparticles indicated antimicrobial activity against both gram negative and gram positive bacteria. Similarly, the antimicrobial potential of silver nanoparticles synthesized by the extract of mint plant leaves was researched by Sarkar and Paul [28]. Their results showed that these nanoparticles have good inhibition activity towards *Escherichia coli* and *Pseudomonas aeruginosa*.

To the best of our knowledge there have been no reports for silver nanoparticles synthesis using *L. officinale* root aqueous extract. The dried root of Lithospermum species is used as an herbal medicine in many countries. So, the aim of this research was to synthesize and characterize the silver nanoparticles using *L. officinale* root aqueous extract as a reducing, capping and stabilizing agent. In addition, the antioxidant property of the synthesized silver nanoparticles was investigated.

2. Material and Methods

2.1. Synthesis of silver nanoparticles

The silver nanoparticles were synthesized using *L. officinale* roots collected from Azarshahr, (37° 45' N, 45° 57' E) eastern Azarbaijan province, Iran. The roots were cleaned, weighed (20 g) and cut into small pieces. After adding the roots to 200 ml autoclaved double distilled water and shaking for 30 minutes at room temperature, the mixture was centrifuged for 2 minutes and then filtered through a 0.45 μm filter. The experiments were performed at different silver ion concentrations (0.25, 0.50, 1.0, 2.0, and 5 mM), temperatures (25, 40 and 60 ºC), pH (from 3 to 13 using HCl or NaOH), and the ratios of extract to silver nitrate (1:9; 3:7, and 5:5 v/v) in order to obtain the optimum condition for synthesis. The primary observation of synthesis indicated a color change from yellow to brown, which was confirmed by UV-Visible spectroscopy. After centrifuging the nanoparticles solution at 15000 rpm, the precipitation was washed with distilled water to eliminate all impurities and dried in a 50 °C oven for 48 h. The obtained powder was put into a refrigerator for further analysis.

2.2. Characterization of silver nanoparticles

The synthesis of nanoparticles was initially observed by the color change and later confirmed using UV-Vis spectrum. The samples were monitored as a function of reaction time by a UV-Visible spectrophotometer (PG, UK) between 300-700 nm at a resolution of 10 nm. In order to determine the stability and size distribution of the nanoparticles, zeta potential and DLS were measured using a Zetasizer Nano ZS 3600 (Malvern Instrument Ltd, UK). The synthesized silver nanoparticles were then scanned with an AFM (Nano Surf® AG, Switzerland, Product: BTO 2089, BRO). Also, the morphology of nanoparticles was determined using the TEM technique (JEM-2100F, Jeol Ltd., Japan) operating at 200 kV. The crystalline nature of the silver nanoparticles was characterized by the XRD technique using an X-Ray diffractometer (Bruker AXS D8 ADVANCE). A Perkin Elmer infrared spectrophotometer (model FT-PC-160) was used to determine surface chemistry and functional groups, such as hydroxyls and carbonyls, which attach to the surface during the synthesis of the nanoparticles.

2.3. Assay of antioxidant activity

The antioxidant activity of the samples was evaluated using the DPPH method [29]. Briefly, the samples in different concentrations ranging from 50 μg/ml to 300 μg/ml were added to tubes containing 1ml of DPPH solution (0.1 mM in methanol). After vigorous shaking, the mixtures were kept in the dark for 30 min (at room temperature) and then measurement of the absorbance was done at 517 nm against a control (L-Ascorbic acid as the control). The radical scavenging capacity was calculated based on Eq. (1).

$$\text{Inhibition } \% = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \qquad (1)$$

2.4. Total phenolic content

The Folin-Ciocalteau method was used to determine the total phenolic content [30]. In brief, 200 μL of the sample (1 mg/ml) was mixed with 200 μL of Folin-Ciocalteu reagent (10 % v/v). Three minutes later 2 ml of sodium carbonate (20% w/v) was added, and the sample was allowed to stand for 2 hours (in the dark and at room temperature). After measuring the absorbance at λ= 650 nm, the total phenolic contents was calculated using gallic acid as a standard.

2.5. Statistical analysis

The data were statistically analyzed by Statistical Analysis System (SAS) software and are the mean ±SD of three replications.

3. Results and Discussion

The presence research describes silver nanoparticles synthesis using *L. officinale* root aqueous extract. To obtain silver nanoparticles with antioxidant activity, the reaction time and temperature, pH, silver nitrate concentration, and the ratio of extract to silver nitrate were optimized.

3.1. Biosynthesis of silver nanoparticles

3.1.1. Effect of reaction time

As shown in Figure 1a, when the reaction time was extended beyond 10 minutes, the solution started to turn darker and the intensity of the SPR peak (at 418 nm) increased significantly during 2 hours, this was due to the continuous formation of the nanoparticles with the passage of time. In other words, an increase of the SPR peak intensity indicated an enhancement in the silver nanoparticles synthesis. The SPR peak increased and shifted somewhat to higher wavelengths with reaction time increment until it remained constant after 6 hours. The red-shift in the peak wavelength showed that the particles size increases as the reaction time increases. These results were in agreement with Verma and Mehata's [31] report, which showed controllable synthesis of silver nanoparticles using Neem leaves. Therefore, it could be concluded the optimum time for the synthesis of nanoparticles was around 6 hours.

3.1.2. Effect of reaction temperature

To find out the effect of temperature, the silver nanoparticles synthesis was done at 25, 40, and 60 °C. The results shown in Figure 1b reveal that increasing temperature increased the intensity of SPR peaks and the color of solution changed to yellowish brown, which may be due to a faster reaction rate at higher temperature. Also, as the temperature increased a blue-shift appeared from 460 nm to 405 nm because of a reduction in the silver nanoparticles size. The average kinetic energy of silver ions is related to the temperature reaction. As the temperature increases the silver ions would have a more dispersed range of speeds, this causes more collisions between the silver ions and secondary metabolites present in the extract and increases the consumption of silver ions. So, the possibility of particle size growth decreases and uniform size silver nanoparticles form. These results agree well with the findings from Ibrahim [17], Verma & Mehata [31], and Kasture et al. [32] who

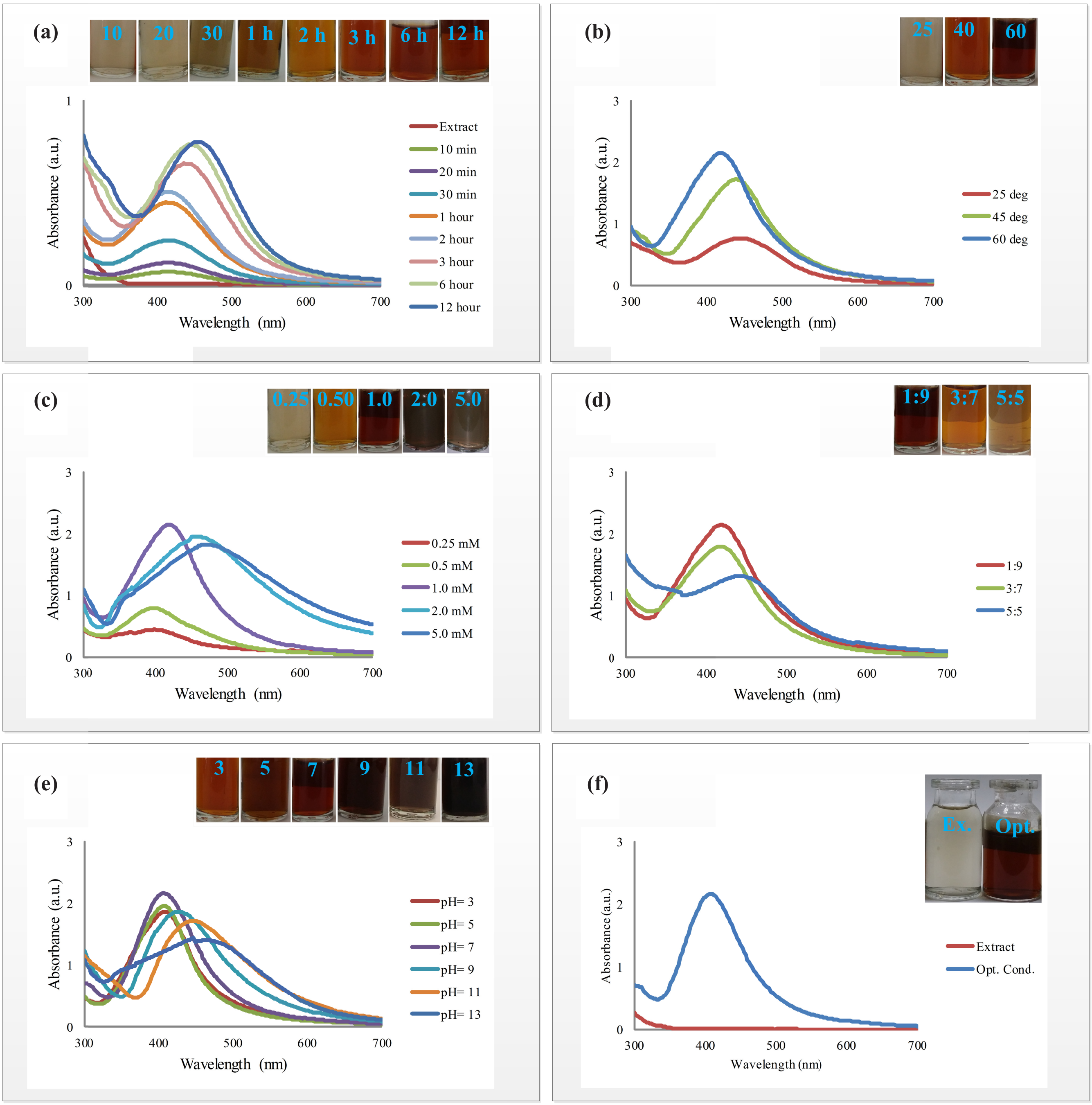

Fig. 1. Visual observation and UV-Vis absorption spectra of the silver nanoparticles at (a) different reaction time, (b) different reaction temperature, (c) different silver nitrate concentration, (d) different concentration ratio of the extract and silver nitrate, (e) different pH, and (f) optimum condition including: reaction time, 6 hours; silver nitrate concentration, 1 mM; temperature, 60°C, extract pH, 7; and concentration ratio of the extract and silver nitrate, 1:9 v/v.

all concluded that as the reaction temperature increased the reaction rate also increased, which may result in smaller-sized nanoparticles. Therefore, the optimum temperature for achieving smaller-sized nanoparticles was 60 °C.

3.1.3. Effect of silver nitrate concentration

As shown in Figure 1c, when the silver nitrate concentration increased from 0.25 to 1 mM the SPR peak intensity significantly increased, which indicated that the silver nanoparticles synthesis occurred at higher rate. But, further increasing the silver nitrate concentration from 1 to 2 mM and above caused reduction of peak intensity, further broadening of the peak, a red-shift from 405 nm to 490 nm, and settlement of particles. The formation of large-scale micro particles may be due to the presence of large amounts of silver atoms in a small volume of the solution, which causes a high attraction between silver atoms and the agglomeration of nanoparticles. Hence, the rapid settling of particles may be related to their large size [33]. So, 1 mM of silver nitrate was selected to achieve a large amount of silver nanoparticles with low dispersion.

3.1.4. Effect of extract and silver nitrate ratio

Optimization of the root extract and silver nitrate ratio was carried out with a change in the volume of root extract and silver nitrate (1 mM). When the volume of the extract to silver salt was in the ratio of 5:5 v/v, a broad peak of less intensity (at 460 nm) was observed (Figure 1d), which might be because of more reduction processes on the surface of the formed nuclei in the presence of higher concentration of reducing agents [34]. A similar result was also observed in the case of gold nanoparticles synthesis using *Cocos nucifera* extract [18]. As the volume of the extract was decreased from 5:5 to 3:7 v/v a narrow peak with a blue shift (from 460 to 407 nm) occurred (Figure 1d), which might be due to a reduction in the particles' size. Also, the absorption intensity increased as the volume of the extract decreases from 3:7 to 1:9 v/v, indicating an enhancement in the silver nanoparticles synthesis. Therefore, the optimum ratio of the extract and silver nitrate for the silver nanoparticles synthesis was evaluated at 1:9 v/v.

3.1.5. Effect of pH

Figure 1e shows the effect of pH on the synthesis of silver nanoparticles. The pH of the extract can influence the electrical charges of natural products which might act as capping and stabilizing agents. So, the size and shape of the particles can be changed by the pH of the extract. The results (Figure 1e) show that by increasing the pH values from 3.0 to 7.0, the intensity of the SPR peaks increased and maximum silver nanoparticles production occurred. In addition, the SPR peak became broader and shifted toward the long wavelength region as the pH value increased. Any shift of the SPR peak toward the longer wavelength is accompanied by an increase in the size of the synthesized silver nanoparticles, and the broadening of the SRP peak indicates the presence of a wider range of particle size in the solution. Also, the decrease of the SRP peak intensity showed the decrement in the silver nanoparticles synthesis. This may happen due to the ionization of natural phenolic compounds in *L. officinale* root extract. So, the results suggested that neutral pH (pH= 7.0) was more suitable to obtain nanoparticles with a small size. Afreen and Vandana [35] synthesized silver nanoparticles at neutral conditions (pH=7) using aqueous extract of Rhizopus stolonier. Similarly, Elemike et al. [36] reported that silver nanoparticles showed maximum stability at neutral pH (6.8-7.0) using an aqueous extract of *Lasienthra africanum*. These findings were in a close agreement with Figure 1f in the present study.

3.2. Characterization of silver nanoparticles

The particle size and morphology of the nanoparticles synthesized by *L. officinale* root aqueous extract was determined by TEM (Figure 2a). It is evident by the morphology of the nanoparticles which was almost spherical and the particles diameter was from 2 to 11 nm with an average size of 7 nm. These results agree well with the particle size calculated from XRD analysis. The results obtained from DLS clearly indicated a narrow range of size distribution from 7 to 22 nm with an average mean size of 9 nm (Figure 2b). The size obtained from DLS was usually larger than that from TEM. This may be due to the surface adsorption of natural products on the nanoparticles, the aggregation of nanoparticles, and the surface adsorption of water onto the silver nanoparticles stabilized with *L. officinale* root aqueous extract. All of these reasons may be have a negative effect on the results obtained by the DLS method [37]. Also, the size of silver nanoparticles was confirmed by AFM (Figure 2c) and it was found to be less than 15 nm. The zeta potential of the synthesized silver nanoparticles was measured and showed a sharp peak at -15.66 mV. So, the repulsive forces between negative charges of the nanoparticles caused the stability of the nanoparticles in the suspension.

The XRD analysis was done to determine the crystalline nature of the synthesized nanoparticles by *L. officinale* root aqueous extract. The XRD spectrum (Figure 3) showed four distinct separate peaks at $2\theta=38.08°$, $44.47°$, $64.17°$ and $77.57°$ corresponding to lattice plane values indexed at (111), (200), (220), and (311) planes, respectively, which were in agreement with the database of face centered cubic (FCC) structures from the Joint Committee Powder Diffraction Standards (JCPDS) file No. 04-0783.

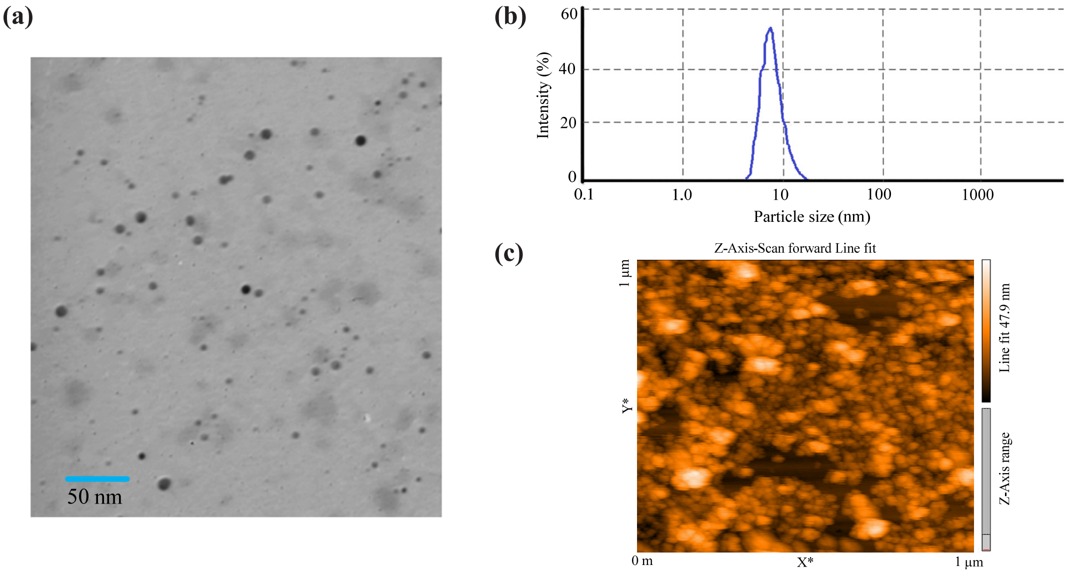

Fig. 2. a) TEM micrograph, b) Size distribution, and c) AFM image of the silver nanoparticles synthesized using *L. officinale* root aqueous extract.

The phytochemical analysis of *Lithospermum* species showed the presence of naphthazalin skeleton metabolites such as shikonin and its derivatives in the outer surface of the roots [38]. The presence of these metabolites may play an important role in silver ion reduction. FT-IR spectroscopy (Figure 4) was used to determine the major functional groups on the *L. officinale* extract which may be responsible for the synthesis and stability of the silver nanoparticles. The –OH and C=O peaks at 3423 cm^{-1} and 1744 cm^{-1}, respectively, were due to the presence of shikonin and its derivatives in the extract. Also, the peak at 1625 cm^{-1} was related to the C=C stretching vibration of aromatic rings. So, the FT-IR spectrum of the silver nanoparticles indicated an increase of the C=O peak and a decrease of the –OH peak, which may be related to the oxidation of –OH groups to C=O groups. So, the proposed mechanism may be due to the presence of shikonin and its derivatives in *L. officinale* extract (Figure 5), which play an important role in the reduction of silver ion to silver nanoparticles. Also, the trace shift in the absorption peaks of the FT-IR spectrum indicated the presence of the natural products on the surface of the silver nanoparticles.

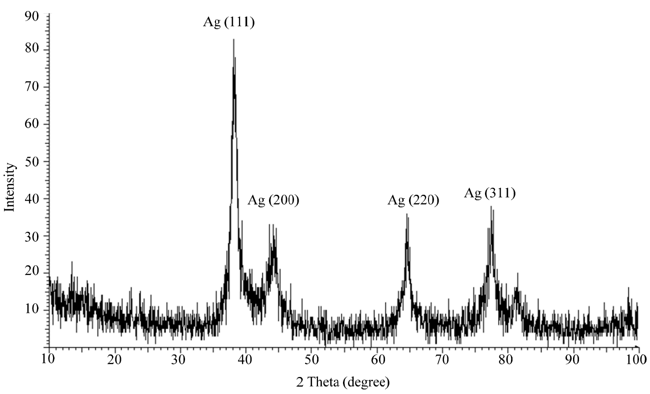

Fig. 3. X-ray diffractometer of the silver nanoparticles synthesized using *L. officinale* root aqueous extract.

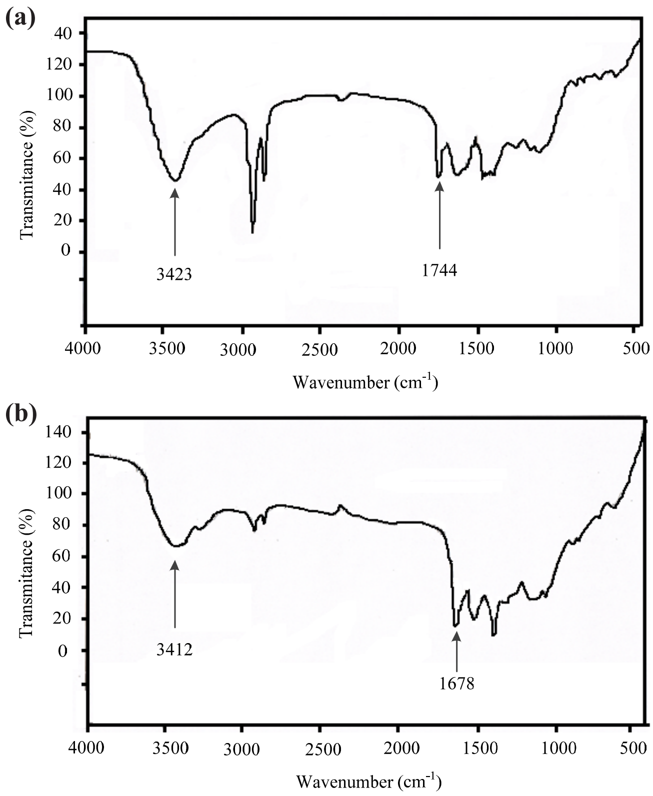

Fig. 4. FT-IR Spectrum of A) *L. officinale* root aqueous extract and B) biosynthesized silver nanoparticles.

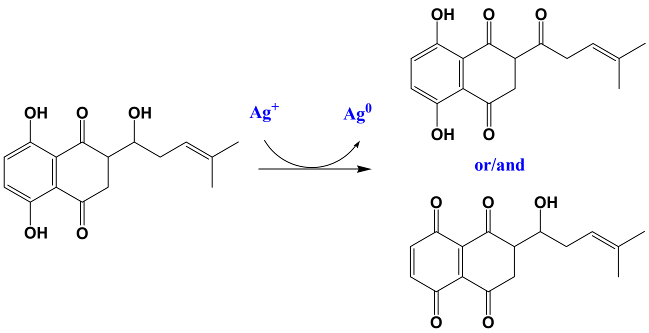

Fig. 5. The proposed mechanism for the synthesis of silver nanoparticles, due to the presence of shikonin and its derivatives.

3.3. Antioxidant activity of synthesized nanoparticles

The antioxidant potential of the synthesized nanoparticles was assayed using the DPPH method and compared to *L. officinale* root aqueous extract. The results shown in Table 1 indicated that the nanoparticles had more antioxidant activity in comparison with *L. officinale* root extract, and the recorded IC50 values were 79±0.9 and 142±2.1 µg/ml, respectively. The enhanced antioxidant capacity of the synthesized silver nanoparticles may be because of the phenolic compounds present on the surface of the nanoparticles, which could cause antioxidant activities via transferring a hydrogen atom or an electron to the reactive species [39]. Also, the analysis of the total phenolic content (TPC) showed (Table 1) that the TPC of the synthesized silver nanoparticles was higher (76.83±0.15 mg/g) compared to the extract (57.9±0.23 mg/g), which had a positive correlation with the antioxidant capacity. Phull et al. [9] evaluated antioxidant activity of the synthesized silver nanoparticles from crude extract of *Bergenia ciliate* and suggested that the synthesized nanoparticles can be used as an antioxidant. Abdel-Aziz et al. [8] reported the presence of strong antioxidant activity of nanoparticles-containing leaf extract compared to *Chenopodium murale* leaf extract. So, the antioxidant potential of the synthesized nanoparticles could be attributed to phenolic compounds which adhered to them and which originated from the extract, and these nanoparticles could be applied as natural antioxidants to cure degenerative diseases which are related to oxidative stress.

4. Conclusion

The synthesis of the silver nanoparticles was done on the base of a green chemistry method, in which *L. officinale* root aqueous extract was used as a reducing and stabilizing agent. In this process, one ml of the extract reduced 9 ml of silver ions (1 mM) into silver nanoparticles during heating of the reaction mixture (60 ºC) for 6 hours at pH 7.0. The synthesized nanoparticles had a uniform and spherical shape with an average particles size around 7 nm, which indicated significant antioxidant activity. So, this study provided a better and faster method for silver nanoparticles synthesis with antioxidant activity.

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