Zahra Abbasi, Sholeh Feizi, Elham Taghipour and Parinaz Ghadam*

Green synthesis of silver nanoparticles using aqueous extract of dried *Juglans regia* green husk and examination of its biological properties

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Abstract: Silver nanoparticles (AgNPs) have widespread applications. Recently, the synthesis of NPs using plant extract has attracted much attention. In this study, with an easy and rapid process at room temperature, AgNPs were produced by the aqueous extract of dried *Juglans regia* green husk, which is considered as a waste. The first sign of AgNPs synthesis was the color change of the sample from light green to yellowish brown. In addition, the production of AgNPs was examined by UV-Vis spectroscopy, dynamic light scattering (DLS), energy dispersive X-ray (EDX), Fourier transform infrared (FT-IR), X-ray diffraction (XRD) and transmission electron microscopy (TEM) analysis. The antibacterial effect of AgNP on *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus* and anticoagulation activity were determined. Synthesized silver colloid had electromagnetic absorbance at approximately 450 nm. The hydrodynamic diameter of monodisperse AgNPs was approximately 67 nm. The face center cubic metallic silver, which was spherical in shape, was in the range 3–50 nm with the average size of 7 nm. The extract showed antioxidant activity against 1,1-diphenyl-2-picryl hydrazyl (DPPH). Phenolic compounds, tannins, flavonoids, reducing sugars, proteins, ascorbic acid and carotenoids existing in the extract acted as reducing agents. AgNPs may be capped by biomolecules having O-H, C=O, N-H and other stabilizing functional groups.

Keywords: antibacterial activity; anticoagulation activity; antioxidant activity; *Juglans regia*; silver nanoparticles.

***Corresponding author: Parinaz Ghadam,** Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran, e-mail: pghadam@alzahra.ac.ir
Zahra Abbasi and Sholeh Feizi: Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran
Elham Taghipour: Department of Botany, Faculty of Biological Sciences, Alzahra University, Tehran, Iran

1 Introduction

Recently, many advances in the field of nanoparticle (NP) synthesis from various materials and strict controls on their size, composition and uniformity have been conducted [1–3]. The biological production of NPs has considerable advantages such as high efficiency, ease of NPs production, use of waste material, for instance skin of fruits and vegetables for production, cost-effectiveness and eco-friendliness [4].

NPs have high surface to volume ratio, active multicentral surface and high surface interactions [2]. These properties cause NPs, especially silver, to have a catalytic effect, electrical conductivity in ceramics and magnetic nanocomposites, an effect on increasing permeability of biological fences (membranes and blood-brain barrier) anticancer activity [5], antioxidant [6] and antibacterial effects [3].

In this study, AgNPs were produced by dried *Juglans regia* green husk aqueous extract. At first, AgNPs production was examined via visual changes, then, it was analyzed with the help of visible spectroscopy, dynamic light scattering (DLS), energy dispersive X-ray (EDX), Fourier transform infrared (FT-IR), X-ray diffraction (XRD) and transmission electron microscopy (TEM). Reducing biomolecules and antioxidant activity of extract was determined. Finally, the effect of AgNPs on *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus* by the micro-broth dilution method and its coagulation was examined.

2 Materials and methods

2.1 Biosynthesis of AgNPs by aqueous extract of dried *J. regia* husk

Green husk of *J. regia* was collected from Karaj Gardens (Karaj, Iran) and the mud was removed by soap and tap water. It was then washed three times with distilled water and air-dried in the shade. Some 58 ml of boiling distilled water was added to 1.6 g of dried husk powder of *J. regia* in a Meyer flask and its lid was closed by parafilm

and aluminum foil. After that, it was placed in a 100°C water bath for 10 min. The extract was filtered by Whatman paper no. 1. Finally, different volumes of extract including 30 µl, 60 µl, 90 µl, 120 µl and 150 µl were added to 10 ml $AgNO_3$ (Merck) solution separately.

2.2 Visible spectrum analysis

Optical properties of obtained silver colloid were recorded by a SPEKOL 2000 spectrophotometer in the range of 400–700 nm at a resolution of 5 nm.

2.3 DLS analysis

Hydrodynamic diameter, polydispersity index and distribution of AgNPs hydrodynamic diameter were examined by a NanoPhox 90-246V instrument (Sympatec GmbH, Clausthal-Zellerfeld, Germany).

2.4 EDX analysis

In order to confirm the presence of silver atoms in the reaction colloid, EDX analysis was operated using a VEGA3 TESCAN instrument on freeze-dried silver colloid.

2.5 TEM

The shape and size of dried silver NPs (AgNP) were determined using Philips CM30. In addition, the distribution of AgNPs size was calculated using Digimizer software version 4.1.1.0.

2.6 XRD analysis

XRD analysis was done to determine the presence and crystal structure of AgNPs. The colloidal silver was freeze-dried and analyzed by Rigaka (40 kV, 40 mA, step size = 0.02°).

2.7 FT-IR analysis

To examine the biomolecules that may reduce and cap the AgNPs, FT-IR analysis was operated on both freeze-dried extract and silver colloid by a TENSOR 27 spectrophotometer in the range of 400–4000 cm^{-1}.

2.8 Determination of reducing biomolecules

To investigate the effective biomolecules that play a role in biosynthesis of AgNPs, phenolic compounds, tannins, flavonoids, total sugars, reducing sugars, ascorbic acid, total chlorophyll, and protein content were inspected by the Folin-Ciocalteau colorimetric method for both phenolic compounds [7] and tannins [8], aluminum chloride colorimetric assay [9], phenol-sulfuric acid method [10], Somogy method [11], method given by Sadasivam and Manickam [12, 13] for both ascorbic acid and total chlorophyll, Bradford method [14], respectively. Finally, equations of Lichtenthaler and Wellburn were used to determine concentration of carotenoids [15].

2.9 Determination of free radical-scavenging activity of extract using the 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay

The DPPH assay was performed for antioxidant activity determination of extract. Absolute ethanol was added to different volumes of extract (400-600) to final volume of 1 ml. Then, 2 ml of DPPH solution (0.1 mM) was added and well blended in. After keeping for about 1 h in a dark place at room temperature, the absorbance at 520 nm was recorded by a Milton Roy spectronic 601 spectrometer. Finally, the inhibition of DPPH free radicals was computed by Eq. (1).

$$EC50 = \left(\frac{A_{control} - A_{sample}}{A_{control}} \right) \times 100 \quad (1)$$

where $A_{control}$ is the absorbance of DPPH solution (0.1 mM) at 520 nm. Absolute methanol was used as the blank [16].

2.10 Antibacterial activity of AgNPs

Antimicrobial activity of silver colloid was surveyed on four different bacteria including *E. coli* and *B. subtilis* as Gram-negative and *P. aeruginosa* and *S. aureus* as Gram-positive bacteria. According to the micro-broth dilution method based on Cinical and Laboratory Standard Institutes [17], 100 µl fluid Nutrient broth culture was added to the first nine wells of the microtiter plate. Then, 200 µl silver colloid and dried husk of *J. regia* aqueous extract were added to the first well in different rows and diluted to the ninth well by transferring 100 µl of sample from the first well and removing 100 µl sample from the ninth well, respectively. Some 100 µl of different bacterial suspensions with 0.5 Mcfarland concentration diluted to 0.01 were added to the first nine wells. The last three wells were the control wells that contained 100 µl sample +100 µl fluid Nutrient broth culture as sample control, 200 µl fluid Nutrient broth culture as culture control and 100 µl bacteria +100 µl fluid Nutrient broth culture as bacterial control, respectively. Microtiter plates were kept in an incubator at 37°C for 24 h. The lowest concentration of AgNPs that act as bacterial growth inhibitors is considered the minimum inhibition concentration (MIC) of AgNPs.

2.11 Anticoagulation activity of AgNPs

Blood samples were collected from Alzahra university students (25–27 years). AgNP colloid was serial diluted in different glass tubes. The blank was plant extract. Some 0.5 ml of blood sample was added to each tube and shaken [18].

3 Results and discussion

J. regia is a walnut tree species native to the region from the Balkans to China. It has a green husk as a waste material which contains a great amount of antioxidants. Therefore, in this research, the aqueous extract of dried green husk was chosen for AgNP synthesis.

3.1 Characterization of AgNPs

The reduction of Ag+ to AgNP changed the color of the sample to yellowish brown due to surface plasmon resonance (SPR) excitation in metal NPs [19]. In general, the mixture of $AgNO_3$ and plant extract can synthesize the NPs, but there are some reports that show that extra energy like sunlight is necessary for synthesis [6]. In this study, the addition of a silver nitrate solution to an aqueous extract of dried *J. regia* green husk immediately changed the color of solution to brown (Figure 1A) which revealed that AgNP synthesis had occurred.

Also, the oscillation of silver electrons (SPR) causes AgNPs to absorb electromagnetic wavelengths at the range of 380–460 nm [19]. The visible spectrum of synthesized AgNPs had the SPR peak at approximately 440 nm, which indicated the creation of AgNPs, so that 150 µl of extract had the most absorbance (Figure 1B). The shoulder of the SPR peak shows a little polydispersity in the particles [19]. For more analysis about the shape and distribution of particles, DLS analysis was done and it was recognized that the hydrodynamic diameter of 90% of them was less than 69.09 nm. The amount of volume mean diameter/surface mean diameter was about 1 which confirmed AgNPs were spherical. The amount of Sv = 89.48 m^2/cm^3 showed that in 1 cm^3 of the silver colloid, the surface of AgNPs is 89.48 m^2 therefore, the size of AgNPs is small. Based on the amount of X90-X10 (3.87) and polydispersity index (1.88), the AgNPs were actually monodisperse (Figure 2). DLS showed the hydrodynamic diameter, but the exact size, distribution of size and shape of AgNPs should be determined using TEM images. It showed that the AgNPs were spherical in shape, the average size of them was 7 nm and AgNPs were distributed between 3 nm and 50 nm (Figure 3).

In order to determine the presence of Ag, the freeze-dried AgNPs created by green husk of *J. regia* extract were analyzed with an EDX spectrum. The peaks of Ag including AgLα and AgLβ confirmed the presence of Ag atoms. Other peaks were related to elements, which belong to the extracted biomolecules (Figure 4).

The XRD pattern of AgNPs in Figure 5 indicated that AgNPs had crystalline structure. The peaks at 38.117°, 44.279°, 64.428° and 77.475° can be attributed to the (111), (200), (220) and (311) Miller indices, respectively, which reflected the face center cubic structure of metallic silver [20, 21].

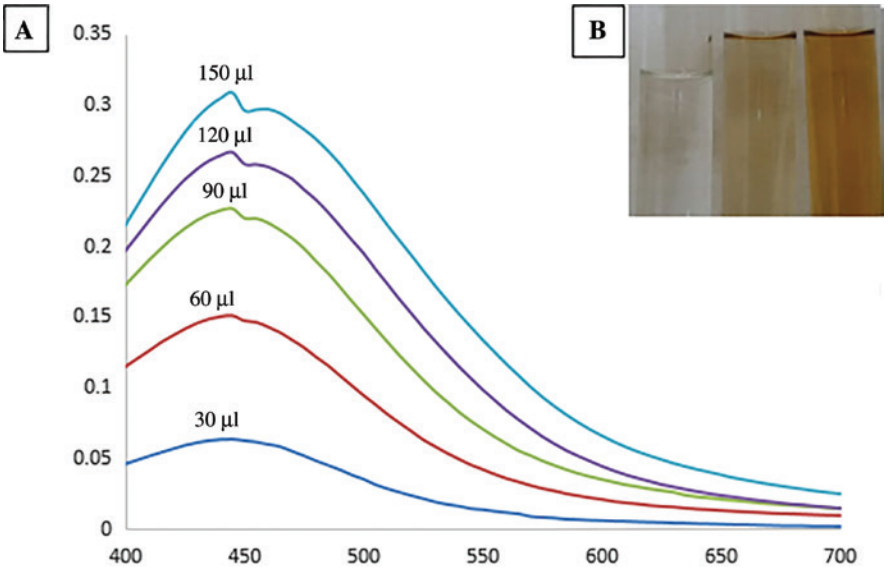

Figure 1: (A) Visible spectrum of silver nanoparticles colloid with increasing concentration of extract (2 h after beginning of the reaction). (B) The color change of extract (the left tube is $AgNO_3$ solution, the middle one is plant extract and the right one is silver nanoparticles colloid).

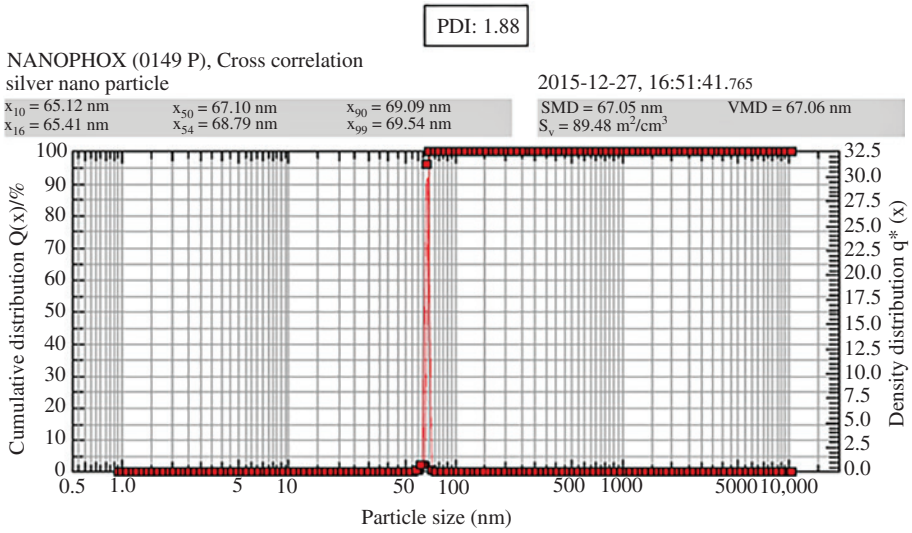

Figure 2: Dynamic light scattering (DLS) spectrum of silver nanoparticles colloid.

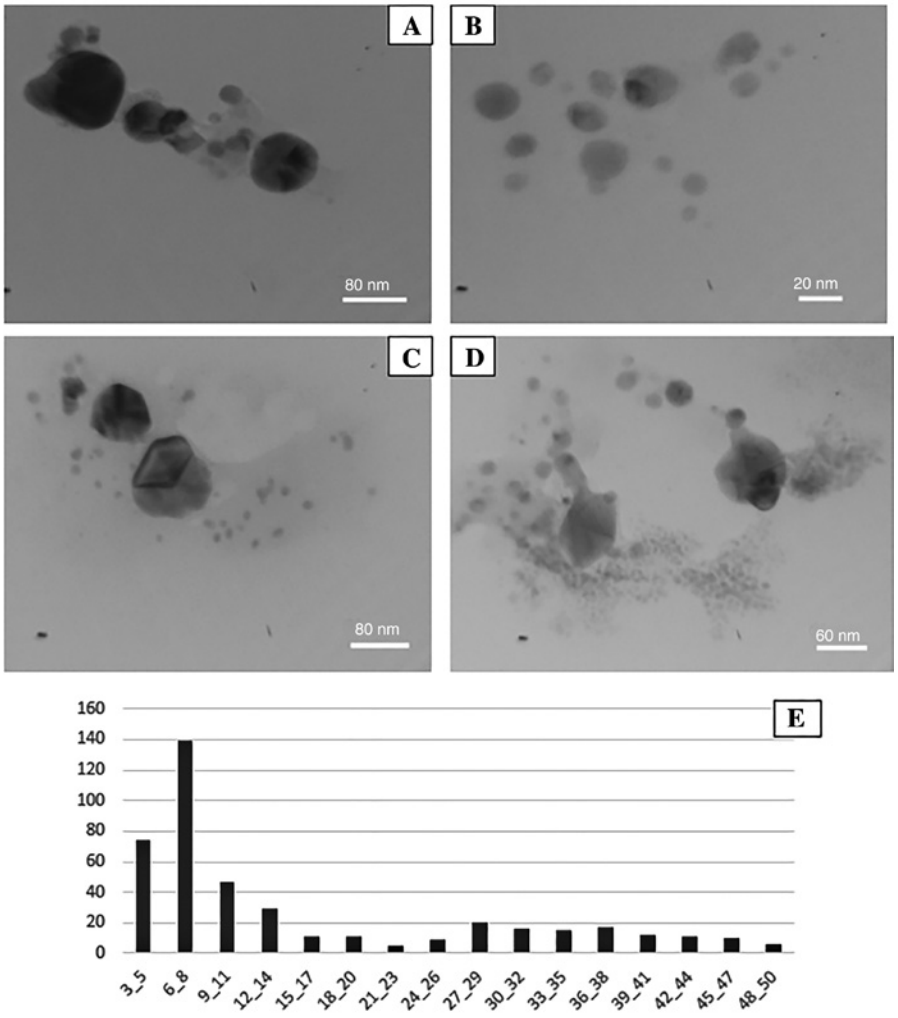

Figure 3: (A–D) The transmission electron microscopy (TEM) images of silver nanoparticles (AgNPs). (E) Histogram of particle size distribution of AgNPs.

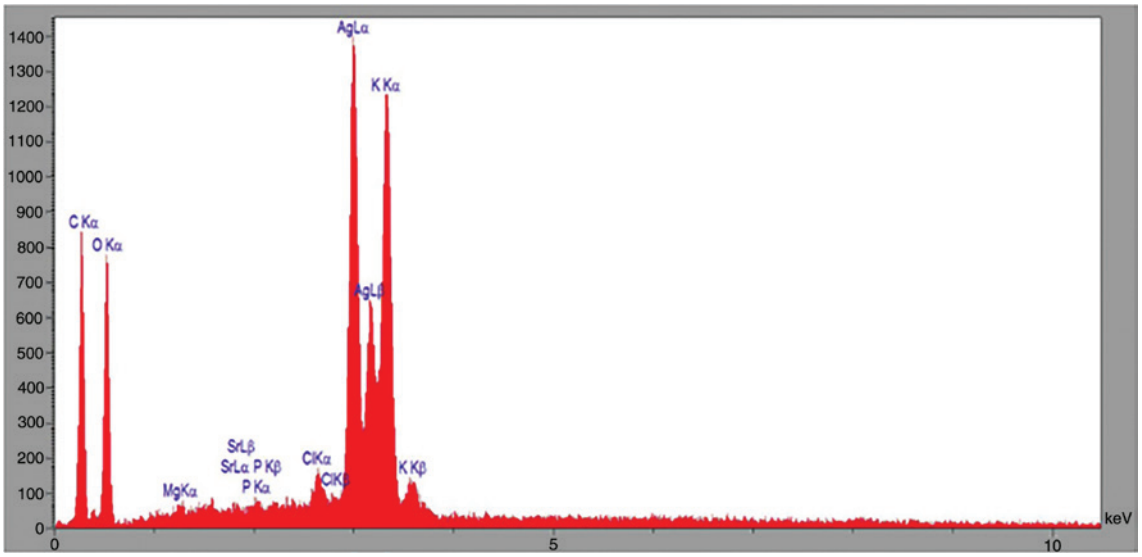

Figure 4: Energy dispersive X-ray (EDX) spectrum of silver nanoparticles colloid.

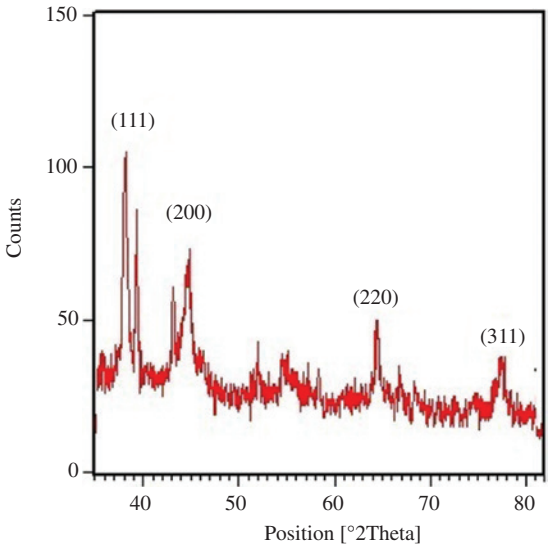

Figure 5: X-ray diffraction (XRD) pattern of silver nanoparticles (AgNPs) obtained by green husk of *Juglans regia* extract.

The surface properties of synthesized NPs were shown by FT-IR analysis. This contributed to determining probable molecules that act as capping and reducing agents. As indicated in Figure 6, the FT-IR spectra of the freeze-dried extract of *J. regia* dried green husk and freeze-dried synthesized AgNPs were similar to each other and changes were observed in the peaks magnitude and numbers (Figure 6). The distinct peaks at 3410 cm^{-1} and 3407 cm^{-1} may be attributed to the OH functional groups of alcohols and phenolic compounds [20]. The recorded peaks at 2923 cm^{-1} and 2924 cm^{-1} could correspond to the C-H stretching of alkanes [22]. The peak at 1704 cm^{-1} existing in AgNPs spectrum was probably related to C=O stretching [23]. Two obvious peaks at 1561 cm^{-1} and 1598 cm^{-1} may be associated with C=C stretching vibration and C=O stretching frequency, respectively [24, 25]. The marked peaks at 1366 cm^{-1} and 1398 cm^{-1} could be due to C-H deformation and binding of AgNPs with hydroxyl and carboxylate groups of proteins residues, respectively [26, 27]. One peak at 1051 cm^{-1} existing in extract spectrum was divided into two peaks including 1079 cm^{-1} and 1046 cm^{-1} in AgNPs spectrum. They may be, respectively attributed to C-stretching of ether groups [28], N-C bond stretching of aliphatic amine groups [20] and C-O vibrational frequency [29]. The weak peak at 926 cm^{-1} in the extract spectrum was probably due to the C-H stretching of alkene groups [28]. One peak at 875 cm^{-1} existing in extract spectrum was divided into two 869 cm^{-1} and 831 cm^{-1} peaks in the AgNPs spectrum. The 875 cm^{-1} peak can be related to the stretching vibration of β-type of glycosidic linkages (carbohydrate) [30] and the 831 cm^{-1} peak may be correspond to the in-plane and out-plane bending for the benzene ring [31]. The weak peaks including 776 cm^{-1} and 672 cm^{-1} in the spectrum of AgNPs and 773 cm^{-1} and 671 cm^{-1} in the spectrum of extract were probably due to the bending vibration of N-H [32]. Furthermore, the weak peaks existing in 635 cm^{-1} and 595 cm^{-1} in the spectrum of extract and 596 cm^{-1} in the spectrum of AgNPs can be assigned to the CH bend of alkynes [26]. As a conclusion, we could mention that any of the biomolecules containing these bonds can act as a reducing or capping agent.

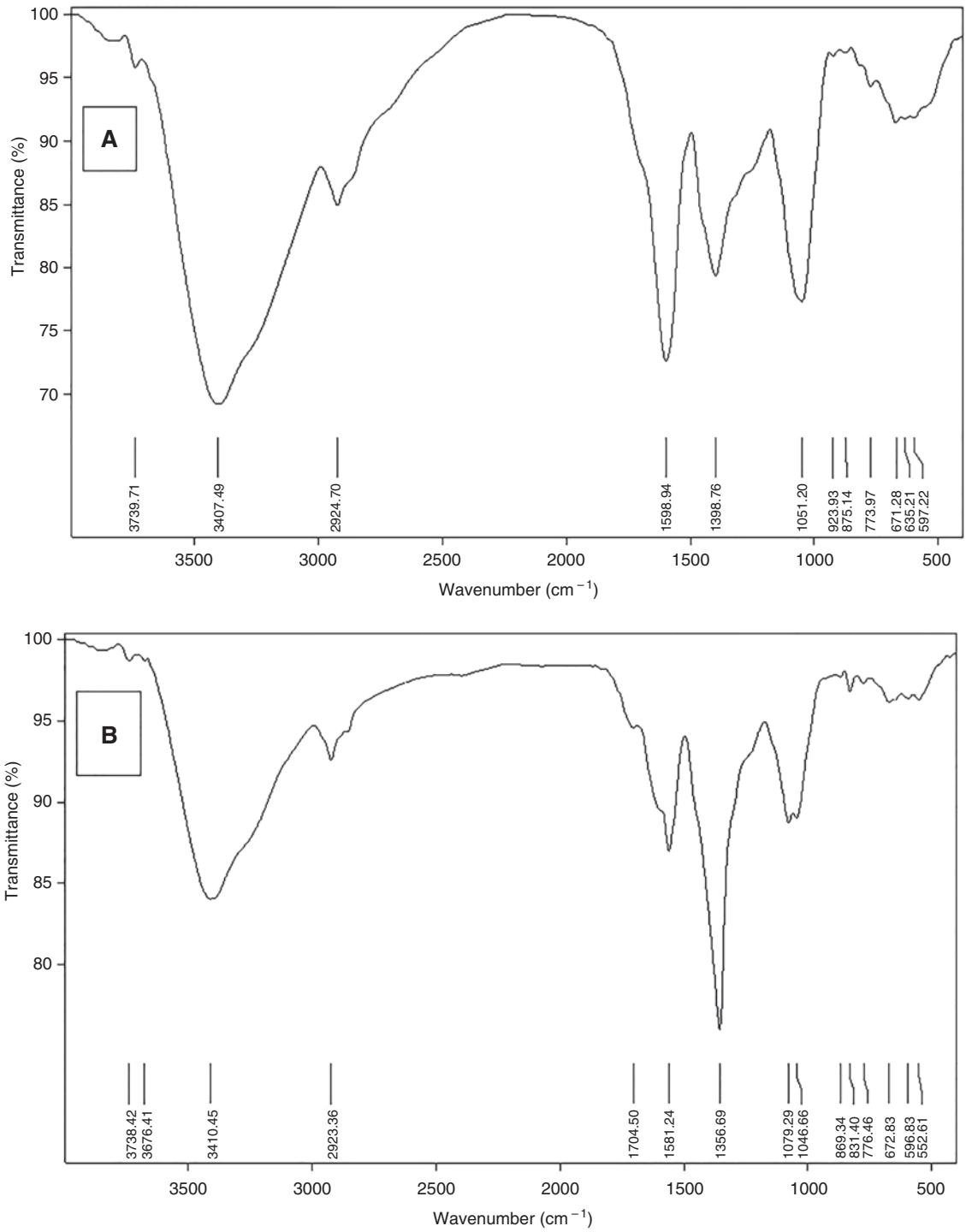

Figure 6: (A) Fourier transform infrared (FT-IR) spectrum of extract. (B) FT-IR spectrum of silver nanoparticles (AgNPs).

3.2 Reducing biomolecules and antioxidant activity of extract

In order to determine reducing agents, the amount of dried *J. regia* green husk aqueous extract of biomolecules was estimated. As shown in Table 1, phenolic compounds, tannins, flavonoids, total sugar, reducing sugars, proteins, ascorbic acid and carotenoids can probably be the reducing and capping agents of the extract involved in the biosynthesis and covering of AgNPs.

The DPPH test was carried out to determine the dried *J. regia* green husk aqueous extract antioxidant activity.

Table 1: Amount of effective biomolecules for biosynthesis of silver nanoparticles (AgNPs).

Substance	Concentration (µg/ml)
Phenolic compounds	8657.4
Tannins	1222.2
Flavonoids	24.6
Total sugar	55
Reducing sugars	2.7
Proteins	11.6
Ascorbic acid	14.2
Carotenoids	0.115
Chlorophylls	0

DPPH free radical is purple when dissolved in methanol, which has absorbance at approximately 520 nm. While they receive an electron from any substance that can give them an electron, like antioxidants, its (DPPH) color changes from purple to yellow. Thus, the absorbance at 520 nm will be reduced. The more the percentage of antioxidant activity, the more DPPH free radicals will change to DPPH molecules [33]. Based on the result, 510 µl of effective substance (150 µl extract +10 ml water) for the synthesis of AgNPs could inhibit 50% of DPPH free radicals activity.

3.3 Antibacterial activity of AgNPs

According to research, AgNPs depending on size and environmental conditions (pH, ionic strength) and capping agents have dose-dependent antibacterial activity, which was examined against both Gram-negative and Gram-positive bacteria [34]. Generally, the antibacterial activity of AgNPs is due to Ag+ or Ag atoms. Their mechanisms include electrostatic attraction between Ag+ releasing from AgNPs in the presence of moisture and any part of bacterial cells with negative charge, like DNA and proteins, as well as Ag atoms binding to proteins. Unlike other reports which confirm more resistance of Gram-positive bacteria to AgNPs rather than Gram-negative ones due to their cell wall structure [35], it was shown that AgNPs synthesized by dried *J. regia* green husk aqueous extract had the same effect on both Gram-negative and Gram-positive bacteria (Figure 7) (Table 2).

3.4 Anticoagulation effect of AgNPs

The anticoagulation mechanism of AgNPs is probably due to the inhibition of integrin-mediated platelet functional responses to immobilize fibrinogen. The purpose of AgNPs anticoagulation activity is its application for modern medical science and humans. AgNPs produced by biological methods are good choices as anticoagulants, since they are compatible with the environment and human metabolism and the production of harmless byproducts in comparison with chemical methods [18]. As shown in Figure 8, dried *J. regia* green husk aqueous extract did not, but AgNPs did have an anticoagulation effect. It was also observed that the greater concentration of AgNPs had greater anticoagulation effect. The anticoagulation effect of AgNPs was followed until 72 h.

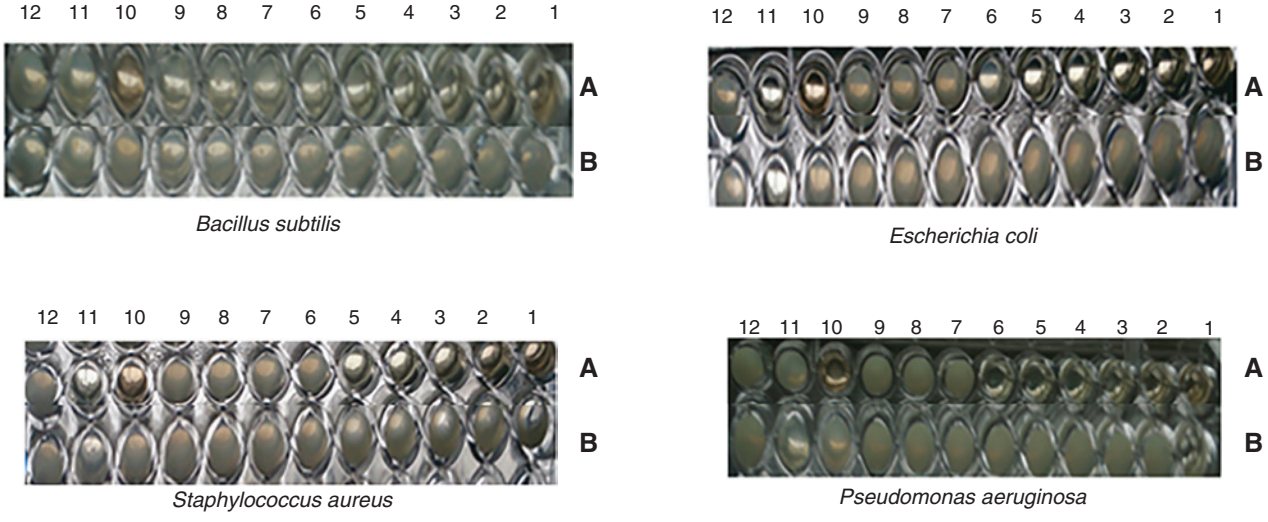

Figure 7: Antibacterial study of silver nanoparticles (AgNPs) on *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* by micro-broth dilution method. (A) Sample, (B) control. Columns 1–9 show the serial dilution.

Table 2: Minimal inhibitory concentration of silver nanoparticles (AgNPs) and plant leaf extract were determined by micro-broth dilution method (growth of bacteria [+] and the inhibition of bacterial growth [–]).

AgNPs volume (µl)	Leave extract volume (µl)	Gram negative bacteria				Gram positive bacteria			
		Escherichia coli		*Pseudomonas aeruginosa*		*Bacillus subtilis*		*Staphylococcus aureus*	
200	200	–	+	–	+	–	+	–	+
100	100	–	+	–	+	–	+	–	+
50	50	–	+	–	+	–	+	–	+
25	25	–	+	–	+	–	+	–	+
12.5	12.5	–	+	–	+	–	+	–	+
6.25	6.25	+	+	–	+	+	+	+	+
3.125	3.125	+	+	+	+	+	+	+	+
1.5625	1.5625	+	+	+	+	+	+	+	+

Figure 8: Anticoagulation effect of silver nanoparticles (AgNPs). Left tube: blood + extract; right tubes: blood + AgNP (with increasing of AgNP dilution).

4 Conclusions

In this study, biocompatible AgNPs were produced through a green, eco-friendly, easy, rapid and cost-effective process using the aqueous extract of dried *J. regia* green husk, considered as a waste, at room temperature. Previously, Baghkheirati et al. [36] produced AgCl-NPs using the ethanolic extract of dried *J. regia* green husk, but we produced AgNPs by aqueous extraction, with an easier, more cost-effective and less time-consuming process. The extract contained many antioxidants including phenolic compounds, tannins, flavonoids, total sugars, reducing sugars, proteins, ascorbic acid and carotenoids, which can play a reducing role to convert Ag+ to Ag0. Moreover, they may act as capping agents and stabilize AgNPs. The 510 µl of extract (150 µl extract + 10 ml water) could inhibit 50% of DPPH free radicals activity. The color change of the extract and the appearance of electromagnetic absorbance at 440 nm at the beginning of the reaction indicated the powerful ability of the extract to produce AgNPs. Based on the DLS result, the hydrodynamic diameter of AgNPs was approximately 69 nm and they were monodisperse. According to TEM images, AgNPs were spherical in shape and distributed in the range 3–50 nm with the average size of 7 nm. XRD determined the presence of face center cubic in the structure of metallic silver. The presence of silver atoms was confirmed by EDX analysis. It was also observed that synthesized silver colloid had the same antibacterial effect on both Gram-negative and Gram-positive bacteria, including *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*. The rate, simplicity, potent anticoagulation effect and wide range of antibacterial activity of synthesized AgNPs with aqueous extract of dried *J. regia* green husk make it an excellent candidate for nanobiotechnology usages.

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Bionotes

Zahra Abbasi

Zahra Abbasi obtained a MS degree in Biochemistry from Alzahra University and an BS degree in Microbiology from Azad University.

Sholeh Feizi

Sholeh Feizi obtained a MS degree in Biochemistry from Alzahra University, and an BS degree in Zoology from Shahid Beheshti University.

Elham Taghipour

Elham Taghipour obtained her BS and MS degrees in Botany from Alzahra University. Currently, she is a PhD student in Plant Biotechnology at the National Institute of Genetic Engineering and Biotechnology.

Parinaz Ghadam

Parinaz Ghadam is an associate professor at the Department of Biotechnology, Alzahra University, Tehran, Iran.