Biosynthesis of silver nanoparticles from juniper tree extracts

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Research Article

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Abstract

In this study, silver nanoparticles were synthesized via a green and inexpensive method, from leaf and twig extract of Juniperus excelsa as a reducing agent for reduction of Ag^{+} to Ag°. The first sign of the production of Ag nanoparticles was the color change from yellow to brown. The effective synthesis parameters such as concentration of silver nitrate, extract volume, pH, reaction time and temperature were investigated and controlled by UV-visible spectroscopy. The silver nanoparticles were synthesized under the optimal conditions of 2 mM silver nitrates, 10 ml extract volume, pH = 12, temperature = 70 ˚C and reaction time of 3h. This sample was characterized using X-Ray diffraction (XRD), field-emission scanning electron microscope (FE-SEM), transmission electron microscope (TEM), and dynamic light scattering (DLS) techniques. The findings revealed that the synthesized silver nanoparticles had spherical morphology and an average size of 40 nm. Evaluation of antibacterial activity by the disc-diffusion assay shown that Ag nanoparticles effectively inhibited the growth of both Escherichia coli and Staphylococcus aureus. The MICs against E. coli and S. aureus was 6.25 and 25 µg/ml, respectively. These results demonstrated that the synthesized nanoparticles had stronger antibacterial activity against E. coli.

Introduction

Amongst different metal nanoparticles, silver nanoparticles are more interesting due to their distinctive properties such as high electrical conductivity, chemical stability, catalytic and antibacterial activity. In regard to the effect of metal nanoparticles' size and shape on their respective properties, a wide range of researches have been focused on optimization of their physical and chemical properties, and likewise manipulation of their size and shape [1].

Among the parameters affecting on the size and shape of nanoparticles, synthetic methods hold a particular importance. Various methods namely electrochemical reduction, photochemical reduction, and thermal evaporation have been applied for the synthesis of silver nanoparticles. Biological processes have also been recently addressed for the synthesis of the mentioned nanomaterial [2]. Chemical methods have diverse shortcomings such as application of toxic solvents, production of dangerous by-products, and high energy consumption. Biological processes are more advantageous as compared to the conventional chemical ones in terms of their cost effectiveness and eco friendliness [3].

Green synthesis of nanoparticles through microorganisms [4], enzymes [5], and plant extract [6] is assumed as a biological method. Application of plant extract is more cost effective than other biological process since, besides increasing safety, it diminishes reaction time by a couple of hours to some days [7].

Synthesized nanoparticles via plant extracts may be used for various biological purposes such as hormones, antibodies, proteins, antibiotics, and targeted release of medicines [8]. The extract derived from different plant organs like leaf, root, seed, stem, and fruits containing antioxidants have been used for biosynthesis of silver nanoparticles [9]. Plant extracts can serve as reductive as well as stabilizing

agents in synthesis of nanoparticles [7]. The existing literature confirms that silver nanoparticles synthesized by plant extracts hold promising medical applications [9].

Juniperus spp. has several species in Iran including *Juniperus excels*, *J. communis*, *J. foetidissima*, and *J. Sabina*, and is observed both as tree and shrub forms. Juniper tree (*Juniperus excelsa* M.Bieb. *subsp*. polycarpos (K. Koch) Takht.) is among the endemic conifers of Iran mainly existing in most of the humid Hyrcanian regions, deserts (Iran-Turan), interior elevations of Iran, western forests, and Oman gulf in the south of Iran [10]. This genus is the second highly distributed one after *Pistacia* amongst the endemic trees of Iran. Different species of the given species are remarkably invaluable in terms of industrial, preservative, and protective aspects. Juniper species naturally appear as scattered individuals in the central regions of Zagros (altitudes of 1800 to 3000 m) and the southern parts of Iran (altitudes above 3400 m). The mentioned habitats, termed as Juniper forests, cover an area of about 1.3 million hectares [10, 11]. Considering all mentioned, the present research is aimed at application of green chemistry for the synthesis of silver nanoparticles as one of the most important and highly applied nanoparticles. To do so, the extract of Juniper tree (*Juniperus excelsa*) existing in Parvar protected area (Semnan province, Iran) was used as the reductive agent. The parameters affecting on synthesis were assessed, and the optimum conditions were so determined. Silver nanoparticles were synthesized under optimum conditions, and their respective structural and antibacterial properties were analyzed.

Results And Discussion

The first sign of the synthesis of silver nanoparticles is the color change of the reaction solution. The reason behind this change is the reduction of silver ions to silver nanoparticles. The addition of juniper extract to $AgNO_3$ (under specific temperature and pH) leads to the color change of the resulting solution from yellow to light brown in a short time, and by the end of the reaction time, the color gradually grows darker. The increase in the intensity of solution color indicates the presence of higher amounts of silver nanoparticles. The observed color change is not only due to the reduction of Ag^+ to Ag^0 by the biomolecules present in the mentioned extract but is also due to the stimulation of surface plasmon resonance (SPR). The absorbance of the solutions was measured via UV-visible spectroscopy at 400–420 nm wavelength spectrum. The highest absorbance was observed at 400 nm indicating that the given wavelength is the maximal wavelength of the studied silver nanoparticles.

The addition of plant extract to $AgNO_3$ brought about a color change from brownish yellow to dark brown in the reaction products [12]. The same color change was also observed in the present research in 10 minutes indicating the formation of silver nanoparticles. After 10 minutes of stirring the reaction solution, the Ostwald ripening (precipitation) phenomenon occurred letting the particles have size homogeneity [12]. The experienced color change may be due to surface plasmon resonance, since the reduction of particle size is associated with increased stimulation of the external surface electrons, a phenomenon known as SPR [13]. The continuous absorbance intensification (as a function of time) indicates the performance of the given plant extract as a reductive agent.

The parameters influencing on the synthesis of silver nanoparticles were evaluated as follows to obtain the optimal conditions:

To optimize the concentration of $AgNO_{3,}$ six solutions of the mentioned salt, having concentrations of 1, 2, 3, 4, 5, and 6 mM, were prepared. A fixed amount of the extract (2 cc) was added to each of the solutions to have the reactions done at the room temperature. UV-Vis spectrum was taken from all the solutions at 400–420 nm after 2 hours.

As observed in Fig. 1, the maximum absorbance was obtained for 2 mM $AgNO_3$ concentration. Thus, 2 mM concentration of silver nitrate was chosen as optimal concentration for the synthesis of silver nanoparticles.

Ali et al. [14] carried out a research on the green synthesis of silver nanoparticles using the blue extract of sagebrush (*Artemisia absinthium*) and concluded that a solution of 2 mM silver nitrate and 10 mg/ml extract had a marked effect on the size, stability, and performance of silver nanoparticles. They found out that the biosynthesis of silver nanoparticles with sagebrush extract was highly dependent on the relative concentration of plant extract and silver nitrate, such that the higher concentrations of the mentioned materials led to the production of larger nanoparticles. In another research conducted by Sanchez et al. [15], the application of 2 mM silver nitrate resulted in the production of pure nanoparticles with relatively smaller sizes.

The optimization of the extract volume was carried out through preparing 6 solutions of 2 mM silver nitrate and different volumes (2, 4, 6, 8, 10, 12, and 14 cc) of the studied plant extract. All the solutions were stirred at the room temperature for 2 hours. After the completion of the reactions, UV-Vis spectrum was taken from all the studied solutions at 400–420 nm. The sample containing 10 ml extract volume showed a higher absorbance as compared to those of the 2, 4, 6, 8, 10, and 12 ml volumes (Fig. 2). Thus, the optimum extract volume leading to the highest production efficiency of silver nanoparticles was 10 ml.

The amount of plant extract has a crucial role in controlling particle size, such that larger amounts lead to the production of smaller size particles [16]. Sun et al. [17] reported that the volume of tea extract (as the reducing agent) can affect the production rate of the resulting bio-synthesized silver nanoparticles. Similarly, Iravani et al. [18] stated that the volume of *Pinus eldarica* bark extract was an important parameter in optimizing nanoparticles with intended morphology and size. They reported that larger amounts of the mentioned extract resulted in enhanced absorbance rate in UV-Vis spectroscopy, as higher concentrations of the extract could increase the production rate of the thereof nanoparticles; however, the mentioned parameters did not show a linear correlation. Moreover, a reduction of size was also observed in the silver nanoparticles by increasing the extract volume.

To optimize the reaction time, a solution of silver nitrate (2 mM) and extract (10 cc) was prepared, and then UV-Vis spectrum was measured at 30, 60, 90, 120, 150, 180, 210, and 240 min. Results demonstrated

that the time of 3 hours was the best for the synthesis of silver nanoparticles. As observed in Fig. 3, the concentration of silver nanoparticles increases until 3 hours and is gradually reduced after this time.

Reaction time can also play a role in the size and morphology of the synthesized silver nanoparticles. The results of a research conducted by Sánchez et al. [15] on the bio-synthesis of silver nanoparticles derived from *Peumus boldus* extract are in line with those of the mentioned study. In that research, as well, the best reaction time was reported as 3 hours, which led to the production of spherical, homogeneous nanoparticles with average size about 18 nm.

To study the effect of pH, a solution containing the optimum amounts of $AgNO_3$ (2 mM) and extract (10 cc) was prepared. The initial pH of the given solution was 6.4, and the studied pH values were 2, 4, 8, 10, and 12. HCl and NaOH solutions were used to change the pH to acidic and alkaline ranges, respectively. The prepared solutions were stirred for 3 hours and then UV-Vis spectrum was measured. The obtained results indicated that the efficiency of silver nanoparticle production increases in alkaline pHs (Fig. 4). Maximum absorbance was observed in pH = 12.

These results are congruent with those reported by Iravani et al. [18]. They concluded that after optimizing the pH, the higher pHs of the reaction solution lead to the higher absorbance. It seems that pH plays a role in the production rate of homogeneous nanoparticles. Moreover, it was found that, in contrast with the acidic pHs, the alkaline pHs led to the production of the smaller nanoparticles. Studying on the bio-synthesis of silver nanoparticles from stevia, Siddiqui et al. [19] stated that smaller size nanoparticles were produced at pH 8.5. In another study conducted by Heydari et al. [20] on the biosynthesis of silver nanoparticles from acorn shell extract, the optimization of pH was investigated at the range of 2–11. The results showed that the production of silver nanoparticles sped up at pH 9. They attributed this behavior to the possible ionization of the phenolic and tannin compounds present in the mentioned extract. Similar results have also been reported on the effect of alkaline pH on the synthesis of silver nanoparticles by Andreescu et al. [21]. They concluded that the quick and complete reduction of silver ions occurs only at pH = 12 and above 50 ˚C. Thus, they reported the given pH as the optimum value for the production of silver nanoparticles with the highest efficiency.

To optimize the reaction temperature, 7 solutions obtained from the aforementioned parameters (i.e., 2 mM $AgNO_3$, 10 cc extract, pH 12). The temperature of each solution was then fixed at the specific value. The studied temperatures were room temperature (22 ˚C), 30, 40, 50, 60, and 70 ˚C. The optimum reaction time was considered as 3 hours based on the conducted assessments. Temperature rise could increase the efficiency of silver nanoparticle production, and likewise the samples' absorbance (Fig. 5). This upward trend continued up to 80 ˚C. The comparison of the absorbance data at 70 and 80 ˚C reveals that, in spite of the existing high similarity in some of the absorbance data, the above 404 nm absorbance rates pertaining to 70 ˚C are more than those of 80 ˚C. Thus, 70 ˚C was considered as the optimum temperature.

Sun et al. [17] 2014 evidenced that the production of silver nanoparticles increased at higher temperature ranges. Heydari et al. [20] 2015 investigated the reaction temperature at 4 to 60 ˚C and concluded that the synthesis efficiency of silver nanoparticles was higher at 45 ˚C. Iravani et al. [18] found out that increase in reaction temperature from 25 to 150 ˚C brings about smaller size silver nanoparticles.

Considering the obtained results, the optimal conditions for the synthesis of silver nanoparticles were determined as follows: $AgNO_3$ concentration: 2 mM, extract volume: 10 cc, reaction time: 3 hours, pH = 12, and reaction temperature: 70 ˚C. The silver nanoparticles were synthesized under the given conditions, and characterized via different techniques. Finally, the antibacterial activity were studied.

Characterization of the silver nanoparticles

The X-ray diffraction pattern of the synthesized silver nanoparticles is shown in Fig. 6. The obtained pattern had been compared with the standard powder diffraction card of JCPDS silver file (98-005-0882). Four peaks at 38º, 44º, 64º, and 77º corresponding to the (111), (200), (220), and (311) planes, respectively. No peak was observed relative to impurities; thus, the synthesized sample is completely pure.

The XRD results reported by Forough et al. [22] are similar to this rsearch, and the silver nanoparticles had a high purity. Also, the results of another study conducted by Dong et al. [23] on the bio-synthesis of silver nanoparticles from the fruit extract of *Lycium barbaru*m confirmed the crystalline nature of the synthesized nanoparticles.

As seen in the FE-SEM and TEM images (Fig. 7), the produced nanoparticles are spherical and structurally homogeneous; this can be considered as one of the advantages of the method employed for the bio-synthesis of this nanoparticle. The agglomeration of nanoparticles observed in the images arise from the high intensity of the nanoparticles' surface energy leading to their respective cohesion. According to the mentioned images, the mean diameter of the synthesized nanoparticles is approximately 40 nm.

Different morphologies of nanoparticles may be due to the varied amounts and properties of the inhibitory factors present in the extract of miscellaneous plants. The results reported by Rasheed et al. [12] on the bio-synthesis of silver nanoparticles from *Artemisia vulgaris* extract showing that the obtained nanoparticles were spherical, with a mean size of 42 nm. Heydari et al. [20] conducted a research on the bio-synthesis of silver nanoparticles from the extract of cork acorn and the evaluation of the effect of nanoparticles' toxicity on MCF-7 cells. The SEM micrographs revealed that the produced silver nanoparticles had a spherical structure.

Figure 8 displays the results obtained from DLS analysis. The results indicate that there is only one peak pertaining to nanoparticles of about 50 nm diameter. In other words, it can be concluded that the synthesized nanoparticles were identical in size. This is in agreement with the obtained results from SEM and TEM images.

Antibacterial activity

To evaluate the antibacterial activity of the synthesized sample, *Escherichia coli* (the cause of urinary, blood, and hospital infections) and *Staphylococcus aureus* (the cause of eye, skin, bone, and joint infections) bacteria were used. As observed in Table 1, the minimum growth inhibitory concentrations of silver nanoparticles against *Escherichia coli and Staphylococcus aureus* bacteria are 6.25 and 25 µg/ml, respectively. The mean non-growth halo determined for these amounts of MIC analysis in both kinds of the bacteria equals 15 mm. These results denote that the silver nanoparticles show a favorable antibacterial activity against both of the given bacteria. Furthermore, the antibacterial activity of the synthesized nanoparticles against *Escherichia* coli is stronger than that of *Staphylococcus aureus*, since the former bacterium was inhibited at lower concentrations of silver nanoparticles. This difference is due to the cell wall structure of gram-positive and gram-negative bacteria causing the infiltration in latter bacteria's cell wall to be easier. The wall thickness of *Escherichia coli* is 7–8 nm, and that of *Staphylococcus aureus* is 20–80 nm.

Table 1
MIC (µg/ml) and Non-growth halo *(mm)* of the silver nanoparticles

Bacteria species	**Minimum inhibitory concentration (µg/ml)**	**Non-growth halo (mm)**
Escherichia coli	6.25	15
Staphylococcus aureus	25	15

The antibacterial effect of nanoparticles can be studied in two parts: 1- affecting on cell wall and the outer part of bacterium cell, 2- infiltration in cell and affecting on the inner parts of bacterium cell. In the first part, the nanoparticles, holding a positive electrical charge, are absorbed by the negative-charge outer surface of bacterium cell; this absorption disrupts the electrolyte balance of the bacterium and can affect the reparatory cycle of the bacterium [24]. The second part entails the effect of nanoparticles on proteins and enzymes which have a crucial role in cell growth [25]. Although the mechanism of the effect of silver nanoparticles on bacteria is not fully known, attaching to the surface of the bacterium cell and the resulting disruption of cell wall function has been mentioned as the foremost mechanism of bacterial death by silver nanoparticles [26]. Imanzade and Hadi [27], conducting a research on the biosynthesis of silver nanoparticles from *Brassica oleraceae* extract reported that the derived nanoparticles had a favorable antibacterial activity against *Escherichia coli.* Studying the bio-synthesis of silver nanoparticles from *Datura stramonium* leaf extract, Gomathi et al. [28] demonstrated that the given nanoparicles showed antibacterial activity against *E. coli* and *S. aureu*, but the antibacterial property against the former bacterium was stronger, similar to that of this research. Same results were also reported by Dhand et al. [29] who biosynthesized silver nanoparticles from the seed extract of *Coffea Arabica.* They stated that the intended antibacterial activity was more severe against the gram-negative *Escherichia coli*, and the antibacterial activity of silver nanoparticles was identical to that of penicillin medicine.

Conclusion

The main purpose of this study was to produce silver nanoparticles using a green chemistry, inexpensive, safe and environmentally friendly method. To achieve this goal, extracts of leaves and twigs of *Juniperus excelsa* were used as a reducing agent to produce silver nanoparticles for the first time. The results showed that the maximum production efficiency of silver nanoparticles is created using 10 ml of plant extract with 2 mM silver nitrate solution, in 3 hours at pH = 12°C and 70°C conditions. The results of various identification techniques indicated that the synthetic sample is completely pure, with a spherical morphology and average dimensions of about 40 nm. Furthermore, due to the results of prominent antibacterial activity of the synthesized silver nanoparticles against Escherichia coli and Staphylococcus aureus, this type of nanoparticles can be recommended for application in disinfectant filters, coatings and food packaging.

Experimental

Preparation of plant extract

To provide the plant extract and reductive agent, the leaves and the just sprouted branches of juniper tree (*Juniperus excelsa*) were collected from Parvar protected area (Semnan province, Iran), thoroughly rinsed with distilled water to remove dust, and dried at room temperature (22 ˚C). An amount of 2 g of the mentioned leaves and branches was then poured into a beaker containing 100 ml distilled water. The obtained mixture was boiled at 100 ˚C for 15 min and then cooled to the ambient temperature. The plant extract was obtained after filtering the mixture and stored in a refrigerator (4 ˚C).

Synthesis of silver nanoparticles

A 2 mM solution of $AgNO_3$ was prepared and mixed with 10 cc juniper extract. The pH of solution was adjusted to 12 using 0.1 M NaOH solution. To obtain the highest production efficiency, the reaction was carried out at 70 ˚C for 3 h. The formation of nanoparticles was primarily confirmed by the color change of the solution from yellow to light brown and gradually to darker brown. The resulting mixture was centrifuged after cooling, and the obtained precipitation was dried at ambient temperature.

Antibacterial activity

To investigate the antibacterial activity, two widely-known pathogenic bacteria namely *Stapylococcus aureus* (as gram-positive) and *Escherichia coli* (as gram-negative) were used. The amount of the given bacteria's non-growth halo was evaluated through disk diffusion method in agar. A suspension of the live bacteria was prepared in a sterilized atmosphere and adjusted according to Mac Farland standard (CFU/ml 108). Then, a Mueller Hinton Agar culture medium was prepared and sterilized in Petri dishes placed into an autoclave. *Escherichia coli* and *Staphylococcus aureus* were separately placed on the mentioned Mueller Hinton Agar culture medium. Then, 100 ml of the colloidal silver nanoparticle solution (100 µg concentration) was poured into the sump. All the Petri dishes were kept at 37 ˚C for 18–24 h. The

non-growth halo was measured several times, and the mean value of the mentioned halo was ultimately reported.

Minimum Inihibitory Concent (MIC)

Microdilution method was employed to determine the *MIC* of the silver nanoparticle solution. Briefly, 100 µl of the Mueller Hinton sterilized medium was added to 96 circular, multi-sump plates (microplates) that below surfaces had been disinfected. The silver nanoparticle solution (at 100 µg/ml concentrations) was poured into the sump and distilled fractionally in the Mueller Hinton sterilized medium. *Stapylococcus aureus* and *Escherichia coli* bacteria were then cultured in a nutrient culture medium at 37 ˚C for a period of one night. 20 µl of each bacterium (CFU/ml 10 1.5) was taken and added to the Mueller Hinton sterilized medium. The positive and negative controls were considered as follows: (1) with the silver nanoparticles solution and without the bacterium inoculation substance and (2) without the silver nanoparticles solution and with the bacterium inoculation substance. The microplates were covered with sterilized lids and placed in an incubator at 37 ˚C for 18–24 h.

Characterization

The small angle X-ray scattering (SAXS) patterns were recorded with a model Hecus S3-MICROpix SAXS diffractometer with a one-dimensional PSD detector using Cu Kα radiation (50 kV, 1 mA) at the wavelength 1.542 Å. The SEM and TEM images were taken using Oxford LEO 1455 V STEM and Philips EM-208 100 KV, respectively. The UV/Vis absorption spectra were recorded using a Ray light, UV 1600 spectrophotometer.

Declarations

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Authors’ contributions

Current manuscript is part of a master thesis is conducted by S Jabini under supervision of Dr D Kartoolinejad and Z Bahrami and advised by Dr R Naghdi and A Sadeghipour. All antibacterial tests performed by Dr. Sara Minaeian.

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Availability of data and material

Due to the ethical issue of Semnan University, authors do not want to submit the raw data. During the review process, however, editors and reviewers have complete authority to check and verify the raw data.

Competing interests

The authors declare that they have no competing interests.

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Figures

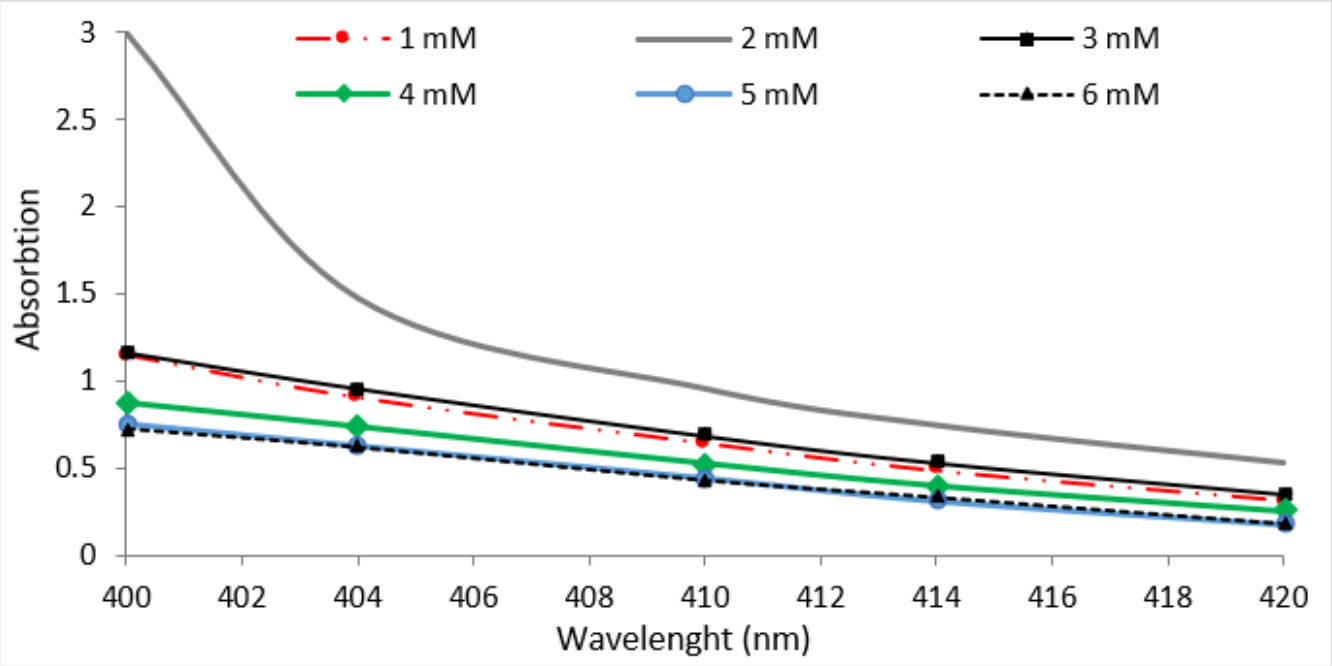

Figure 1

UV-Vis spectrum for optimization of silver nitrate concentration

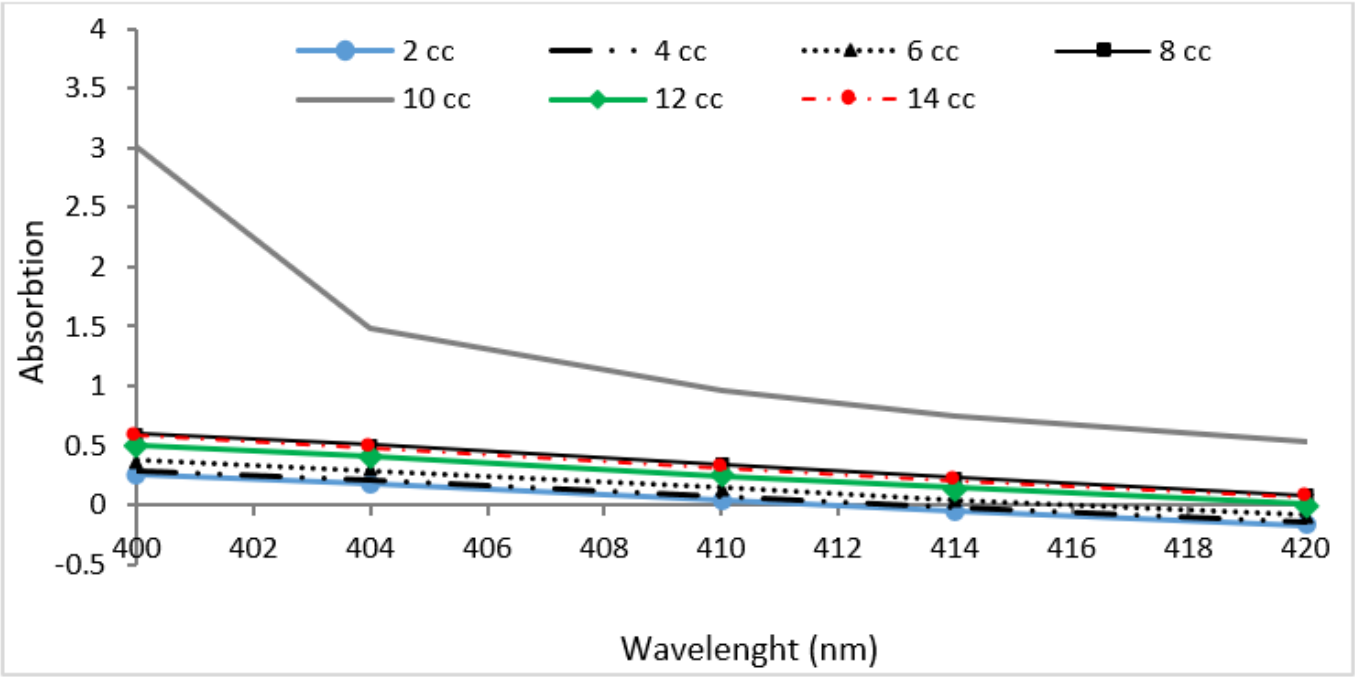

Figure 2

UV-Vis spectrum for optimization of extract volume

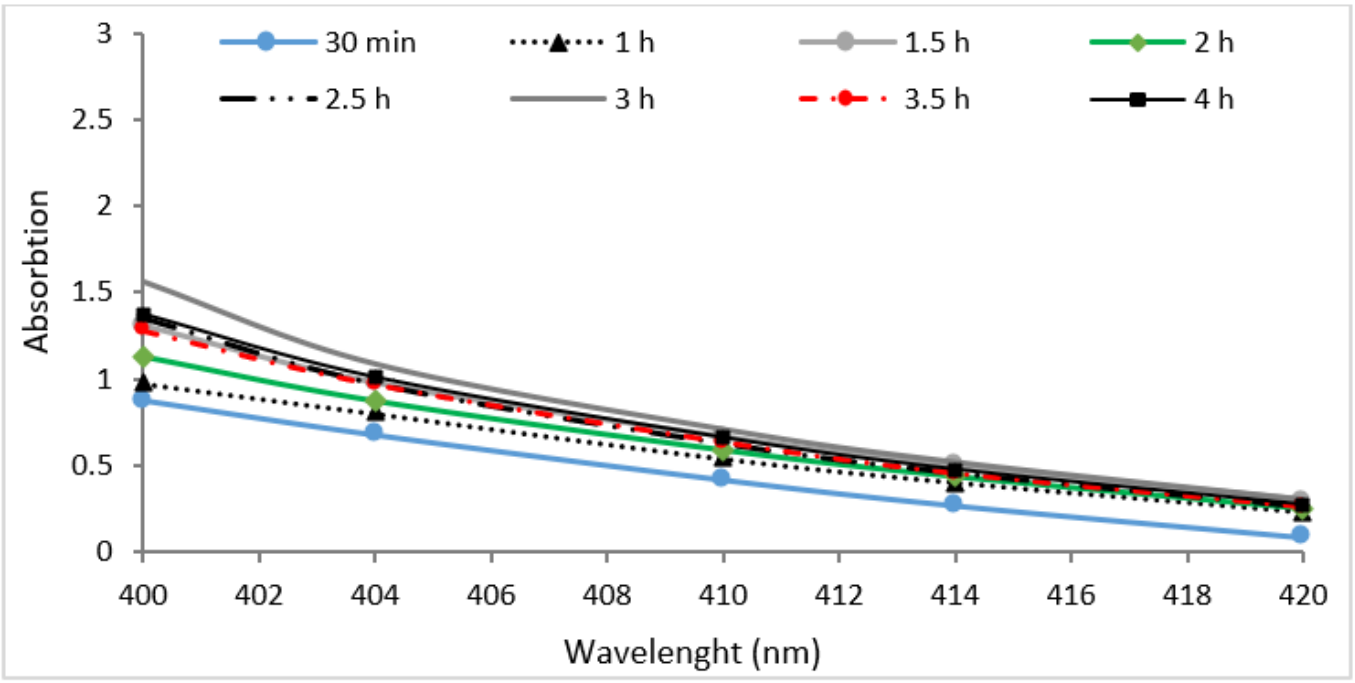

Figure 3

UV-Vis spectrum for optimization of reaction time

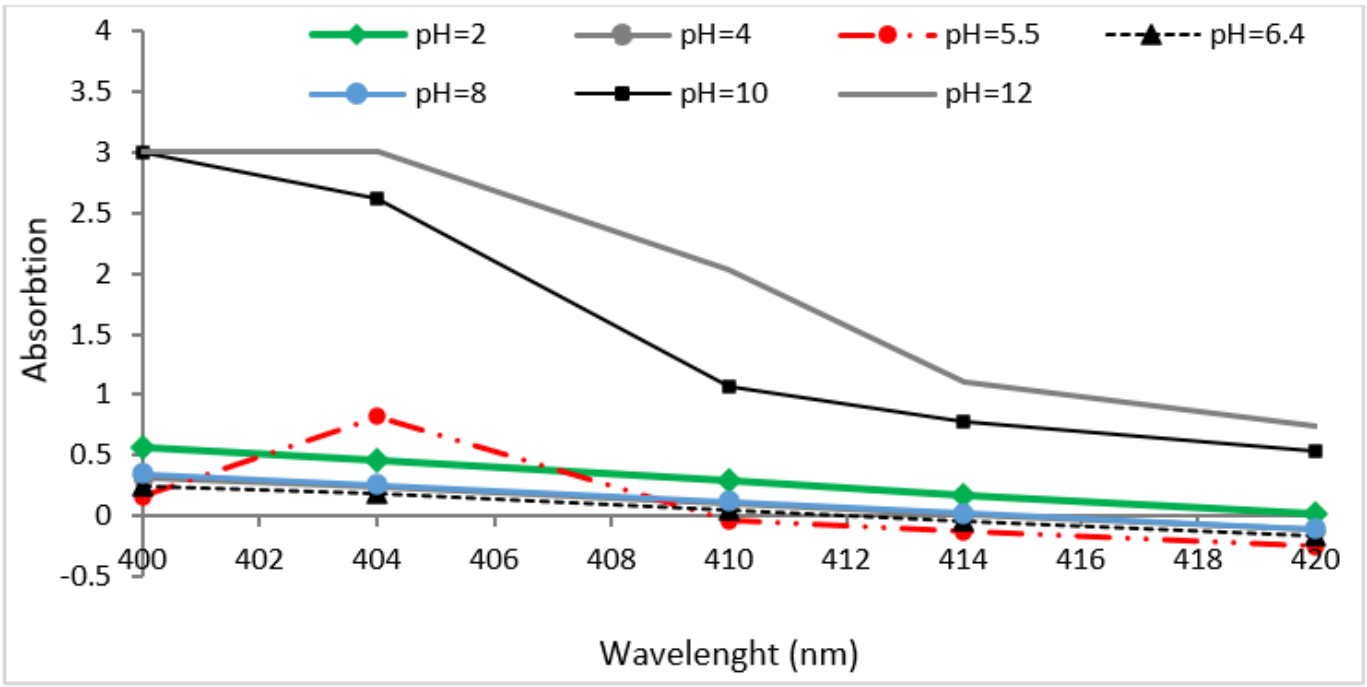

Figure 4

UV-Vis spectrum for optimization of pH

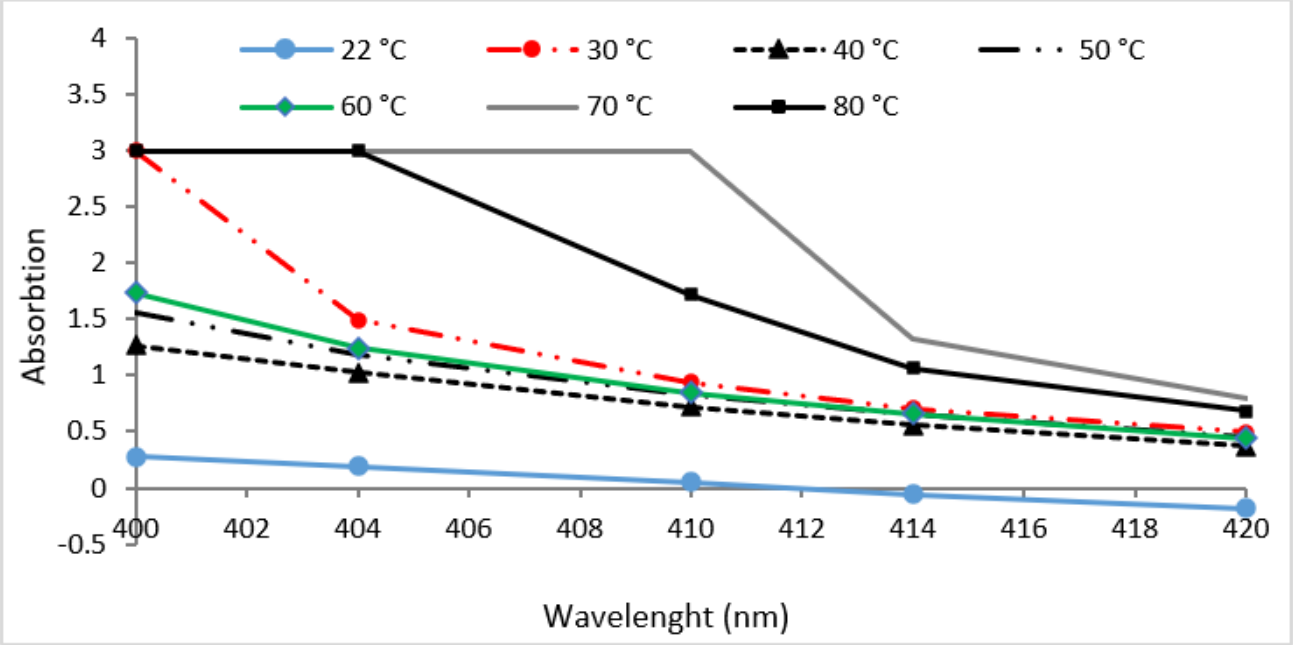

Figure 5

UV-Vis spectrum for optimization of solution temperature

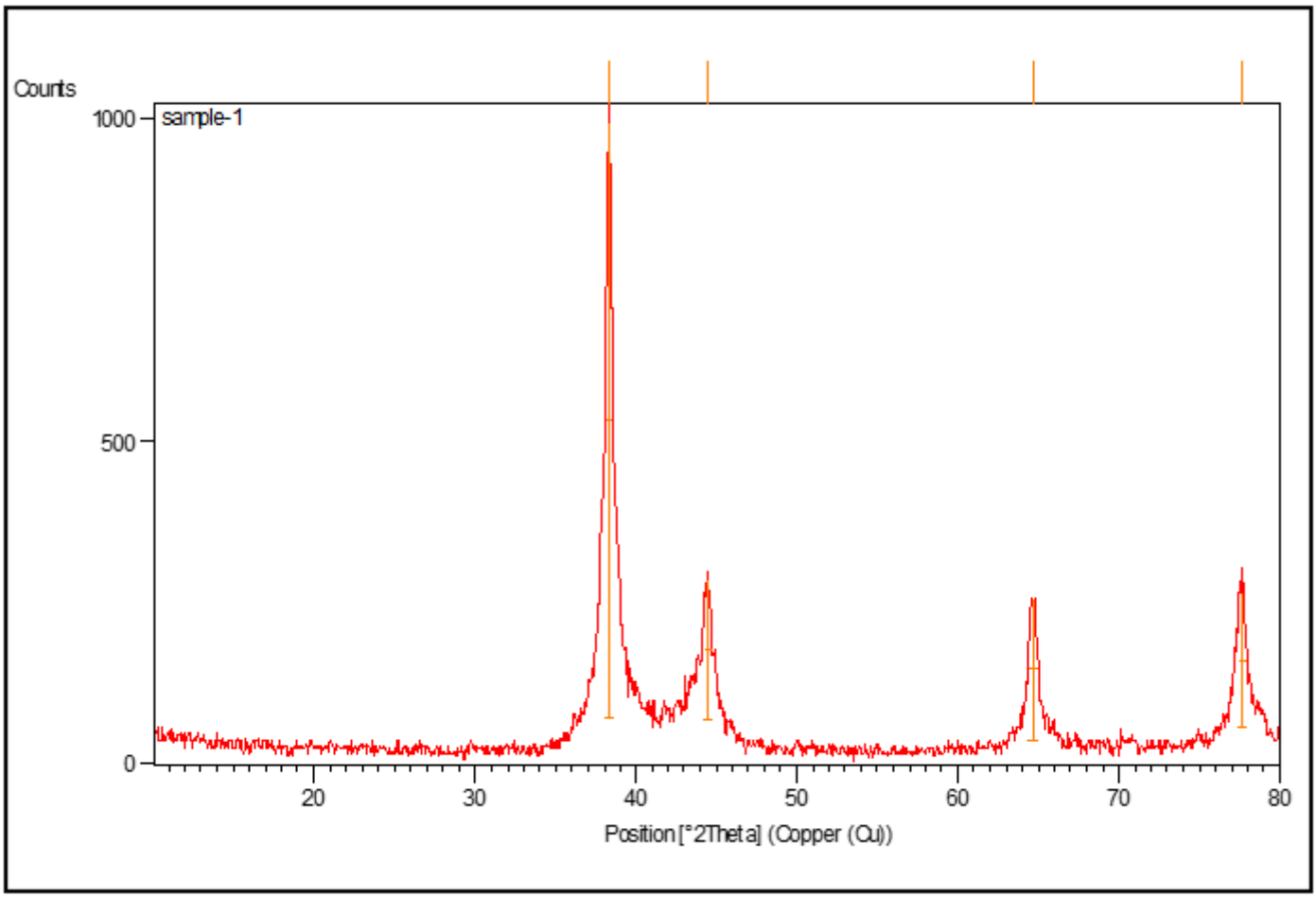

Figure 6

XRD pattern of Ag nanoparticles

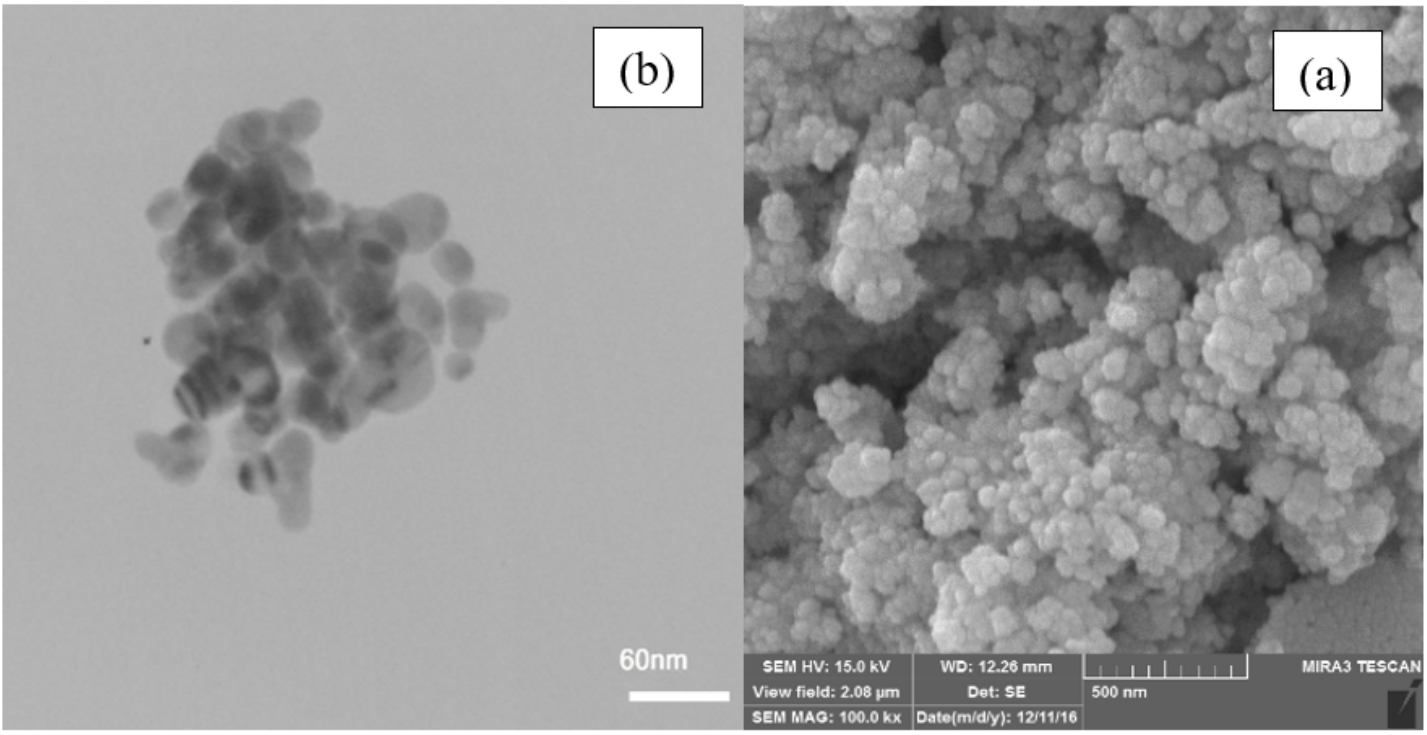

Figure 7

(a) SEM and (b) TEM images of silver nanoparticles

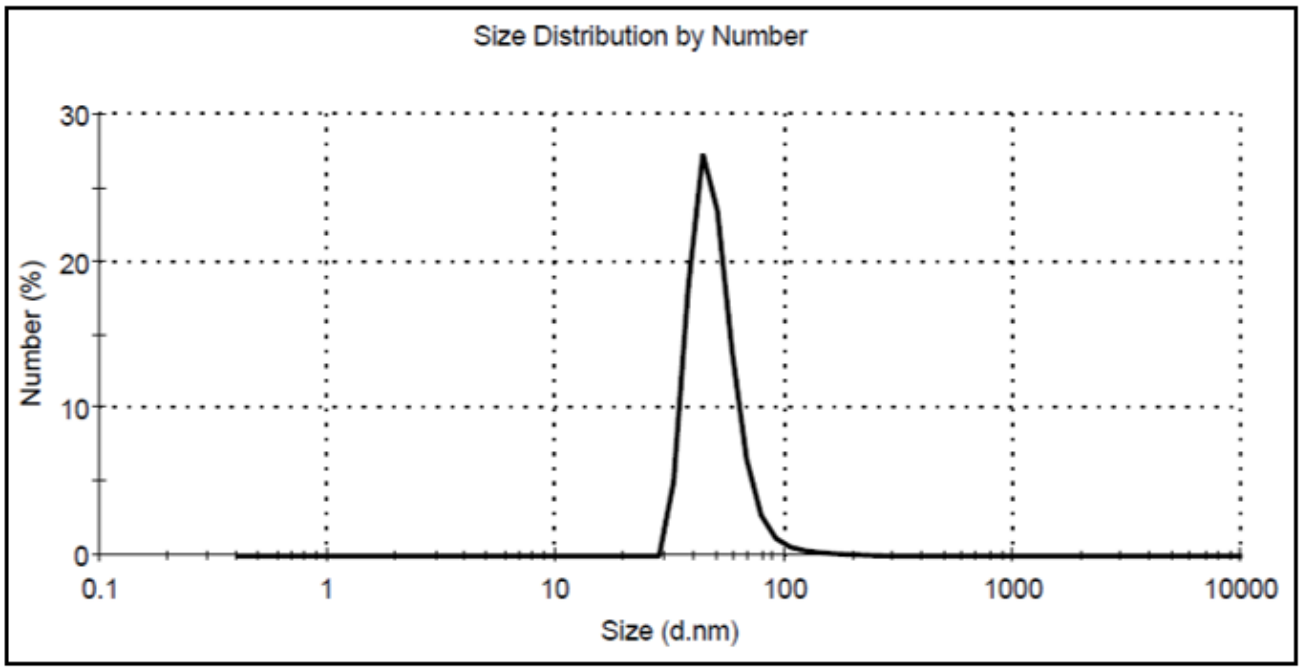

Figure 8

DLS spectrum of the silver nanoparticles