Synthesis and characterization of antibacterial silver nanoparticles using essential oils of crown imperial leaves, bulbs and petals

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Abstract
In this research, the essential oils obtained from leaves, bulbs and petals of crown imperial plant are used as reducing agents to fabricate silver nanoparticles. The aromatic hydrocarbons, alkenes, ketenes and alkaloids are among the compounds identified in the essential oils as detected by gas chromatography mass spectrometry. The characteristics of silver nanoparticles are investigated by field emission scanning electron microscopy, transmission electron microscopy and XRD. The chemical groups and surface resonance characteristics of the samples are revealed by Fourier transform infrared spectroscopy and UV-Visible spectroscopy, accordingly. The field emission scanning electron microscopy results show that the silver nanoparticles form nanoscale spheres with a mean diameter of $27 \pm 14$ nm in case of the sample obtained by essential oil of petals as confirmed by transmission electron microscopy. This sample shows a UV-Visible absorption band at 450 nm. The antibacterial activity of the silver nanoparticles shows a remarkable inhibition capability against all of the tested bacteria, with the sample obtained from petals exhibiting the strongest antibacterial effect in agreement with the obtained minimum inhibitory concentration and minimum bactericidal concentration values. The cell viability assay using Vero cell line reveals a nearly constant viability rate above 125 $\mu$g/mL of silver nanoparticles.

1 INTRODUCTION

Nanotechnology is a rapidly growing field in which nanoscale materials are introduced with different forms, distributions and potentials to be innovatively used in various applications. Although conventional physical and chemical preparation methods are known to be successful in producing nanomaterials, they are generally expensive, time-consuming and potentially hazardous to the environment [1]. Therefore, continuous effort to find cost-efficient green procedures is essential when it comes to applications of metal nanoparticles. Biosynthetic processes mediated by micro-organisms such as fungi [2], bacteria, yeasts [3] and actinomycetes [4] or macroorganisms such as plants [5] and algae [6] are considered as novel approaches for producing metal nanoparticles. Plant mediated synthesis of metal nanoparticles not only is a facile and cost-efficient procedure, but also involves biomolecules that lower the risk of toxicity in humans and ecosystems [7, 8].

Silver is recognized in nanoscience as one of the materials with remarkable antimicrobial, physical and chemical properties [9, 10]. Many studies have investigated the activity and engrossing application of AgNPs in diverse fields such as water treatment, textiles, food packaging, cosmetics, ointments, photocatalysis etc. [11, 12]. As AgNPs have found remarkable biomedical applications, green synthesis of AgNPs has been extensively pursued by researchers [12–14].

Essential oils are aromatic substances present in particular parts of the plant structure such as secretion ducts, secretory glands or resin ducts. These oils exhibit antimicrobial properties and are becoming increasingly popular in biomedicine [15]. The essential oil yield of a plant is usually very low, but in some cases such as Syzygium aromaticum (clove), relatively large amounts could be obtained [16]. By applying heat and pressure, the oil contained in plant cells is released. Different methods such as steaming and distillation have been suggested for obtaining essential oils from different plants [17]. Among other methods,
hydro-extraction and steam extraction approaches usually offer more efficient yields [18].

Crown imperial (Fritillaria imperialis L.) is a 60–120 cm tall perennial plant with thick cylindrical brownish stems. It is a bulbous plant belonging to the Liliaceae family with a fast growth rate. The inflorescence grows in the form of a plain umbrella consisting of 5 to 8 red or dark orange flowers. The peduncles are downward and its petals are large and solitary. It has narrow leaves with sharp tips that cover the stem in form of bunches. The bulb is large and scaly with either solitary or overlapping yellowish scales [19]. This genus can produce a variety of steroidal alkaloids with pharmaceutical properties [20]. A limited number of studies have been published so far on the pharmaceutical effects of crown imperial. The crown imperial extracts exhibit antiproliferative effects in vitro against human liver cancer (LCL-PI 11) and breast adenocarcinoma (MCF-7) cell lines due to the synergistic effects of extract compounds, which is comparable to the results of conventional 5-FU drug of chemotherapy [21].

The AgNPs prepared using medicinal plants exhibit enhanced properties that can be applied fruitfully in biomedicine [22–24]. Synthesis of AgNPs using the extract of Ficus carica showed that these nanoparticles have a considerable cytotoxic effect on MCF7 cancer cells, which was suggested to be due to the effect of reactive oxygen species (ROS) on cellular components [25]. Green synthesis of AgNPs has been reported using an aqueous extract of green tea leaves [26]. The authors found that the resulting AgNPs exhibit a promising antimicrobial activity compared to aqueous, methanolic and ethanolic extracts of green tea. In another study, AgNPs were made by a solution of Ricinus communis L. leaf extract [27], which showed enhanced antiviral activity against Coxsackie virus B3.

The present study is concerned with obtaining AgNPs using crown imperial essential oils. The chemical compositions of the obtained essential oil as well as the antibacterial activity of the resulting AgNPs are studied. Production of AgNPs using bulbs, leaves and petals of this plant is evaluated by XRD, transmission electron microscopy (TEM), field emission scanning electron microscopy (FE-SEM), FT-IR and UV–Visible spectroscopy.

2 MATERIALS AND METHODS

2.1 Materials

Considering the phenological growth stages of the species at their full petaling stage, crown imperial specimens were collected in mid-May from rangelands of Bid Valley in Freidan, located in the west of Isfahan province, Iran. The taxonomic identity of the collected specimens was verified by comparing them with well-known herbarium species. Analytical grade AgNO₃ powder was purchased from Merck (Germany) and distilled water was used as solvent. Four bacterial strains including Escherichia coli (ATCC 35218), Salmonella typhimurium (ATCC 14028), Staphylococcus aureus (ATCC 29213) and Bacillus cereus (ATCC 14579) were selected for testing antibacterial activity of the AgNPs.

2.2 Preparation of the essential oils

Samples were collected from crown imperial plant organs of bulbs, leaves and petals. A total of 10 plants were taken from the site. The collected samples were dried at room temperature for 2 weeks and were ground subsequently. The essential oil extraction was carried out by steam distillation method using a Clevenger apparatus (Figure 1). The powdered bulbs, stems, leaves and petals were separately heated with water in the balloon of the apparatus. In each run, a powder sample mass of 10 g was added to 300 mL of distilled water. Typically, an orange to brown essential oil with a strong aroma was obtained after 12 h. The chemical compounds of the resulting volatile oils were identified by gas chromatography mass spectrometry (GC-MS, Agilent Technologies 7890A). This instrument was equipped with a mass detector having an HP-5 MS capillary column (0.25 mm × 30 m with a film thickness of 0.25 in) containing 5% polymethyl phenyl-siloxane. The injection volume of the extracted oil was 1 μL. The flow rate of the helium carrier gas (99.999%) was 1 mL/min and the column temperature was 60 °C followed by an increase to 270 °C with a rate of 3°C/min. Other parameters were set as follows: an ionization potential of 70 eV, an ion source temperature of 240 °C and a mass resolution of 1000.

2.3 Synthesis of samples

In order to prepare AgNPs, 100 mL AgNO₃ aqueous solution was prepared with a concentration of 0.1 mol/L. Then 10 mL of the plant essential oil was added to the solution. The solution turned black in 5 min, indicating that the AgNPs have been synthesized. The obtained AgNPs were
dried in an oven at 50 °C for 24 h and stored for further experiments.

2.4 Characterization of samples

UV-Visible spectra were recorded on a SUV-S2100 spectrophotometer (USA). Fourier transform infrared spectroscopy (FT-IR 680 plus, Japan) was used in a wavenumber range of 400–4000 cm⁻¹ by KBr pellet method to identify the functional groups and chemical bonds. The crystalline nature of the AgNPs was investigated by XRD. Morphological characteristics of the specimens were explored using a FE-SEM (HITACHI S-4160, Japan) and TEM (Philips CM30). The particle size distribution was determined by Digimizer software.

2.5 Antibacterial activity of the AgNPs

Agar disk diffusion method was used to study the antibacterial activity of the AgNPs prepared by all three plant organs of bulbs, leaves and petals were evaluated. Four bacterial species of *B. cereus*, *S. aureus*, *E. coli* and *S. typhimurium* were individually introduced to Muller–Hinton agars with a concentration of 0.5 McFarland using sterile cotton swabs. The positive control (0.1 mol/L pure AgNO₃ solution), negative control (distilled water) and sample disks (0.1 g/L aqueous AgNPs solutions) were placed on agars prior to a 24 h incubation at 37 °C. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests were performed, such that nine tubes were selected for each bacterial species in a typical MIC test. In a sterile condition near a flame, 1 mL of Laura Bertani culture medium was placed inside each tube. Then, 1 mL of the AgNPs was introduced to the first tube and was thoroughly shaken. Next, 1 mL of the first tube was transferred to the second tube and was shaken to be completely mixed. This process continued until the eighth tube. Finally, the ninth tube was chosen to only contain the culture medium as a blank control. A specific amount of each bacterial suspension with a concentration of 0.5 McFarland was introduced to each tube before incubation for 24 h at 37 °C. In the MBC tests, the blank test tube was examined for each bacterial species after incubation where the blurred color of the blank content indicated bacterial growth. An amount of bacteria was taken from three test tubes with no sign of blurring and cultured. The specimens from each test tube were then introduced to Laura Bertani media and incubated for 24 h.

2.6 Cell viability study

The MTT assay was carried out to study the cytotoxicity of the AgNPs in contact with epithelial Vero cells. Vero cells with a density of 2 × 10³ cells/mL were seeded into a 96-well microplate (100 μL/well) to be cultured for 12 h at 37 °C in a 5% CO₂ atmosphere. Consequently, the cell cultures were treated with various concentrations of AgNPs (62.5, 125, 250, 500 and 1000 μg/mL) and incubated for 24 h. Next, the MTT reagent ([3-[4, 5-Dimethyl thiazol-2-yl]-2, 5-diphenyltetrazolium bromide]) was added to the wells and the cells were incubated for another 3 h at 37 °C prior to dissolving the appeared purple formazan crystals with 100 μL dimethyl sulfoxide (DMSO). Eventually, the colour intensity was measured at 570 nm by a Stat Fax 2100 Microplate Reader (Awareness Technologies, USA). The experiments were performed three times and optical density (OD) of the samples were plotted against their respective concentrations.

3 RESULTS AND DISCUSSION

3.1 Chemical composition of samples

The chemical compounds of the extracted essential oils from crown imperial were studied by GC-MS. A total of 237, 192 and 205 chemical compounds were identified in the essential oils obtained from bulbs, leaves and petals, respectively. The main chemical components included hydrocarbon groups, nitrogen compounds, alcohols, ketenes, terpenes, ethers, acids, esters and aldehydes.

3.2 UV-Visible spectra

The change in colour of the AgNO₃ solution from pale pink to black indicates formation of AgNPs. The UV-Visible absorption peaks can be sensitive to size and shape of the particles and also to the surface species. Figure 2 shows the UV-Visible spectra for AgNPs synthesized using essential oils of crown imperial organs, which exhibit strong absorbance peaks at 418 (bulbs), 408 (leaves) and 450 (petals) nm. The results are in
agreement with the well-known surface plasmon resonance of silver [28].

### 3.3 | XRD analysis

The XRD pattern of the synthesized AgNPs using the petals sample is presented in Figure 3. The AgNPs sample from petals was selected based on its more porous structure revealed by FE-SEM. It is established that more porous nanostructures offer higher contact areas with their media. The observed characteristic peaks at $2\theta = 38.08^\circ$, $35.34^\circ$, $64.24^\circ$ and $60.67^\circ$ are attributed to the crystalline planes of (111), (200), (220) and (311), respectively. The pattern could be matched with the JCPDS reference pattern No. 04–0783 with a face-centered cubic (FCC) crystal structure [29]. The intense reflection from (111) implies the dominant growth direction of silver nanocrystals. No extra reflections other than AgNPs were found, which indicates the nanoparticles remained structurally unaffected by other components of the essential oil.

### 3.4 | FT-IR analysis

The FT-IR spectra of the samples are used to analyse the chemical bonds and functional groups associated with them. The FT-IR results revealed various groups that are presumably responsible for reducing and stabilizing AgNPs. Figure 4 shows the FT-IR spectra of the plant organs. In the spectrum of leaves, the peak at 3422 cm$^{-1}$ represents O H stretching vibrations of alcohols, the peak at 2923 cm$^{-1}$ is associated with C H stretching vibrations of carboxylic acids and alkanes and the peak at 1632 cm$^{-1}$ is related to C C stretching vibrations of alkene groups. The 1384 cm$^{-1}$ peak is related to C C vibrations of aromatic rings and the last peak at 1030 cm$^{-1}$ is attributed to C N stretching vibrations of amine groups [13, 30]. The petals spectrum shows a strong peak at 3433 cm$^{-1}$, which is attributed to stretching vibrations of O H. The peak observed at 2923 cm$^{-1}$ is due to stretching vibrations of C H, and is attributed to the carboxyl and alkane groups. The peak at 1379 cm$^{-1}$ is assigned to C C vibrations of amide compounds. The last peak at 793 cm$^{-1}$ is associated with C C H bonds of aromatic rings.

### 3.5 | FE-SEM analysis

Morphological features of the AgNPs prepared by the essential oils were studied by FE-SEM. Figure 5 shows that all of the AgNPs are formed in spherical shapes and are well dispersed. The nanoparticles show porous structures with the petals sample showing more porosity.

### 3.6 | TEM analysis

The nanoparticle morphology of the petals sample was further studied by TEM (Figure 6). To determine the size distribution, each discernible particle was taken into account twice, such that its lowest and greatest dimensions are measured. It is found that these particles are mostly spherical with a mean diameter of $26.59 \pm 14.37$ nm. The results show that the essential oil of petals can lead to almost spherical nanometer-sized AgNPs. Spherical AgNPs offer a higher surface area than other shapes, providing more contact with the target bacteria. It is found that in addition to their size, the shape of nanoparticles is important in their antibacterial properties.
3.7 | Antibacterial activity

The AgNPs showed promising biocidal activity against Gram-positive (*B. cereus* and *S. aureus*) and Gram-negative (*E. coli* and *S. typhimurium*) bacteria (Figure 7). The results of the three plant organs (Table 1) show that all antibacterial activities of the AgNPs were stronger than AgNO₃ solution. The Gram-positive bacteria showed greater resistance to the AgNPs as figured from their smaller inhibition zones and higher MIC and MBC values (Table 2). This may be due to the Gram-positive cell wall structure, which is composed of a thick peptidoglycan layer [17]. Also, the biocidal effect of the AgNPs could be ascribed to a release of ROS that can affect the biomolecular structure of bacterial cell membranes [12].

3.8 | Cytotoxicity of the AgNPs

Based on the MTT assay results shown in Figure 8, the cell viability decreased significantly at the concentrations of 62.5, 125, 250, 500 and 1000 μg/mL (corresponding to 45.21%, 35.57%, 26.93%, 18.29%, and 9.57%, respectively). The MIC and MBC results of the synthesized AgNPs against tested bacteria are shown in Table 2.

### Table 1: Diameters of inhibition zone (mm) for AgNPs prepared using the essential oils of bulb, petals and leaves of crown imperial

| Components       | Zone of inhibition (mm) |
|------------------|-------------------------|
|                  | *S. aureus* | *B. cereus* | *E. coli* | *S. typhimurium* |
| Leaves           | 10.4        | 10.4        | 10.6      | 10.0            |
| Petals           | 10.4        | 11.8        | 11.7      | 11.4            |
| Bulbs            | 9.3         | 11.3        | 11.7      | 10.5            |
| AgNO₃            | 8.7         | 8.90        | 8.9       | 8.6             |
| Distilled water  | 6.4         | 6.4         | 6.4       | 6.4             |

### Table 2: The MIC and MBC results of the synthesized AgNPs against tested bacteria

| Components | *S. aureus* | *B. cereus* | *E. coli* | *S. typhimurium* |
|------------|-------------|-------------|-----------|------------------|
| MIC        |             |             |           |                  |
| Leaves     | 3.12        | 3.12        | 0.8       | 0.8              |
| Petals     | 6.25        | 6.25        | 1.6       | 1.6              |
| Bulbs      | 6.25        | 6.25        | 1.6       | 1.6              |
| MBC        |             |             |           |                  |
| Leaves     | 6.25        | 6.25        | 1.6       | 1.6              |
| Petals     | 12.5        | 12.5        | 3.12      | 3.12             |
| Bulbs      | 12.5        | 12.5        | 3.12      | 3.12             |
FIGURE 7  Zones of inhibition for AgNPs synthesized using (a) leaves, (b) petals and (c) bulb extracts of crown imperial together with (d) AgNO₃ solution (positive control) and (e) distilled water (negative control)

40.42%, 25.90%, 23.79% and 21.04% viability, respectively). The low cytotoxic performance of the AgNPs has been previously reported [31]. The AgNPs prepared by Penicillium aculeatum Su1 showed high biocompatibility with normal human cells (HBE cells) in contrast to the silver ions that exhibited high cytotoxic effects [32]. Considering these studies, it is figured that the cytotoxic effects can be lowered by using green synthesis methods such as various plant extracts and oils.

4 | CONCLUSION

The present study showed that the essential oils of bulbs, leaves and petals of crown imperial could effectively contribute to the production of spherical AgNPs. This method is considered as a synthesis route in green chemistry. Formation of AgNPs was confirmed by UV-Visible, XRD, FE-SEM, TEM and FT-IR tests. The UV-Visible characteristic peak for all three organs of bulbs, leaves and petals matched the plasmonic resonance characteristic of silver. XRD study confirmed the crystalline structure of the synthesized AgNPs. The morphological features of the AgNPs were explored by FE-SEM, showing a more porous structure for petals sample. The shape of the particles obtained from petals sample was mostly spherical with a mean size of about 27 nm as revealed by TEM. Also, all of the prepared AgNPs showed a strong antibacterial activity against Gram-positive (B. cereus and S. aureus) and Gram-negative (E. coli and S. typhimurium) bacteria. Therefore, crown imperial essential oils
could be considered as effective components for fabricating AgNPs, and this protocol could be considered as an environmentally friendly method of obtaining these nanoparticles.

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