The effect of volume and concentration of AgNO₃ aqueous solutions on silver nanoparticles synthesized using Ziziphus Spina–Christi leaf extract and their antibacterial activity

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Abstract: In the present study, silver nanoparticles (SNPs) were synthesized using an aqueous leaf extract of Ziziphus spina–Christi (ZSC). The volume and the concentration of the aqueous silver nitrate (AgNO₃) were studied to evaluate their effects on the synthesized SNPs. A various AgNO₃ volume of (10, 20, 30, 40, and 50 mL) having a constant concentration were mixed separately with a fixed concentration of ZSC leaf extract. Moreover, AgNO₃ with various concentrations (0.5, 1, 2, 4, 6, 8, and 10 mM) were investigated to synthesize SNPs. The optical, surface morphological, and antibacterial properties were studied for these SNPs. The optical properties were characterized using UV-Visible spectra. The particle size and morphology were checked using a dynamic light scattering (LDS) and Transmission Electron Microscopy (TEM). All the synthesized particles were spherical in shape and well-dispersed with average sizes (21-42 nm). The SNPs prepared by varying AgNO₃ volumes have an average size of (23 nm). The variation of AgNO₃ concentration has a redshift in the surface plasmon resonant (SPR) band which indicates an increase in the size of particles (25-42 nm) as confirmed by TEM. The biosynthesized SNPs exhibited good antibacterial activities against Staphylococcus aureus (S. aureus) and Escherichia coli (Esch. coli) bacteria.

Keywords: Silver nanoparticles, Ziziphus spina–Christi, Antibacterial activity, Staphylococcus aureus, Escherichia coli.

1. Introduction
Silver nanoparticles (SNPs) have captured the attention of many researchers because of their various and fascinating applications in different fields. SNPs are among the most attractive nanomaterials that help to expand the commercialization of silver-based industrial products. SNPs are involved in many applications such as engineering, biomedical and agricultural sciences [1, 2, 3, 4]. Several methods and approaches for the synthesis of SNPs have now been documented using chemical [5], physical [6], photochemical [7], and biological routes [8]. These methods have advantages and disadvantages in expense, scalability, particle size and size distribution, and so on as popular problems. Besides,
physical and chemical methods employ toxic substances that restrict their applications [9]. Therefore, the usage of the green approach to synthesize SNPs is preferred and may be suitable for large-scale synthesis of nanoparticles, given the lower cost and limited availability of the main material. In this method, most plant extracts can be used because they have the majority of antioxidants that could act as reducing agents [10]. A lot of research work, therefore, needs to be done in synthesizing SNPs using the different leaf of plants, and their suitability in some applications is required to be discovered.

*Ziziphus spina-Christi* (ZSC), also known as Sidr or Nabq, draws the interest of many researchers for its widespread use in nutritional and medicinal aspects [11, 12, 13, 14, 15]. The plant belongs to the family of Rhamnaceae and these trees have an immense length of life and can withstand and thrive in desert areas at high temperatures [14,16]. Their leaves and cortex are used in the treatment of wounds and skin diseases. Plant extract is also active against certain pathogenic bacterial strains and, thus, the plant extract can be useful for treating the disease caused by these bacteria [13]. ZSC tree imposes itself as an important plant for microbiological studies and it can be said that positive results would be predicted when ZSC assists in the preparation processes for antibacterial compounds.

Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli) live on the body surface of mammals and sometimes cause infection to them. The bacteria are among the most widespread gram-positive and gram-negative bacteria, respectively [17]. Infections of these bacteria pose a significant threat to humans as well as to animals and can cause a wide variety of infections [17, 18, 19]. As an antimicrobial substance, several materials have been used against S. aureus and Esch. coli [20, 21, 22, 23, 24, 25]. SNPs have high microorganism cytotoxicity and are widely used as an antibacterial agent [26]. The antimicrobial activity of SNPs is comparatively better than most commonly used antibiotics worldwide [27]. Researchers recently discovered that the use of plants with a medicinal history in the SNP green synthesis process offers significant outcomes in microbiology [28].

The goal of this research is to investigate the use of ZSC leaf extract in SNP synthesis and to assess the antibacterial activity of synthesized SNPs against *S. aureus* and *E. coli*.

2. Materials and Methods

2.1 Materials

Fresh and healthy ZSC leaves were identified by life sciences department, college of science. The leaves were collected in summer 2018 from campus garden of Hadhramout University, Mukalla Yemen. Analytical-grade silver nitrate (AgNO₃) was purchased from Fisher Scientific, UK. There was no further purification of the chemicals and they were used as produced. For rinsing and other aqueous solutions preparation, deionized (DI) water (18 MΩ resistivity, Milli-Q method, Millipore Inc.) was used.

2.2 Crude Extracts Preparation

The leaves of ZSC were refluxed into flowing tap water and washed several times with DI water. Once the debris and other contaminating organic materials were completely removed, 10 g of cleaned and chopped leaves were put in a beaker holding 100 ml of DI water and boiled at 60°C for 60 min. When the mixture color became greenish-yellow, the mixture was allowed to cool down to room temperature. After grinding the mixture, the Whatman No.1 filter paper (pore size 25 μm) was used to revise the product. The leaf extract was accompanied by centrifuging (Clifton Refrigerated Centrifuge NE040) procedure at room temperature and 4000 rpm for 10 min to isolate and extract insoluble fractions. The centrifuged ZSC leaf extract was stored at 4°C before further analysis was needed.

2.3 Green synthesis of SNPs

In a typical reaction procedure, 10 mL of aqueous AgNO₃ solution (1 mM) was stirred. About 1 mL of ZSC leaf extract was added gradually to the aqueous AgNO₃ solution. The reaction was completed at a constant temperature of 30°C (room temperature). An identical procedure, with no modifications to AgNO₃ concentration (1mM), was replicated with a different volume of AgNO₃ (20, 30, 40, and 50 mL). Also, to examine the influence of the AgNO₃ concentration on the SNPs synthesis, 50 ml of AgNO₃ with various concentrations (0.5, 1, 2, 4, 6, 8, and 10 mM) were mixed...
separately with 1 ml of leaf extract.

2.4 Characterization techniques

The size, morphology, and composition of the generated nanoparticles were analyzed using various characterization techniques. Using a quartz cuvette with an optical path of 1 cm, the UV –vis absorption spectra of SNPs dispersions were confirmed on a spectrophotometer (Jasco V-670) within the range of 200–900 nm. In all measurements, the silverless leaf extract solution was used as a reference sample for background absorption. The scale and structure of the SNPs were investigated using high-resolution electron microscopy transmission (HRTEM, JEOL JEM2100) running at a 200 kV accelerating voltage. The dynamic light scattering or DLS (PSS-NICOMP 380- ZLS particle sizing system St. Barbara, California, USA) was used to measure particle size.

2.5 Antibacterial Activity

To examine the antibacterial activities of the biological synthesized SNPs, the cup-plate agar diffusion method was applied to study the antibacterial activity of the synthesized silver nanoparticles with a minor modification. 1 mL of standardized S. aureus and Esch. coli bacterial stock suspensions (108–109) CFU/ml was thoroughly mixed with 250 ml of sterile nutrient agar medium. 20 ml of the inoculated nutrient agar medium was distributed into sterile Petri dishes. The agar was left to set and was cut with a sterile cork borer in each plate from 3 to 4 cups, 10 mm in diameter and the agar disks were withdrawn. Each cup was filled with 80 μl of samples and allowed to diffuse at room temperature for 2hrs. Then the plates incubated in the upright position at 37°C for 24 hrs. The investigation of the antibacterial activity for all samples was achieved by measuring the inhibition zone. The diameter of the inhibition zone was measured averaged and the mean values were recorded [29].

2. Results and Discussion

3.1 Variation of AgNO₃ volume

When the SNPs were prepared using the mixture of AgNO₃ as a metal source and ZSC leaf extract as a reducing and capping agent, a change in mixture color was noticed. The mixture began with faint light green, and in the second stage, the color changed to a dark brownish which probably related to the reduction of Ag⁺ to Ag₀. Before completing 1 h of the stirring process, the solution color stabilized to its final color which is dark orang (Fig. 1) [30]. It can be noted that the change in mixture color is carried out due to the excitation of surface Plasmon vibrations for the existed silver nanoparticles in the mixture. Moreover, no observable change was noticed in the mixture color during the investigation period of more than 12 months.

Like all metals, silver has free electrons on the surface of the particle, the SPR band of absorption happens due to the simultaneous vibration of these electrons with the incident light, which must be in resonance with the wave of the incident lights. The bioreducing process of silver ions to SNPs was tracked using absorption data recorded by UV-vis spectroscopy. The UV-vis absorption spectra of SNPs prepared by different volumes of AgNO₃ (0, 10, 20, 30, 40, and 50 mL) are illustrated in Fig. 2a. The spectra, for all the samples, show mostly a single and narrow absorption peak except for pure ZSC leaf extract where no peak is observed. The surface plasmon resonance (SPR) band was found to be between 428-430 nm for each sample. The identical SPR band could confirm that all the samples have almost the same average particle size [31]. However, when a Gaussian fitting for the localized peak (330- 550 nm) was applied to the recorded UV-vis data (inset Fig. 2b), the intensity of the SPR band decreases with the increase in the AgNO₃ quantity (Fig.2a). While the full width at half maximum (FWHM) increases with the increasing in AgNO₃ quantity until gets saturated. The sample of 10 mL recorded the highest intensity and lowest FWHM (150 nm), and the sample prepared from 50 mL showed the lowest intensity and highest FWHM (157 nm).
It can be noted that the present method showed nearly no change in average particle size (23 nm) when the quantity of the precursor AgNO₃ was changed from 10 to 50 mL. These results were confirmed using the DLS analysis (inset Fig.2e). If the UV-vis intensity is a function of the Ag₀ₗ, a remarkable change was observed in the UV-vis intensity which indicates that a higher number of silver ions were converted to SNPs. Also, if the FWHM of SPR is considered to be a good indicator to identify the uniformity of the particle size (size distribution), Fig.2b shows that the sample prepared by 10 mL has a narrow SPR peak. This narrow peak suggests that the 10 mL sample has better particle size uniformity among all the samples. Moreover, the SPR peak was getting wider when AgNO₃ increased.

In its chemical structure, *Ziziphus spina-Christi* (ZSC) contains several compounds, such as Steroids, β-sitosterol, β-D-glucoside, and condensed tannins. Free sugars, including fructose, raffinose, sucrose, glucose, galactose, and rhamnose, are also present [32]. Using the fact that ZSC leaf extract contains enzymes and protein, the obtained UV-vis results could be interpreted. The enzymes react with silver nitrate by converting Ag⁺ to Ag₀ resulting in silver nanoparticles. The protein works as a capping agent preventing more silver atoms to combine in order to form bigger silver nanoparticle size [33]. In the present case, a fixed concentration of AgNO₃ produces mostly SNPs with constant average particle size. Similar results were observed when a constant concentration of AgNO₃ (50mL and 1 mM) was mixed with a variable quantities (10, 20, 30, 40, and 50 mL) of the ZSC leaf extraction (Fig. 2c).

The 10 mL sample (Fig. 2a) shows higher intensity in the SPR band which is probably due to the comparable higher concentration of the enzyme. When 20 to 50 mL of AgNO₃ mixing with 1 mL leaf extract, no enough comparably enzyme in the mixture was able to convert all the available Ag⁺, and as a sequence, a low number of silver nanoparticles is produced (lower absorption intensity). Furthermore, in the case of the 10 mL, Ag capping takes place faster because of the higher concentration of the protein, and that makes silver particles have less chance to agglomerate. On the other hand, increasing the quantity of AgNO₃ from 20 to 50 is expected to decrease the capping agent due to protein dilution, so agglomeration takes place and as a sequence, the shape of the SPR peak becomes broader. This could explain why the SPR peak of the 20, 30, 40, and 50 mL sample is broader.
than that of the 10 mL sample. Fig.2 d, illustrate a snapshot of all the suspension SNPs samples prepared by changing the quantity of precursor aquatic AgNO₃.

Figure 2: (a) UV-vis absorption spectra for SNPs prepared by different volume of AgNO₃, (b) Gauss fit of SPR, (c) UV-vis absorption spectra of SNPs prepared by different volumes of ZSC extract, (d) Dispersive SNPs prepared variable AgNO₃, (e) TEM of SNPs synthesized by mixing 50 mL of AgNO₃ (1mM) with 1 mL of leaf extract.

Silver nanoparticles are more widely used for medical purposes because of their antimicrobial characteristics. The results of the antibacterial activity effect of ZSC were simply determined by the cup plate method. The diameter (in mm) of the inhibition zone was determined after 24 h incubation at 37°C. Fig.3 demonstrates the antibacterial test results of the SNPs synthesized from various AgNO₃ aquatic solutions against the E. coli and S. aureus. No zone of inhibition was observed for gram-negative E. coli and gram-positive S. aureus bacteria when ZSC leaf extract (No silver presence) was tested. Also, the SNPs (prepared from the 10 mL) create inhibition zone when was tested. However, when SNPs prepared from 20 mL were applied, an inhibition zone of 12 mm for E. coli was observed, whereas no inhibition zone was produced for S. aureus by the sample prepared by 20 mL. When the SNPs prepared by 50 mL of AgNO₃ was applied to E. coli and S. aureus, the maximum inhibition region was 15 mm and 10 mm respectively (Fig. 3c). Therefore, further studies are then required to improve this sample (50 mL) by changing the concentration of precursor aquatic solution AgNO₃.
3.2. Variation of AgNO$_3$ concentration

When 50 mL of AgNO$_3$ (1mM), showed the maximum inhibition zone for both E. coli and S. aureus, attempts were made to investigate the effect of AgNO$_3$ concentration on synthesizing SNPs. By mixing 1 ml of ZSC leaf extract with 50 mL of AgNO$_3$ having different concentrations (0.5, 1, 2, 4, 6, 8, and 10 mM), new seven samples of SNPs were produced. The UV-vis data of these samples are illustrated in Fig. 4a. The UV-vis shows a slight change in localized SPR band when the AgNO$_3$ concentration varies from 0.5 mM to 4 mM with an average of $\lambda_{\text{max}} = 430$ nm. The maximum wavelength $\lambda_{\text{max}}$, the FWHM of the SPR bands, and the average particle size for all the samples are summarized in table 1. The maximum wavelength $\lambda_{\text{max}}$ was observed between (429-446 nm). According to the calculated FWHM of the localized peak in the UV-vis data, the monodispersity was enhanced by increasing the concentration of AgNO$_3$. The UV-vis exhibits a redshift in the SPR band, which raises when AgNO$_3$ concentration varies from 6 to 10 mM. The redshift in SPR band suggested that excess Ag$^+$ ions would contribute to SNPs growth and lead to increased particle size. These results could be attributed to aggregation and/or agglomeration [34], which is likely to contribute to a particle diameter rise, as verified by the DLS data seen in Table 1.
Figure 4: SNPs synthesized using different concentrations of AgNO₃ (0.5-10 mM and a constant extract quantity (50 ml)), (a) UV-Vis for all concentrations, (b) Estimated FWHM of SPR peaks, TEM image for sample prepared with (c) 0.5 mM and (d) 10 mM.

The TEM images of all the SNPs samples show that the particles are nearly spherical with smooth surface morphology. Table 1 shows that the size of the synthesized silver particles increases with the rising concentration of AgNO₃. Such observations correspond well with the UV-vision spectra obtained, in which the peak of absorption shifted towards the higher wavelength of visible light, indicating that the particle size is increasing. The results also matched well with what Zayed reported despite the use of methanol in the extraction method, the tested quantity of the leaf extract and the AgNO₃, and the higher percentage of the extract to a metal that used (0.04) [34].

| AgNO₃ Cons.(mM) | SPR Band (nm) | FWHM(nm) | Diameter (nm) |
|----------------|---------------|-----------|---------------|
| 0.5            | 429           | 171.55    | 25            |
| 1              | 429           | 148.39    | 26            |
| 2              | 430           | 138.99    | 29            |
| 4              | 430           | 128.25    | 30            |
| 6              | 434           | 121.25    | 32            |
| 8              | 437           | 117.08    | 38            |
| 10             | 446           | 112.51    | 42            |

Table 1.: Variation of average particles size and SPR band for SNPs prepared by different concentration of AgNO₃.

Figure 4 a-c shows the antibacterial activities of SNPs synthesized by changing the precursor AgNO₃ concentrations. Fig 4a showed an inhibition zone of (0 mm) millimeters and (19 mm) for E. coli using leaf extract and SNPs prepared by 10 mM of AgNO₃. Also, Fig 4b shows an inhibition zone of (0 mm) and (17 mm) for S. aureus using leaf extract and SNPs were prepared by 4 mM of AgNO₃. Fig. 4c shows the antibacterial activity influence of SNP's prepared against E. coli and S. aureus at varying AgNO₃ concentrations. For E. coli and S. aureus, 20 and 18 mm respectively were the
maximum inhibition zones. The SNPs prepared with AgNO₃ (6 mM) showed an inhibition zone of 20 mm, while samples prepared with 2 mM showed an inhibition zone of 18 mm. It is observed that SNPs prepared by 0.5 mM of AgNO₃ do not impact S. aureus, while a 12 mm inhibition zone on E. coli is seen when the same sample was tested. These results could be due to the thick peptidoglycan layer (20–80 nm) found in the gram-positive bacteria (S. aureus) and the thin gram-negative (E. coli) peptidoglycan layer (7–8 nm) [35]. Silver nanoparticles are causing toxicity on bacteria which could be due to the formation of Ag⁺ ions when these SNPs react in the presence of oxygen. The antibacterial behavior of SNPs depends on particle size and the peptidoglycan layer of bacteria requires a high density of lower-sized nanoparticles [36]. So, these facts could clarify why there is no substantial difference in the S. aureus inhibition zone produced by 2 mM and 4 mM samples. The larger particle size produced utilizing higher concentrations of AgNO₃ (> 4 mM) and the lower silver density produced by lower concentrated AgNO₃ (<2 mM) may be the explanation behind the lower inhibition zone of the produced SNPs. In the case of E. coli, the observed result (inhibition zone of 19 mm) that occurred by the sample with the average largest particle size (42 nm) is probably due to these particles along with the large quantity of AgNO₃ that the leaf extract was not enough to oxidize.

![Image](image_url)

**Figure 5:** Anti-bacterial activities of ZSC leaf extract and SNPs against (a) E. coli (b) S. aureus, (c) effect of AgNO₃ concentration on E. coli and S. aureus and (d) effect of SNPs dose.

Different suspension SNPs (10, 20, 40, 60, 80, and 100 µL) were investigated to analyze the lowest dose which could inhibit E. coli and S. aureus bacteria. Fig. 5c reveals that 40, 60, 80 and 100 µL could inhibit the growth of the gram-negative E. coli bacteria with a zone of 20 mm, while only 80 and 100 µL could inhibit the growth of the gram-positive S. aureus.

4. **Conclusion**

The current study shows that SNPs are easily, economically, and greenly synthesized with aqueous Ziziphus spina-Christi leaf excerpts. All the characterization techniques confirmed the successful formation of spherical SNPs with particle size between (23-42 nm). No remarkable change in the average particles size was observed when different volume of AgNO₃ and ZSC leaf extract were mixed with the 1 mM AgNO₃ concentration. A change in particle size was observed when higher concentrated AgNO₃ was used. It was observed that a lower concentration of the percussed AgNO₃ increased the monodispersity of the synthesized SNPs. The SNPs with the spherical shapes particles show different
anti-bacterial activity against the gram- positive *S. aureus* and gram-negative *E. coli* bacteria. However, the SNPs showed much greater anti-microbial activity than the ZSC leaf extract showed and lower than 100 µL dose of SNPs could be enough to inhibit the studied bacteria.

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