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Green synthesis of silver nanoparticles using aqueous extract of saffron (*Crocus sativus* L.) wastages and its antibacterial activity against six bacteria

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ABSTRACT

Objective: To synthesis silver nanoparticles (AgNPs) by using extract of saffron (*Crocus sativus* L.) wastages and to test their antibacterial activity against six bacteria.

Methods: In this paper, the synthesis of AgNPs using aqueous extract of saffron wastage as a green method without any chemical stabilizer and reducer is demonstrated. The synthesized AgNPs were determined by UV–vis spectrum, high resolution transmission electron microscopy, X-ray diffraction, and Fourier transmission infrared spectroscopy analysis.

Results: UV–vis spectrum showed a peak at 450 nm due to excitation of surface plasmon vibrations. Fourier transmission infrared spectroscopy showed that nanoparticles were capped with plant secondary metabolites. X-ray diffraction analysis also demonstrated that the size range of the synthesized nanoparticles was 12–20 nm. Transmission electron microscope image illustrated AgNPs with spherical shape and an average size of 15 nm. The result of antibacterial activities showed that the biosynthesized AgNPs had an inhibiting activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Shigella flexneri* and *Bacillus subtilis*.

Conclusions: The biosynthesized AgNPs showed significant antibacterial effect against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Shigella flexneri* and *Bacillus subtilis*, so, it can be used in biomedical applications.

1. Introduction

Nanotechnology is a new form of technology which has produced a great development in various fields. Due to the unique features and applications of the nanoparticles, they are very useful especially in the field of biotechnology, medical imaging and catalysts. Silver nanoparticles (AgNPs) are among the most widely used nanoparticles that show a broad spectrum of antibacterial activity. Studies have shown that silver nanoparticles prevent replication of the AIDS virus (HIV) and their impact is much greater than that of gold nanoparticles [1]. AgNPs are used in the treatment of wounds. In general, injuries such as cuts, abrasions, burns, warts, fungal diseases, and other skin diseases can be treated with AgNPs [2,3].

Over the past few decades, the use of plants with different applications in medicine and industry has been growing increasingly in the world. In recent years, many environmentally friendly methods have been employed in the synthesis of nanoparticles [4]. The biological methods for AgNPs synthesis using bacteria, fungi, proteins, polypeptides, nucleic acids and plant extracts are simple, nontoxic, affordable and environmentally friendly. These biological methods can be used to generate nanoparticles with acceptable size and morphology [5,6]. There are numerous reports on the synthesis of AgNPs by using plant extract. The reports show that these methods are simpler and nontoxic when compared with physicochemical methods. Synthesis of AgNPs from leaf extract of *Prosopis juliflora* is an example of the biological methods [7]. The synthesis mediated by *Ficus carica* leaf extract is also reported [8,9].

Pourmortazavi *et al.* [10] described the green synthesis of AgNPs by aqueous extract of *Eucalyptus oleosa*. Ramesh *et al.*

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[11], Mohammed [12] and Ajitha *et al*. [13] synthesized AgNPs using fruit extract of *Emblica officinalis*. Green synthesis of AgNPs mediated by *Eucalyptus camaldulensis* and *Lantana camara* leaf extract was also announced. Suman *et al*. [14] reported biosynthesis of AgNPs using *Morinda citrifolia* root extract. Synthesis of antimicrobial AgNPs by callus and leaf extracts from saltmarsh plant, *Sesuvium portulacastrum* L. was reported by Nabikhan *et al*. [15].

Crocus sativus L. (*C. sativus*), also named saffron, is one of the most valuable medicinal plants worldwide. The flowers of *C. sativus* are a combination of six petals, three stamens, and three red stigmas [16]. The only usable part of the plant is stigma and other parts are discarded as wastage. In Iran, there are 70 thousand hectares of saffron farms. South Khorasan Province is the most important region of saffron production. After separation of stigmas (saffron), its petals containing valuable pigments called anthocyanins are thrown away. The color of saffron petals is caused by the compounds called anthocyanins. Anthocyanins are a group of natural compounds and secondary metabolites belonging to the flavonoid family [17,18]. Several compounds are identified in saffron petals. Flavonols such as kaempferol, quercetin, isorhamnetin and anthocyanins such as delphinidin, petunidin and malvidin are isolated from saffron petals [19].

For many years, AgNPs have been well-known for their antimicrobial activity against various groups of bacteria [5,20]. The antibacterial specifications of the produced AgNPs are illustrated by directly exposing bacteria to AgNPs [21]. Researchers reported the biological activity of AgNPs synthesized by using banana extracts. These nanoparticles showed antifungal activity towards *Candida albicans* and *Candida lipolytica* and antibacterial activity towards *Escherichia coli* (*E. coli*), *Enterobacter aerogenes*, *Klebsiella* sp. and *Shigella* sp. [22]. By using carob leaf extract [23], synthesized AgNPs showed an effective antibacterial activity towards *E. coli* pathogen.

Nanoparticles formed from commercially available plant powders showed antibacterial activity against *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *E. coli* and *Klebsiella pneumonia* (*K. pneumonia*) [24]. Ghaffari-Moghaddam and Hadi-Dabanlou carried out synthesis of AgNPs mediated by *Crataegus douglasii* fruit extract [25]. These nanoparticles showed antibacterial activity against *S. aureus* and *E. coli*. Extracts of *Skimmia laureola* leaf showed maximum growth inhibition effect against *K. pneumonia* and *P. aeruginosa* [26]. Previous results indicated that AgNPs formed from aqueous solution of *Azadirachta indica* L. could be used as a promising antibacterial agent against clinical pathogens [27]. The antibacterial activity of synthesized AgNPs with aqueous solution of *Trichoderma harzianum* was evaluated against *S. aureus* and *K. pneumonia* by Ahluwalia *et al*. [28].

In this study for the first time, we've reported the biosynthesis of AgNPs via reduction of silver ions using wastage of saffron extract, and we have studied the antibacterial activity of these biosynthesized AgNPs against six bacteria.

2. Materials and methods

2.1. Materials

Silver nitrate ($AgNO_3$), AgNPs (with average size of 20 nm) and methanol were purchased from Merck Company. Glasswares were washed with deionized water and were put in oven at 160 °C for 2 h, plastic equipment was autoclaved for antibacterial assays before using. Wastage of saffron was collected in November from Birjand farms, South Khorasan, Iran. The samples were stored in a freezer at −15 °C for further studies.

2.2. Preparation of plant extract

To prepare the aqueous wastage extract of *C. sativus*, the frozen sample was cut into small pieces and 12 g was weighed, put into a 250 mL flask. Deionized water (30 mL) was added and the solution was placed on a stirrer in a dark place at the room temperature for 24 h. The solution was separated by filtration with Whatman No.1 filter paper and then centrifuged at 5 500 r/min for 10 min. The upper phase was retained for synthesis of AgNPs.

2.3. Preparation of plant extract and silver nitrate solution for antibacterial assays

All methods for preparing extract are the same as in the previous section, but in order to remove germs, the centrifuged extract was filtered through 0.45 μm filter and then used for further experiment. For more precaution, the solution of silver nitrate was filtered through 0.45 μm filter. To estimate the antibacterial activity of aqueous extract of saffron wastage, the prepared extract was lyophilized and then used for antibacterial assays. In this study, we compare antibacterial properties of purchased silver nanoparticle, biosynthesized AgNPs, and aqueous extract of saffron wastage.

2.4. Synthesis of AgNPs by using the saffron wastage extract

Aqueous extract of saffron wastage was used for the synthesis of AgNPs. To this end, the effect of the amount of extract and the concentration of silver nitrate solution was investigated to obtain the optimum values. The best concentration of the extract was obtained by changing the amount of extract. Therefore, different amounts of 1–10 mL of the extract were added to different concentrations of silver nitrate solutions. Thus, the volume of 5 mL of extract and 2 mmol/L concentration of silver nitrate solution was selected as optimal. Then 5 mL of the extract was added to 20 mL of 2 mmol/L solution of silver nitrate drop by drop. The ultrasonic irradiation was applied to the mixed solution for 3 h. The colorless solution of silver nitrate changed to deep brown, indicating the formation of AgNPs. Evidence suggests that the change in color is due to the formation of AgNPs. After the synthesis of AgNPs, the solution containing nanoparticles was centrifuged at 8 000 r/min for 10 min to separate AgNPs from other composition of solution and the deposit was prepared for relevant analysis. The dried AgNPs were kept in microtube for further study.

2.5. Characterization of AgNPs

There are several ways for characterizing nanoparticles. The first and the most convenient way is the color change of solution. We used the UV–vis (Spectrophotometer UV–vis 2501 PC, Shimadzu, Kyoto, Japan) and Fourier transform infrared

spectroscopy (FTIR; Nicolet 800, Nicolet, Madison, USA) spectrums for determination of optical properties of AgNPs. X-ray diffraction (XRD; D8 ADVANCE, Bruker) operating at a voltage 35 kV and a current of 30 mA with $K_{\alpha 1}$ Cu radiation was used for the phase identification and characterization of crystalline metallic AgNPs. High resolution transmission electron microscopy (TEM; Zeiss EM 900, Jena, Germany, −80 kv) photographs were used for morphological characterization and the size distributions of AgNPs. The size of synthesized AgNPs was assessed by Debye–Scherrer equation:

$$D = 0.94\ \lambda/\beta\ \mathrm{Cos}\theta$$

where D is the average size of synthesized AgNPs, K is constant (K = 0.94), λ is the wavelength of X-ray (0.154 6 nm), β is the width of maximum peak at half of height and θ is diffraction angle (in degrees).

2.6. Antibacterial assays

The antibacterial activity of purchased AgNPs, biosynthesized AgNPs, and aqueous extract of saffron wastage was tested on six bacteria namely *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *K. pneumonia* (ATCC 9997), *S. aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6633) and *Shigella flexneri* (ATCC 12022). We used broth dilution method to determine the minimum inhibitory concentration (MIC) of assayed substances according to the ninth edition of Clinical and Laboratory Standards Institute standard protocol (M07A9, 2012). Briefly, serial twofold concentrations of the purchased AgNPs, biosynthesized AgNPs, and aqueous extract of saffron wastage, ranging from 500.0 to 62.5 μg/mL in Muller–Hinton broth (MHB), were prepared. Inoculate was prepared by suspending of at least 5 young colonies of tested bacteria in normal saline and the turbidity was adjusted to 0.5 McFarland standard, in which the concentration of bacteria was 1.5×10^8 CFU/mL. This suspension was diluted with MHB medium to 10^6 CFU/mL, and 100 μL of diluted suspension was added to each tube containing 1 mL of MHB supplemented with different concentrations of the extracts and nanoparticles. Tubes with MHB were used as control. All test tubes were incubated in shaker incubator 37 °C for 24 h. The MIC was defined as the lowest concentration in which no obvious growth was observed after 24 h. As an additional precaution, subcultures were performed after 24 h incubation for tubes without any obvious growth.

3. Results

3.1. Color change and UV–vis spectroscopy

Reduction of silver ions into AgNPs using aqueous extract of saffron wastage was evidenced by the visual color change of solution from pale pink to deep brown due to excitation of surface plasmon vibrations in AgNPs as shown in Figure 1.

The surface plasmon resonance of AgNPs showed a peak centered near 450 nm at UV–vis spectra which corresponds to the absorbance of AgNPs (Figure 2). This peak indicates the reduction of silver nitrate into AgNPs. There was an increase in intensity till the fifth hour as a function of reaction time without any shift in the peak wavelength as shown in Figure 3.

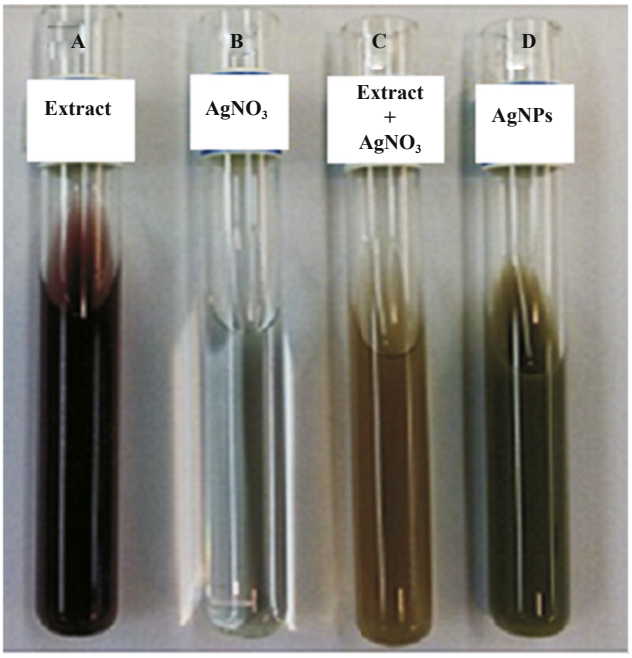

Figure 1. Synthesis of AgNPs by using aqueous extract of saffron wastage. A: Aqueous extract of saffron wastage; B: $AgNO_3$; C: Solution of silver nitrate and aqueous extract of saffron wastage; D: Aqueous extract of saffron wastage treated with $AgNO_3$.

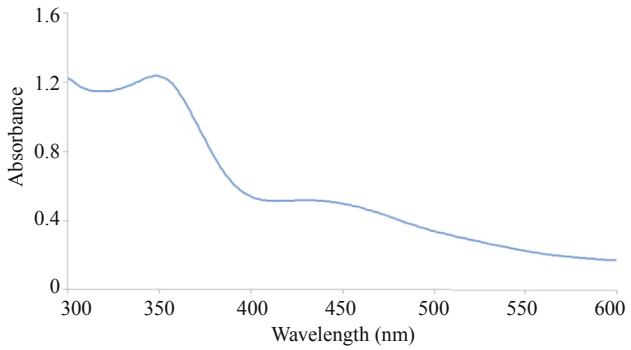

Figure 2. UV–vis spectra of synthesized AgNPs using the aqueous extract of saffron wastage.

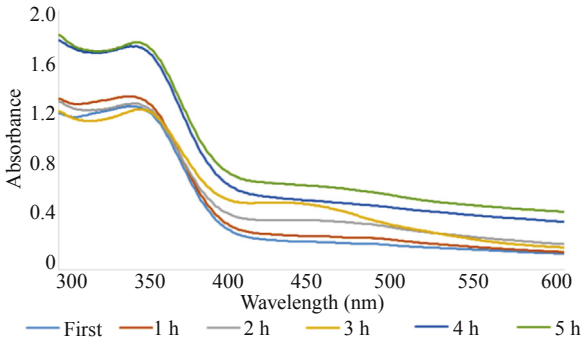

Figure 3. UV–vis spectra recorded as a reaction time function of aqueous solution of silver nitrate with aqueous extract of saffron wastage.

3.2. XRD studies

Figure 4 represents the XRD pattern gained for the synthesized AgNPs using the aqueous extract of saffron wastage. The size, phase identification and crystalline nature of the AgNPs were determined by the XRD analysis.

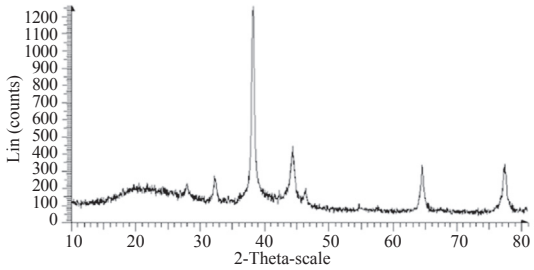

Figure 4. XRD pattern of AgNPs obtained using aqueous extract of saffron wastage.

Four intense and sharp peaks at 2θ = 38.16°, 44.28°, 77.45° and 64.51° can be indexed to the 111, 200, 220 and 311 planes of Bragg's reflection of silver, respectively. The XRD pattern thus clearly indicated that the AgNPs organized by the reduction of Ag^+ ions by the aqueous extract of saffron wastage were crystalline in nature. The size range of the synthesized AgNPs was 12–20 nm with cubic type.

The peaks at 2θ = 27.90°, 32.16°, 46.00° are corresponded to AgCl nanoparticles (Figure 4). It seems that ion chloride is taken from anthocyanins. Anthocyanins were detected in petals and sepals of saffron [29] and were related to crystalline and amorphous organic phases.

3.3. TEM analysis

TEM analysis clearly illustrated that the shape of AgNPs was spherical and the average size of them was 15 nm.

3.4. FTIR studies

FTIR analysis is utilizable for characterizing the surface chemistry of nanoparticles. Organic functional groups like OH, C═O linked to the surface of nanoparticles are found by FTIR [30]. The FTIR spectra of saffron wastage extract are shown in Figure 5. After the synthesis of AgNPs, the solution containing nanoparticles was centrifuged at 8 000 r/min for 10 min to separate AgNPs from other composition of solution and the deposit was prepared for FTIR analysis (Figure 6).

The FTIR spectra of the extract of saffron wastage (Figure 5) displayed peak in the range of 600–680 cm^{-1}, which demonstrated the alkyl halides band especially the C−Cl bond. The peak in the range of 1 640 cm^{-1} that are relevant to C═O bond of the carbonyl group and the stretching vibrations of amides also emerged in this range. The peaks in the range of 3 200–3 500 cm^{-1} were assigned as −OH stretching in alcohols and phenolic compounds with strong hydrogen bonds.

Figure 6 shows the FTIR bands at 1 021 cm^{-1} and 1 108 cm^{-1}, which indicates the C−O phenolic compounds. The presence of these peaks confirmed that the nanoparticles were covered by plant secondary metabolites such as terpenoids, flavonoids, glycosides, phenols, tannins, with functional groups such as ketone, aldehyde, carboxylic acid, and others. The presence of these groups is due to the stability of the nanoparticles. These metabolites prevent clotting and pairing of the nanoparticles. If the amount of antioxidants and flavonoid compounds such as ascorbic acid and gallic acid are high in plant extract, they are more suitable for the reduction of metal salts.

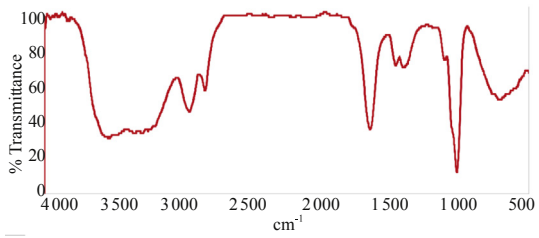

Figure 5. FTIR spectra of aqueous extract of saffron wastage.

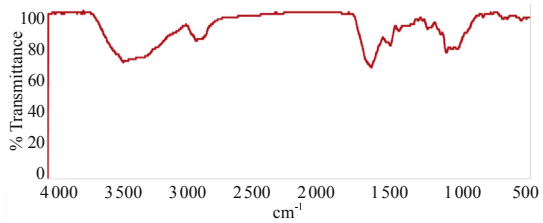

Figure 6. FTIR spectra of capped AgNPs synthesized using aqueous extract of saffron wastage.

3.5. Antibacterial activity of AgNPs

The results of our study showed that the purchased AgNPs and aqueous extract of saffron wastage did not have significant inhibiting activity on all six bacteria in tested concentrations (Figure 7). The biosynthesized AgNPs, inhibited the growth of all tested bacteria except for *S. aureus* in concentration of 250 μg/mL (Figure 8).

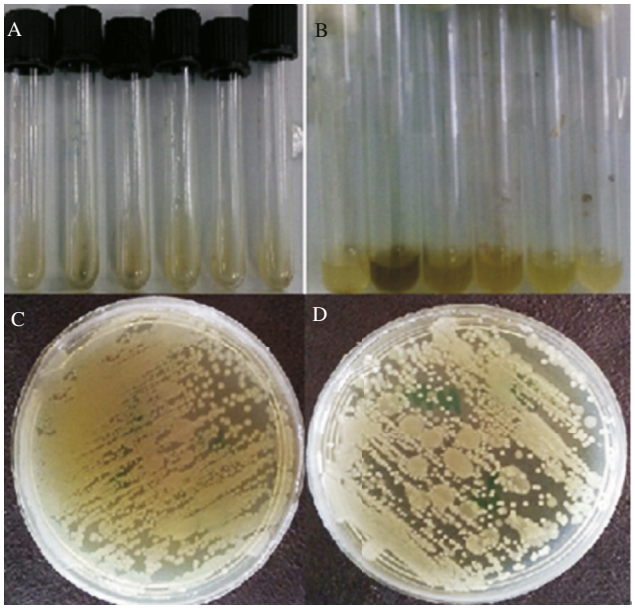

Figure 7. Antimicrobial activity of purchased AgNPs and extract against six bacteria.
A: Merck nanoparticles; B: Aqueous extract of saffron wastage; C: Subculture for purchased AgNPs after 24 h incubation; D: Subculture for aqueous extract of saffron wastage after 24 h incubation.

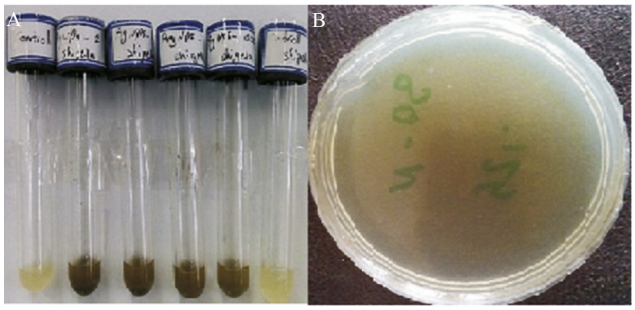

Figure 8. Antimicrobial activity of biosynthesized AgNPs against six bacteria.
A: Biosynthesized AgNPs; B: Subculture for biosynthesized AgNPs after 24 h incubation.

4. Discussion

While metal nanoparticles are being increasingly used in many sectors of the economy, there is growing interest in the biological and environmental safety of their production. The main methods for nanoparticle production are chemical and physical approaches that are often costly and potentially harmful to the environment.

Antimicrobial and antibiotic resistances are an increasingly serious threat to human health. It is necessary to overcome it with the help of nature. Therefore, there is an increase in the investigation of plants as a source of human infectious diseases management [31,32]. Owing to the urgent need for new antibiotics, growing interest is taken into the research on the chemistry of medicinal plants. Medicinal plants have been a potential source of therapeutic agents for thousands of years. An impressive number of modern drugs have been derived from natural sources like plants which have been recognized as a part of the improvement of human healthcare for thousands of years [33]. In addition, within the past decade, it has been demonstrated that many biological systems like plants, can transform metal ions into metal nanoparticles via the reductive capacities of metabolites present in these organisms [34].

Silver has been widely employed for many years in human history. Silver is the only element whose plasmon resonance can be tuned to any wavelength in the visible spectrum. This element has many applications, among which its disinfectant property is very important. AgNPs have been demonstrated to exhibit antimicrobial properties against bacteria with close attachment of the nanoparticles themselves to the microbial cell and the activity being size dependent [35]. Fundamental studies showed that AgNPs exhibit a rare combination of valuable properties, namely, unique optical properties associated with the surface plasmon resonance, catalytic activity, high electrical double layer capacitance, *etc*. Nanosilver has been used extensively as an anti-bacterial agent in the health industry, food storage, textile coatings and a number of environmental applications [36].

In this regard an effective and versatile method was performed for the synthesis of AgNPs using extract of saffron. The samples of *C. sativus* (saffron) were prepared for synthesis of AgNPs. We compared antibacterial properties of purchased AgNPs, biosynthesized AgNPs and aqueous extract of saffron wastage.

Antimicrobial effects of AgNPs have been evaluated in several studies. These studies show different values for the MIC of the particles on the tested bacteria. In most of these studies, the MIC values were lower than the MIC values obtained in our study [37]. However, due to different methods used to assess the MIC in these studies, the results of different studies are not comparable; yet this difference may be due to various factors such as studied organism, synthetic parameters, bacterial strains used, toxicity criterion (growth inhibition or full eradication), composition of the medium and presence of light or oxygen [38].

The ultrasonic irradiation was applied to the mixed solution. The colorless solution of silver nitrate changed to deep brown, indicating the formation of AgNPs. Using ultrasound for generating AgNPs was fast and efficient since there is uniformity in the shape of nanoparticles, as mentioned in materials and methods section. We used the UV–vis and FTIR spectrums for determination of optical properties of AgNPs. The surface plasmon resonance of AgNPs showed a peak centered near 450 nm at UV–vis spectra which corresponds to the absorbance of AgNPs. A comparison of our XRD spectrum with the standard authenticated the AgNPs formed in our experiments as demonstrated by the peaks 2θ values of 38.16°, 44.28°, 77.45° and 64.51°, corresponding to 111, 200, 220 and 311, respectively, for silver. The XRD pattern thus clearly indicated that the AgNPs organized by the reduction of Ag^+ ions by the aqueous extract of saffron wastage are crystalline in nature.

High resolution TEM photographs are used for morphological characterization and the size distributions of AgNPs. TEM analysis clearly illustrated that the shape of AgNPs was spherical and the average size of them was 15 nm.

FTIR measurement was used to identify the role of aqueous extract of saffron wastage as the reducing agent for reduction of Ag^+ ions to the AgNPs and stabilizing agent for these nanoparticles. FTIR analysis also is utilizable for characterizing the surface chemistry of nanoparticles.

Organic functional groups like OH, C═O linked to the surface of nanoparticles are found by FTIR [30]. The FTIR spectra of the extract of saffron wastage displayed peak in the range of 600 cm^{-1} that demonstrated the alkyl halides band especially the C−Cl bond. The peaks in the range of 1 640 cm^{-1} that are relevant to C═O bond of the carbonyl group and the stretching vibrations of amides also emerged in this range. The peaks in the range of 3 200–3 500 cm^{-1} were assigned as −OH stretching in alcohols and phenolic compounds with strong hydrogen bonds. Results showed the FTIR bands at 3 453 cm^{-1}, 2 923 cm^{-1}, 2 876 cm^{-1}, 1 653 cm^{-1}, 1 515 cm^{-1}, 1 108 cm^{-1} and 1 021 cm^{-1}. This illustrated various functional groups linked to the surface of the nanoparticles. On the other hand, the peaks in 1 021 cm^{-1} and 1 108 cm^{-1} indicated the C−O of alcohols and phenols. The presence of these peaks confirmed that the nanoparticles were covered by plant secondary metabolites such as terpenoids, flavonoids, glycosides, phenols, tannins, with functional groups such as ketone, aldehyde, carboxylic acid, and others. The presence of these groups is due to the stability of the nanoparticles.

The present study showed an innovative way to synthesize antimicrobial AgNPs using natural products, which can be used in various biomedical applications. Also a hitherto unreported agricultural waste material for the consistent and quick synthesis of AgNPs was reported. We reported the synthesis of AgNPs using aqueous extract of saffron wastage as a green method without any chemical stabilizer and reducer. The results of our investigation showed that the purchased AgNPs and aqueous extract of saffron wastage did not have significant inhibiting activity on all six bacteria in tested concentrations but the biosynthesized AgNPs, inhibited the growth of all the tested bacteria except for *S. aureus*.

Organisms have a great potential for synthesizing nanoparticles with a vast range of applications. Plant and plant extract are considered as green and effective paths in the synthesis of gold and AgNPs. Synthesis of nanoparticles with plant extract provides acceptable morphology and size of nanoparticles. There are many saffron farms in Iran, and saffron petals are discarded as wastage. The synthesis of AgNPs using saffron wastage provides an easy, and affordable way. The size of synthesized AgNPs was reported to be 12–20 nm by XRD analysis and an average size of 15 nm with spherical shape by TEM images was also reported. FTIR spectra confirmed that the nanoparticles were covered by plant compounds. The biosynthesized AgNPs showed significant antibacterial effect against *E. coli*, *P. aeruginosa*, *K. pneumonia*, *Shigella flexneri* and *Bacillus subtilis*, so it can be used in biomedical applications.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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