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GREEN SYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES USING *Alcea rosea* FLOWER EXTRACT AS A NEW GENERATION OF ANTIMICROBIALS

Article Highlights

- Synthesis of silver nanoparticles was developed using *Alcea rosea* flower extract
- $AgNO_3$ concentration, flower extracts quantity, and reaction temperatures were determined to be significant factors in the AgNPs biosynthesis
- Prepared AgNPs were spherical in shape with 7.2 nm mean particle size
- Oxygen-bearing functional groups in biochemical compounds from *A. rosea* were responsible for reduction of Ag^+
- The *MIC* for AgNPs against *E. coli* and *S. aureus* was determined to be 37.5 μg/mL

Abstract

Green synthesis of silver nanoparticles (AgNPs) was developed by treating Ag^+ with Alcea rosea flower extract. $AgNO_3$ concentration, flower extract quantity, and reaction temperature were found to be significant factors in the bioreduction reaction. Synthesized AgNPs were almost spherical in shape with an average diameter of 7.2 nm. Fourier transform infrared spectroscopy (FTIR) analysis revealed that oxygen-bearing functional groups in the A. rosea flower extract are responsible for reduction of Ag^+. The minimum inhibitory concentration (MIC) of AgNPs against a Gram-positive (Staphylococcus aureus) and Gram-negative (Escherichia coli) bacteria was determined to be 37.5 μg/ml.

Keywords: Alcea rosea; biochemical reduction; biosynthesis; green synthesis; Ag nanoparticles.

AgNPs have found widespread technological applications due to their unique physicochemical, optical and catalytic properties [1,2]. There is an increasing interest for application of AgNPs in households, medicine and industry [3]. Nowadays, there are a lot of commercially available products containing AgNPs, ranging from burn treating materials to antimicrobial fabrics and paints [3]. The increasing application of AgNPs will lead to increased demand for AgNPs production. To date, various chemical and physicochemical techniques have been used for the production of AgNPs [4,5]. However, all these methods suffer from high energy consumption and the use of toxic chemicals which are potentially dangerous to the environment and human health. Therefore, there is a need for development of reliable and green process of AgNPs synthesis.

Green chemistry has emerged as a new concept for development and implementation of chemical processes in order to reduce or eliminate the use of hazardous substances. Chemically synthesized nanoparticles are not colloidal or physicochemically stable in aqueous media and therefore capping agents must be used to increase the particle stability. In biosynthesis reactions, biochemical species attach to the surface of nanoparticles and act as capping and stabilizing agent in a one-pot reaction [6-12]. Bioactive

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compounds from microorganisms and plants have a valuable capability for reduction and capping of AgNPs without the use of any toxic chemicals and harsh reaction conditions [6-8,10]. Carbohydrates and proteins from microbial cells can be effective in reduction of Ag^+ [12]. In comparison to usage of plant extracts, biosynthesis of AgNPs using microorganisms needs an elaborated process of culturing and maintaining microbial cells that, in some cases, could be pathogenic to humans. The use of plant extract has advantages such as ease of handling, availability and a broad viability of metabolites. AgNPs have been synthesized using leaf extract of various plants such as black tea, *Lippia citriodora* (Lemon Verbena), maple (Acer sp.) and eucalyptus [7,8,13,14]. Other parts of plants have also been used such as *Piper longum* and *Crataegus douglasii* fruit extract, coffee powder extract, *Nephelium lappaceum*, orange peel extract, oil of *Plukenetia volubilis* L., C*hrysanthemum morifolium* Ramat extract, *Medicago sativa* and *Sterculia foetida* seed exudate, sorghum bran extract and *Cinnamon zeylanicum* bark extract [6,7,10,11,15-21].

Alcea rosea (*Althaea rosea*) is an important medicinal herb in many countries. It was used traditionally as expectorant, cooling, diuretic and emmenagogue substance. *Alcea rosea* flowers extract is used as an anti-inflammatory, febrifuge, demulcent and astringent agent. Flowers as well as their roots are used in the treatment of inflammation of the kidneys and the uterus. *Alcea rosea* contains high molecular weight acidic polysaccharides (1.3 to 1.6 million Dalton) known as mucilages which are abundant in flowers and leaves. These mucilages are composed of glucoronic acid, galacturonic acid, rhamnose and galactose. It also contains proteins, alkaloids and flavonoids [22]. All of these biochemical compounds are reported to be effective in bioreduction of Ag^+ [6-8,10]. According to our best knowledge there is no report on biosynthesis of AgNPs using *Alcea rosea*. Therefore, the current research work aims to investigate: *i*) the potential of *Alcea rosea* flower aqueous extract for biosynthesis of AgNPs and *ii*) the antimicrobial effect of the prepared AgNPs.

EXPERIMENTAL

Materials

Silver nitrate was purchased from Merck. All glassware have been acid washed and then rinsed with deionised water. All the solutions were prepared using deionized-Millipore water (resistance >18 MΩ cm).

Preparation of flower extract

Dried flowers of *Alcea rosea* were initially washed in deionized water to remove the soil and dust particles. The aqueous extract was consequently prepared by mixing 2.5 g of dried flowers with 100 mL of deionized water in a 250 mL Erlenmeyer flask. The prepared mixture was boiled for 15 min, then filtered through Whatman filter paper (Reeve angel®, grade 201) and stored at -20 °C.

Preparation of $AgNO_3$ solutions

Solutions of $AgNO_3$ were prepared at 100, 50 and 10 mM concentration. For the initial/stock concentration, 1.7 g $AgNO_3$ was dissolved in 100 mL deionized water to obtain 100 mM solution. Solutions with lower concentrations (50 and 10 mM) were prepared by two and 10-fold dilutions, respectively. Desired $AgNO_3$ concentrations in the bioreduction reactions were achieved by adding 1 mL of corresponding solution to the reaction mixture.

Biosynthesis and characterization of AgNPs

Alcea rosea flowers extract was used as a source for reducing and capping agent for synthesis and stabilization of AgNPs in a simplified one-pot reaction. The impact of various parameters such as the amount of *Alcea rosea* flower extract, $AgNO_3$ concentration and the reaction temperature was evaluated by conducting several sets of experiments. The $AgNO_3$ concentration was tested at 1, 5 and 10 mM in 10 mL total reaction volume containing 4 mL (40 vol.%) flower extract at room temperature (27 °C). Impact of various flower extract amounts was also investigated in the range from 10 to 70% of total reaction volume at room temperature and 5 mM $AgNO_3$. The effect of reaction temperature on silver ions reduction was evaluated at 15, 28, 50 and 75 °C using 5 mM $AgNO_3$ and 4 mL flower extract. All the reactions were monitored for 24 h.

The optical properties of the produced particles were analyzed by ultraviolet and visible absorption spectroscopy (T80+ UV/Vis spectrometer, PG Instruments Ltd.) operated at a resolution of 1 nm within the range of 300-700 nm. In each analysis, 0.1 mL of the sample was diluted to 1 mL with deionized water [11]. Further characterizations were done by Transmission Electron Microscopy (TEM, Philips, CM 10; HT 100 kV), Fourier-transform infrared spectroscopy (FTIR, Bruker, Vertex 70, FT-IR spectrometer) and X-ray powder diffraction (XRD, Siemens D5000).

Antimicrobial assay

Escherichia coli PTCC 1399 (ATCC 25922) and *Staphylococcus aureus* PTCC 1112 (ATCC 6538)

were purchased from Persian Type Culture Collection (PTCC). Minimum inhibitory concentration (*MIC*) was determined using standard microdilution method (CLSI M07-A8) [23]. In the experiment, AgNPs suspension was prepared in Mueller-Hinton broth (MHB). To prepare bacterial suspensions for inoculation, bacterial cells were cultured in MHB up to turbidity of the $BaSO_4$ 0.5 McFarland standard (OD600 0.11). Then the 0.5 McFarland suspensions were diluted to 1:20. Finally, 10 µL of the prepared inoculums' suspension was transferred to each well in the 96-well plate containing 90 µL MHB media with AgNPs. For blank wells, 10 µL of fresh MHB was added to 90 µL MHB with AgNPs. After 24 h incubation at 37 °C, the OD600 was measured by microplate reader (BioTek, Power Wave XS2).

RESULTS AND DISCUSSION

Biosynthesis of AgNPs

Reduction of Ag^+ to AgNPs resulted in color change of the reaction solution due to excitation of surface plasmon resonance (SPR) in the AgNPs. AgNPs have a typical surface plasmon band absorption at about 400-450 nm. The UV-Vis spectroscopy, therefore, can be used as an indirect method to examine the formation and to some extend characterisation of AgNPs [8,11]. The UV-Vis spectra of the prepared particles in various concentrations of silver nitrate are shown in Fig. 1. Increasing the silver nitrate concentration from 1 to 5 mM resulted in a major increase of AgNPs content as indicated by the hyperchromic shift in SPR band. No significant increase in AgNPs concentration was observed when using higher $AgNO_3$ concentration (>5 mM).

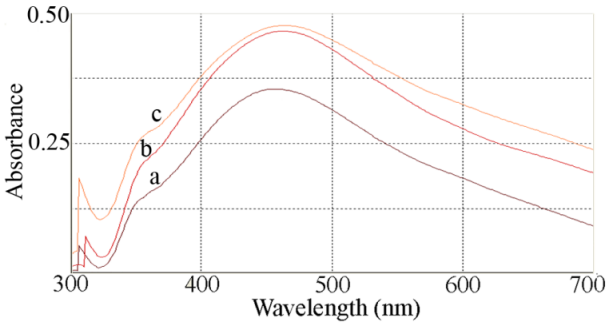

Figure 1. UV-Vis spectra of AgNPs prepared at various concentrations of silver nitrate: a) 1, b) 5 and c) 10 mM.

SPR bands of the prepared AgNPs using different amounts of flower extracts are shown in Fig. 2. By increasing the flower extract up to 40% of the total reaction volume, an obvious hyperchromic shift was observed. However, further increase in the flower extract content, above 40 vol.% resulted in a significant hypochromic shift in SPR band. Synthesis of the metal nanoparticles was conducted in two main steps namely, a) nucleation and b) growth of nanoparticles. Organic compounds presence in the reaction mixture has an inhibitory effect on the particle growth [24-26]. Thus, an optimal value of the *Alcea rosea* flower extract is required for reduction of Ag^+ to AgNPs.

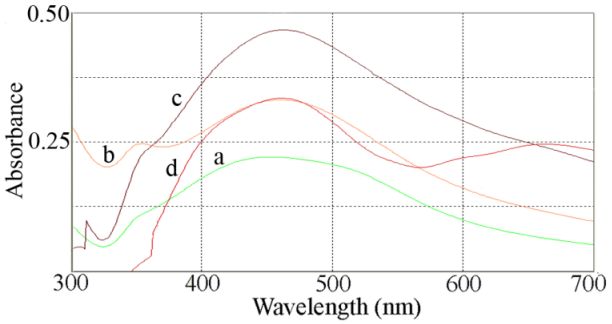

Figure 2. UV-Vis spectra of AgNPs prepared in various amounts of Alcea rosea flower extract: a) 10, b) 20, c) 40 and d) 70% of the reaction volume.

By increasing the volume of flower extract to more than 40 vol.% of the total reaction volume, a second absorption peak appeared at about 660 nm. As shown in TEM micrographs (Fig. 3b), appearance of this second peak is due to the formation of the second population of large particles.

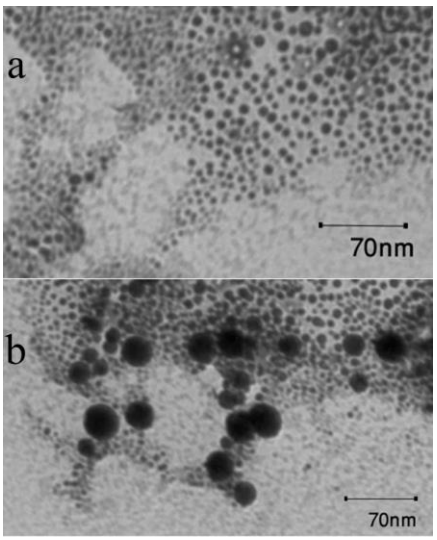

Figure 3. TEM micrographs of the prepared AgNPs at various amounts of flower extract: a) 40 and b) 70 vol.% of the total reaction volume.

The UV-Vis spectra of the prepared particles at different temperatures are shown in Fig. 4. Increasing

the reaction temperature resulted in significant increase of the AgNPs concentration, as shown by the hyperchromic shift in the SPR band. However, the reaction temperature increase resulted in the appearance of a shoulder in UV-Vis absorption spectra, indicating the formation of poly-disperse AgNPs [12]. According to the results, room temperature is the optimal temperature for bioreduction of AgNPs by *Alcea rosea* flower extract. Conducting reaction in the ambient condition could considerably reduce the energy cost, which is one of the most important issues in the scale-up process. A flowchart diagram of the biosynthesis process is illustrated in Fig. 5.

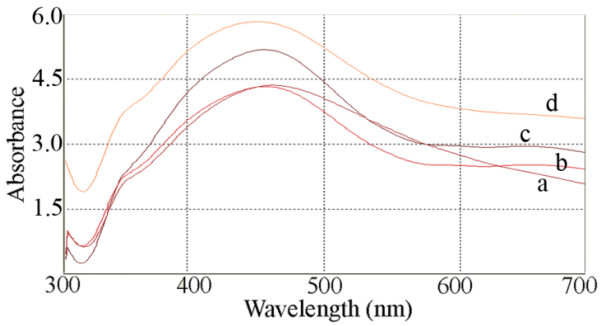

Figure 4: UV-Vis spectra of the prepared AgNPs at various temperatures: a) 27, b) 40, c) 50 and d) 70 °C.

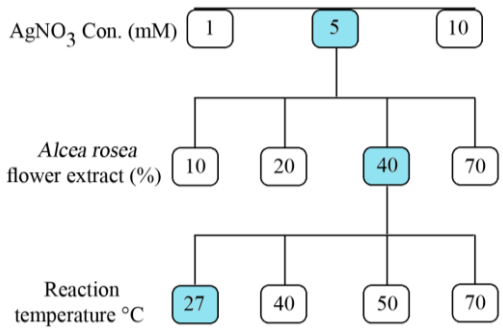

Figure 5. The flowchart diagram of the biosynthesis process.

Characterizations of AgNPs

Particle size distribution was determined by measuring diameters of one hundred nanoparticles randomly selected on the TEM images [27]. As shown in Fig 6, the prepared particles were spherical in shape with the average diameter of 7.2 nm. The prepared particles were spherical in shape with the average diameter of 7.2 nm. The crystallinity of the particles was evaluated by X-ray powder diffraction patterns (XRD, Siemens D5000) using drop coated films on a glass slide [6,9]. As shown in Fig. 7, four main characteristic diffraction peaks for silver were observed at 2θ values 38.2, 44.4, 64.7 and 77.4° due to reflection from the crystal facets of (111), (200), (220) and (311), respectively (JCPDS, silver file No. 04--0783) [11,13]. Three peaks around $2\theta = 32°$ are indicated by asterisks. Some researchers have attributed these peaks to the interaction of silver nitrate with biologic matrixes [28,29].

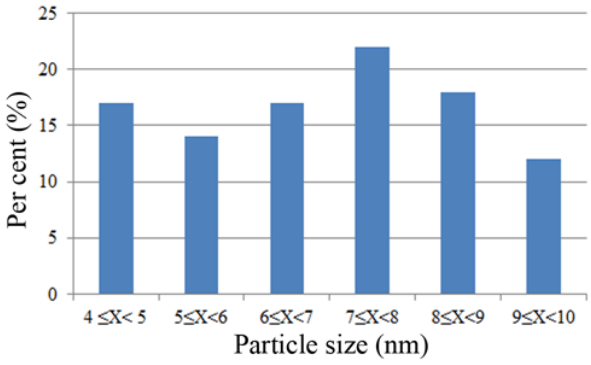

Figure 6. Particles size distribution of the prepared AgNPs.

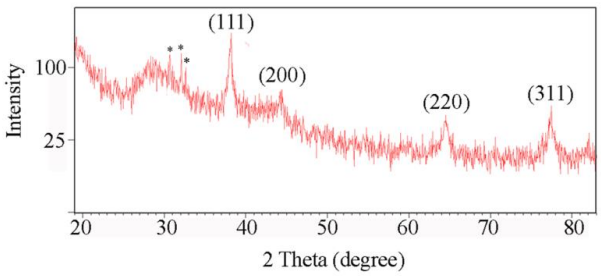

Figure 7. XRD pattern of AgNPs indicating four main characteristic peaks for silver, the peaks indicated by asterisk are from mineral complexes.

FTIR spectra of AgNPs and *Alcea rosea* flower extract are depicted in Fig. 8. The bands at 1059 and 1261 cm^{-1} are from C–O and C–C stretching vibrations, respectively. The peak with medium intensity at 1421 cm^{-1} could be due to C–H bending vibrations. Stretching vibrations of aliphatic C–H absorbed IR radiation at about 2925 cm^{-1}. The absorption peak from carbonyl groups appeared at 1630 cm^{-1}.

The broad absorption peak of hydrogen bonds from O–H groups can be seen at 3394 cm^{-1} which could overlap with the absorption from N–H bonds [23,30-32]. Similarity to the flower extract, AgNPs FTIR spectra indicates that AgNPs are capped with biochemical compounds from *Alcea rosea* flower extract. As it could be observed in the FTIR spectrum, the main peaks come from oxygen-bearing functional groups. It is widely believed that oxygen-containing functionalities are necessary for anchoring of the metal nanoparticles, and silver ions could easily oxidize these groups [25].

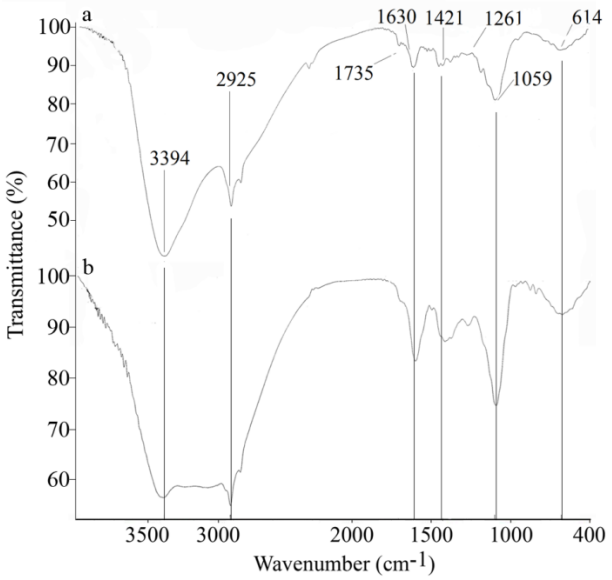

Figure 8. FTIR spectra of AgNPs (a) and Alcea rosea flower extract (b).

Antibacterial assay

Prepared nanoparticles have shown intense effect on the bacterial growth (Fig. 9). The *MIC* concentrations for *S. aureus* and *E. coli* were determined to be 37.5 μg/ml, which is acceptable compared to the previously reported concentrations, 10-60 μg/ml [33–35]. Silver ions and silver based compounds have strong antimicrobial effects and have been used for decades as antimicrobial agents in various fields [36,37]. AgNPs provide high fraction of exposed atoms due to their extremely small size and thus expand the contact surface of silver with microorganisms. It has been confirmed that antimicrobial properties of AgNPs are due to oxidation of the exposed silver atoms and release of Ag^+ from the surface of AgNPs [37]. Exposure to the air promoted Ag^+ release and resulted in 2.3-fold increase in the AgNPs antimicrobial effects [36]. Silver ions are potent oxidants and can destroy variety of cellular structures. The Ag^+ enter into the bacterial cells by penetrating through the cell wall and consequently turn the DNA molecule into condensed form which results in the cell death. In addition, it was also shown that Ag^+ binds to functional groups of proteins, resulting in protein denaturation [35,38]. Metal nanoparticles and particularly AgNPs are able to destroy the permeability of the bacterial membranes [34,39]. Exposure of bacterial membranes to the AgNPs resulted in the leakage of reducing sugars and proteins and induced the respiratory chain dehydrogenases into inactive state [34].

Commonly used antibiotics act very specifically and target exact physiological points in the microorganisms. This precise strategy provides a chance for some mutant strains to escape and distribute. To alleviate this condition and reduce the probability of new resistant strain appearance, multidrug therapies have been developed and used; however, this strategy is ineffective against multidrug resistant strains. Interestingly, released Ag^+ from AgNPs with multi-targeting antimicrobial mechanism of action, significantly reduced the chance for mutation and development of a bacterial resistance mechanism. In addition, AgNPs could increase the potency of the common antibiotics [33,40-42].

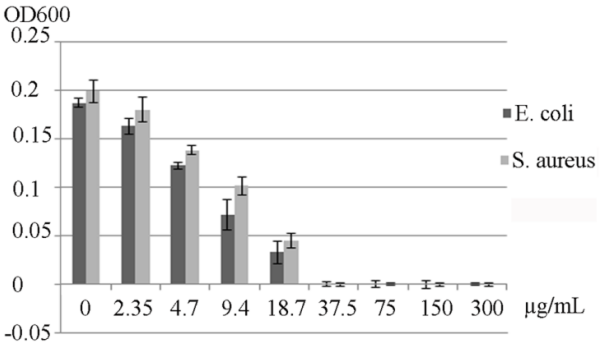

Figure 9. Antimicrobial effect of AgNPs against E. coli and S. aureus.

CONCLUSION

Alcea rosea flower extract contains bioactive compounds that are effective in bioreduction of Ag^+. These biochemical compounds contain oxygen bearing functional groups, which act as an anchor for Ag^+. Subsequently, produced AgNPs are capped with hydrophilic biochemical compounds, which make them colloidally stable. Reaction conditions such as silver precursor concentration, amount of flower extract and reaction temperature are the key factors in preparation of quality-based AgNPs. Therefore, these factors should be controlled in order to produce uniform particles with narrow particle size distribution.

Acknowledgments

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NAUČNI RAD

ZELENA SINTEZA I KARAKTERIZACIJA NANOČESTICA SREBRA POMOĆU EKSTRAKTA CVETA *Alcea rosea* KAO ANTIMIKROBNOG SREDSTVA NOVE GENERACIJE

U radu je razvijena zelena sinteza nanočestica srebra (AgNP) tretiranjem Ag^+ ekstraktom cveta Alcea rosea. Utvrđeno je da su koncentracija $AgNO_3$, količina ekstrakta cveta i temperatura reakcije značajni faktori ove bioredukcione reakcije. Dobijene AgNP čestice su sfernog oblika sa prosečnim prečnikom od 7,2 nm. FTIR analiza je pokazala da su za redukciju Ag^+ odgovorne funkcionalne grupe sa kiseonikom prisutne u ekstraktu cveta A. rosea. Određena je minimalna inhibitorna koncentracija (MIC) AgNP za Gram-pozitivne (Staphylococcus aureus) i Gram-negativne (Escherichia coli) bakterije i ona iznosi 37,5 μg/ml.

Ključne reči: Alcea rosea, biohemijska redukcija, biosinteza, zelena sinteza, Ag nanočestice.