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Green synthesis of non-toxic silver nanoparticles using *Salvia tebesana* Bunge extract: Optimization, cytotoxicity, and antibacterial activities

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ABSTRACT

The remarkable antibacterial activity and potential biomedical applications of silver nanoparticles (AgNPs) synthesized through eco-friendly approaches have garnered significant attention in recent years. This research aimed to introduce a green synthesis method for AgNPs using *Salvia tebesana (S. tebesana)* Bunge extract and to investigate their properties, antibacterial activities, and cytotoxicity effects. The fabricated AgNPs were analyzed with various analyses, including UV–Vis, DLS, XRD, FT-IR, and TEM analysis. The broth microdilution assay was applied to measure the Minimum Inhibitory Concentrations (MIC) of chemical AgNPs, biosynthesized AgNPs, and *S. tebesana* Bunge leaves extract alone against selected standard bacteria strains. The biosynthesized AgNPs displayed a surface plasmon resonance peak at approximately 415 nm, and the AgNPs synthesized using *S. tebesana* Bunge extract had a spherical configuration with an average size in the range of 10–15 nm. Bio-synthesized nanoparticles showed a noteworthy antibacterial activity compared to chemical nanoparticles, particularly against *P. aeruginosa*, with the highest antibacterial activity reported at a MIC value of 39.06 μg/mL. AgNPs synthesized using *S. tebesana Bunge* extract demonstrated a significant decrease in fibroblast cell viability, but only when the concentration reached 2 mg/mL. The findings demonstrate that *S. tebesana* Bunge leaves extract enhances the antibacterial properties of AgNPs, and also represents an appropriate and biocompatible option for the synthesis of these nanoparticles. Our research highlights the potential of employing bio-safe and eco-friendly AgNPs synthesized in the presence of *S. tebesana* Bunge extract, which possess remarkable anti-bacterial properties, for various biomedical applications.

1. Introduction

Nowadays, the rise of antibiotic resistance has become a significant global health concern, with antibiotic-resistant bacteria being recognized as a major factor contributing to hospital and community-acquired infections worldwide. The ever-increasing incidence of these infectious agents leads to limitations in the use of effective antibiotics, and without the development of new antibiotics or the discovery of new antibacterial compounds, there will be no effective treatment options to deal with various infections [1,2].

The rapid advancement of nanotechnology has led to extensive research on the fabrication and usisng of nanoparticles. In the recent years, the nanotechnology (nanoparticle, nanocomposite, nanostructure) has been used in the various fields, including pharmacy [3,4], medicine {Zare-Bidaki, 2023 #50}[5–7], environment [8,9], etc. Among various types of metal nanoparticles, silver nanoparticles (AgNPs) have attracted considerable attention owing to their unique properties and the broad applications. Traditional routs for synthesizing AgNPs involve the use of chemical agents, which can be expensive, hazardous, and environmentally unfriendly [10,11]. In past decades, there has been a growing interest in developing alternative methods for the synthesis of silver nanoparticles using natural sources, which are eco-friendly, cost-effective, and sustainable approach. The utilization of natural extracts as reducing and capping agents offers a promising

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pathway to generate AgNPs with enhanced properties and reduced environmental toxicity [12,13].

In recent years, there has been noteworthy focus on the utilization of plant extracts for the eco-friendly green synthesis of AgNPs. *Salvia tebesana* Bunge, a plant species belonging to the Lamiaceae family, is a medicinal plant widely distributed in many regions and presents a unique opportunity to explore its potential for nanoparticle synthesis. The leaf extract of *S. tebesana* Bunge contains various bioactive compounds, such as phenols, flavonoids, terpenoids, and alkaloids, which have been reported to possess antioxidant, antimicrobial, and anticancer activities. These bioactive compounds can potentially act as stabilizers and reducing agents in the green synthesis of AgNPs [14,15].

AgNPs exhibit potent antibacterial effects due to their high surface area, which enhances direct contact with bacterial cells, and their ability to release silver ions, which can disrupt microbial cell membranes and enzymes. The unique antimicrobial properties exhibited by the AgNPs can potentially pave the way for their application in combating drug-resistant strains and controlling infectious diseases. Furthermore, cytotoxicity assessment of the synthesized AgNPs is essential to determine their potential impact on human cells. The biocompatibility and safety of nanoparticles are crucial factors for their successful use in therapeutic applications [16,17].

Given the importance of using green synthesis technology as a eco-friendly and cost-effective approach for synthesizing AgNPs, as well as the unique properties and activities of AgNPs synthesized through green methods and their various medical applications, this study aims to explore the green synthesis of silver nanoparticles using *S. tebesana* Bunge leaf extract and evaluate their characterization, antibacterial activity, and cytotoxicity.

2. Materials and methods

2.1. Materials and characterization methods

The leaves of the *S. tebesana* Bunge plant collected from the surrounding areas of Tabas City in South Khorasan province (Iran) were identified by a botanist from the agriculture department at Birjand University with the code number 24864. The silver nitrate ($AgNO_3$), sodium citrate ($Na_3C_6H_5O_7$), MTT cell proliferation assay kit, and Tetracycline powder were purchased from Sigma-Aldrich Company (USA). DMEM medium and fetal bovine serum (FBS) was obtained from GIBCO Company (USA). The Pasteur Institute of Iran (Tehran, Iran) provided five standard bacterial strains, which included *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and *Klebsiella pneumonia* ATCC 9997. The identification of functional groups employed Fourier Transform Infrared Spectroscopy (FT-IR) (Perkin-Elmer, USA), direct size and morphological observation of products utilized Transmission Electron Microscopy (TEM) (Zeiss, EM10C, Germany), determination of crystal structure of particles was carried out by X-ray Diffraction (XRD) (Philips X'pert Pro, Netherlands), and hydrodynamic diameter determination of silver nanoparticles involved Dynamic Light Scattering (DLS) analysis (Nano-ZS, Malvern, UK).

2.2. Preparation of S. Tebesana Bunge extract

To prepare the aqueous extract of the plant, 20 g of the *S. tebesana* Bunge leaves powder were soaked in 200 mL of distilled water. The resulting extract was centrifuged at 5500 rpm for 10 min, and the supernatant was filtered using Whatman No. 1 filter paper. The extract was finally stored at 4 °C for future use.

2.3. Synthesis of silver nanoparticles

AgNPs synthesis was conducted using two approaches: (1) through a chemical route, where 9 mg of $AgNO_3$ was dispersed in the 50 mL distilled water. Following that, a solution of 1 % sodium citrate (1 ml) was introduced to the mixture with continuous stirring, maintaining a room temperature. The solution's color changed from colorless to brown within one hour, indicating the successful formation of AgNPs. Within a duration of one hour, the color of the solution transformed from being colorless to brown, which clearly indicates the successful formation of AgNPs. (2) The green synthesis method involved the addition of 24 mg of $AgNO_3$ in a beaker containing 10 mL of deionized distilled water was placed on a stirrer under vigorous stirring at room temperature. At the same time, an aqueous solution of *S. tebesana* leaves extract was made by incorporating 100 mg of the plant extract into 10 mL of double-distill water and setting the pH at 12. The plant extract with a pH of 12 was slowly added drop by drop to the beaker containing the completely dissolved $AgNO_3$ in distilled water while stirring vigorously. The shift in color of the mixture, transitioning from pale yellow to deep brown, indicated the reduction of silver ions to Ag^0 NPs. Notably, several experiments were conducted to determine the optimal conditions for the green synthesis method. In these experiments, the concentration of plant extract remained constant while various parameters such as temperature (room temperature, 55 °C, and 85 °C), time (15, 20, 30, 45, and 60 min), and $AgNO_3$ concentration (5, 10, 15, 20, 25, and 30 mM) were varied. UV–Vis absorption spectroscopy was employed to monitor the biosynthesis process of AgNPs in the wavelength range of 300–500 nm.

2.4. Antibacterial assay

This research explored the antibacterial activity of the aqueous extract of *S. tebesana* Bunge leaves, chemical AgNPs, and green synthesized AgNPs, against several bacterial strains, including *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, and *K. pneumonia* ATCC 9997. The microdilution broth assay following the guidelines set by the Clinical and Laboratory Standards Institute (CLSI) [18] was utilized to determine the minimum inhibitory concentration (MIC). In summary, sterile polystyrene 96-well microtiter plates were used to dispense 100 μL of the test compounds with two-fold serial dilutions in Mueller Hinton broth (Merck, Germany). Subsequently, each well of the plates received an addition of 100 μl of a bacterial suspension containing a final concentration of 5×10^5 cfu/ml. After the treatment, the plates were placed in ambient air conditions and incubated at 37 °C for a period of 18–24 h. The positive and negative control in the experiment involved utilizing the bacterial suspension combined with the medium and solely the culture medium, respectively. In addition, the antibiotic tetracycline as an effective antibacterial agent was used to compare the antibacterial activity of the examined compounds.

2.5. Cell viability/proliferation assay

The MTT cell proliferation viability assay was employed to evaluate the cytotoxic potential of the test compounds, such as the *S. tebesana* Bunge leaves extract and synthesized AgNPs. Briefly, after preparing the single-cell suspension of human primary fibroblast cells and counting them, a concentration of 3×10^3 cells/well was cultured in 96-well microtiter plates containing 10 % FBS-supplemented DMEM medium overnight. Cells were treated with various concentrations of the investigated compounds for 24 and 48 h. After this time, 20 μL of MTT reagent (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. Then, the cell-culture supernatant was removed, and the formazan crystals produced by the living cells were dissolved by adding 100 μL of DMSO. Finally, the absorption of the plates was carefully measured at 570 nm using the Epoch Microplate Spectrophotometer (Biotek Instrument, USA). The estimation of cell proliferation/viability was determined using the following equation: $OD_{Sample}/OD_{Control} \times 100$, where OD was the absorbance of the sample (treated) or control (untreated). It should be noted that all study steps were repeated three times.

2.6. Statistical studies

Statistical analysis was performed using SPSS software version 19 and One-Way ANOVA for the cytotoxic results of the examined compounds. A significance level of $P < 0.05$ was considered for statistical analysis.

3. Results and discussion

3.1. Optimizing the synthesis of green AgNPs

The UV–Vis spectrum within a wavelength range of 300–500 nm was utilized in this study to confirm the creation of AgNPs and evaluate the impact of various factors such as temperature, time, and $AgNO_3$ concentration on their formation. The Surface Plasmon Resonance (SPR) reveals that the nanoparticles are uniformly distributed and not aggregated or sedimented. When the particle size decreases, the peak Plasmon resonance shifts towards shorter wavelengths.

3.1.1. Effect of $AgNO_3$ concentration

In this study, the investigation focused on different concentrations of $AgNO_3$ (ranging from 5-30 mM) to identify the optimal concentration required for nanoparticle formation in the presence of plant extract at room temperature. The absorption spectrum of nanoparticles indicated that augmenting the concentration of $AgNO_3$ up to 10 mM resulted in increased absorption band intensity. However, absorption spectrum decrease occurred in concentrations ranging from 15-30 mM (Fig. 1a), which was could be attributed to the aggregation of nanoparticles [19]. Hence, a concentration of 10 mM $AgNO_3$ was deemed optimal for the green synthesis of AgNPs.

3.1.2. Effect of contact time

The reaction between $AgNO_3$ and *S. tebesana* Bunge leaves extract was monitored for a period of 15 to 60 min at room temperature. It was found that extending the reaction time from 15 min to 30 min resulted in an increase in absorption intensity. However, there were no notable changes in absorption intensity beyond that duration (Fig. 1b). Therefore, duration of 30 min can be considered as ideal timeframe for the synthesis of green AgNPs.

3.1.3. Effect of reaction temperature

The UV/Vis spectra were utilized to investigate the impact of reaction temperature (room temperature, 55 °C, and 85 °C) on the size of nanoparticle products. The analysis of the absorption spectrum of nanoparticles at varying temperatures revealed that the absorption intensity increased as the temperature rose, reaching its peak at 55 °C (Fig. 1c). Additionally, the absorption peak shifted towards shorter wavelengths, signifying a reduction in nanoparticle size. As a result, the temperature of 55 °C was determined to be the optimal condition for the reaction.

3.2. DLS, XRD, FT-IR, and TEM analysis

Zeta potential and DLS techniques were employed to determine nanoparticle size (hydrodynamic droplet) and the surface charge of the fabricated AgNPs using *S. tebesana* Bunge extract. In Fig. 2a, the DLS graph shows that the hydrodynamic diameter of AgNPs was approximately 180 nm. The zeta potential of the biosynthesized AgNPs using *S. tebesana* Bunge was −38.82 mV indicating a negative charge on the surface of AgNPs by *S. tebesana* Bunge (Fig. 2b). In the present study, XRD analysis was utilized to ascertain both the crystal structure and

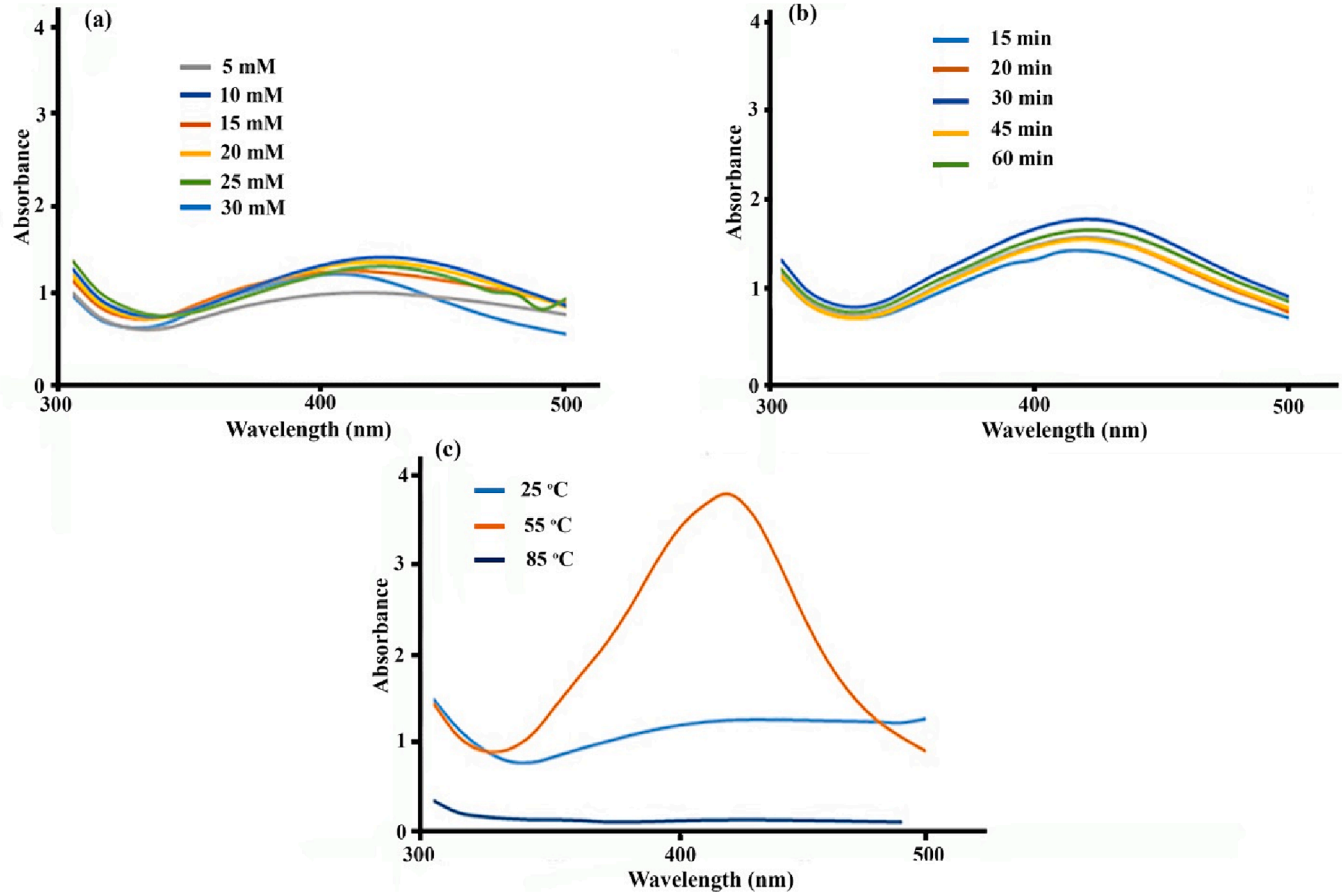

Fig. 1. UV–Vis spectra of AgNPs synthesized using *S. tebesana* Bunge leaves extract at various silver nitrate ($AgNO_3$) concentrations (a), times (b), and temperatures (c).

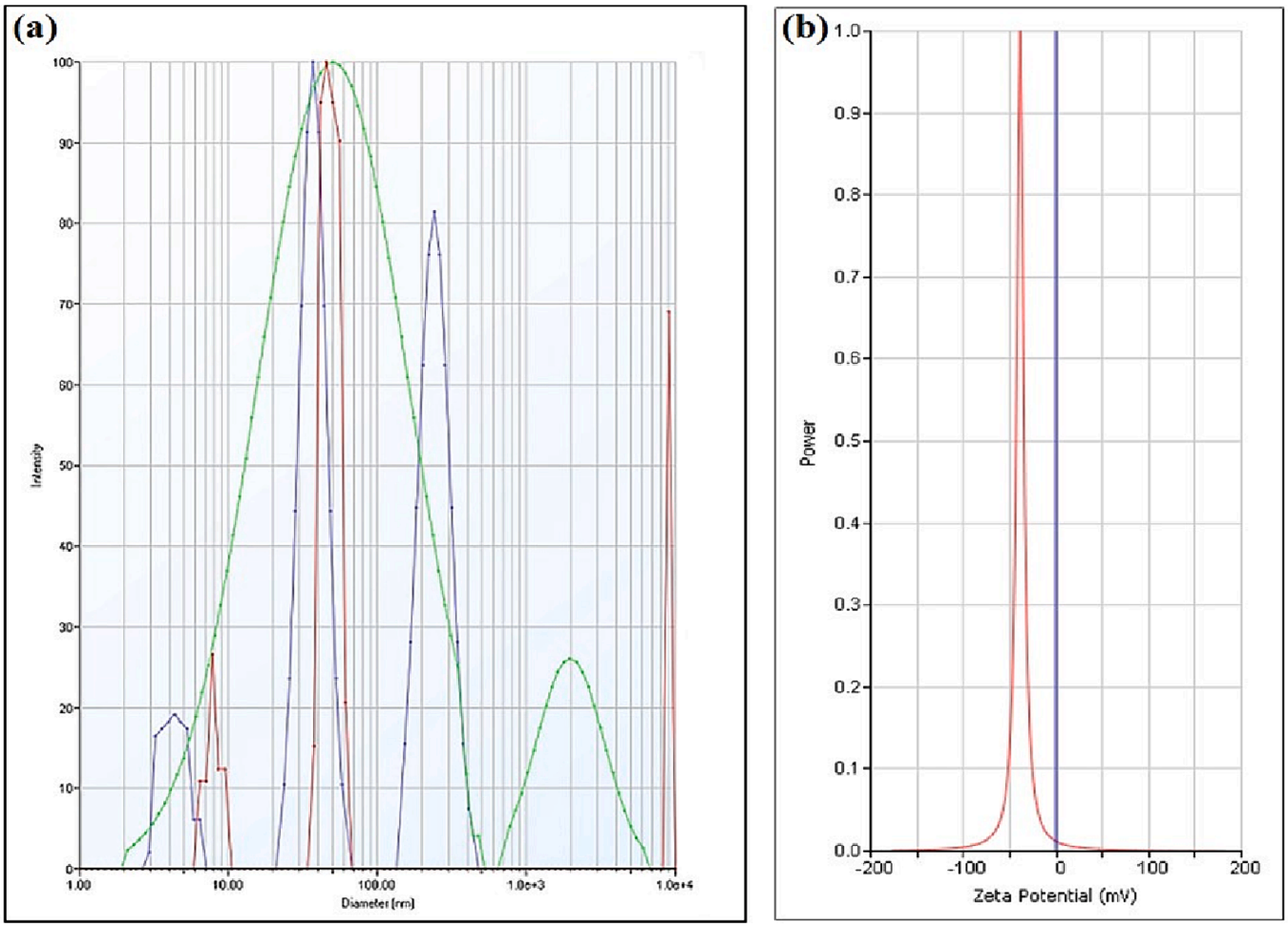

Fig. 2. DLS (a) and zeta potential (b) analysis of AgNPs synthesized using *S. tebesana* Bunge.

purity of the green synthesized nanoparticles. The XRD results demonstrate peaks observed at angles of 38.2°, 44.4°, 64.8°, and 77.8°, corresponding to the (111), (200), (220), and (311) Bragg planes, respectively (Fig. 3). These findings align with the standard diffraction pattern expected from cubic crystal structures of AgNPs (JCPDS 01–087-0717). This information is in accordance with the findings presented in published reports [20,21]. Three extra minor peaks were observed at 28.12°, 55.24°, and 58.5° , which might be associated with impurities of AgCl. The existence of chlorine ions in the solution causes this impurity, as they react with silver nitrate and result in the creation of chloride deposits [22]. Based on the results obtained and the Debye-Scherr equation, the reported size of silver nanoparticles was approximately 10–15 nm. The investigation of the functional groups present in the biosynthesized AgNPs samples was conducted using FT-IR spectroscopy (Fig. 4). The broadband peak observed around 3436.8 cm^{-1} was related to the stretching vibrations of the hydroxyl group in alcohols [23]. The band centered at about 2923.5 cm^{-1} can be attributed to the stretching vibration of the C-H bonds [24]. The intense peak at 1727.3 cm^{-1} is

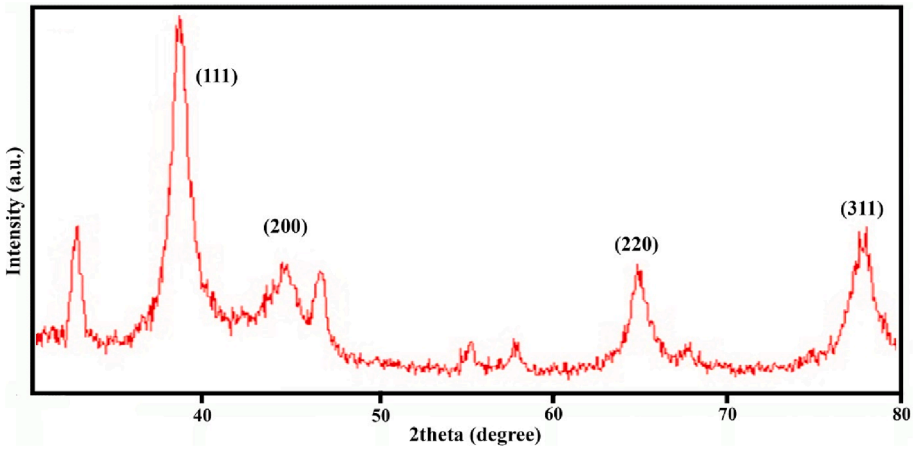

Fig. 3. XRD pattern of AgNPs synthesized using *S. tebesana* Bunge leaves extract.

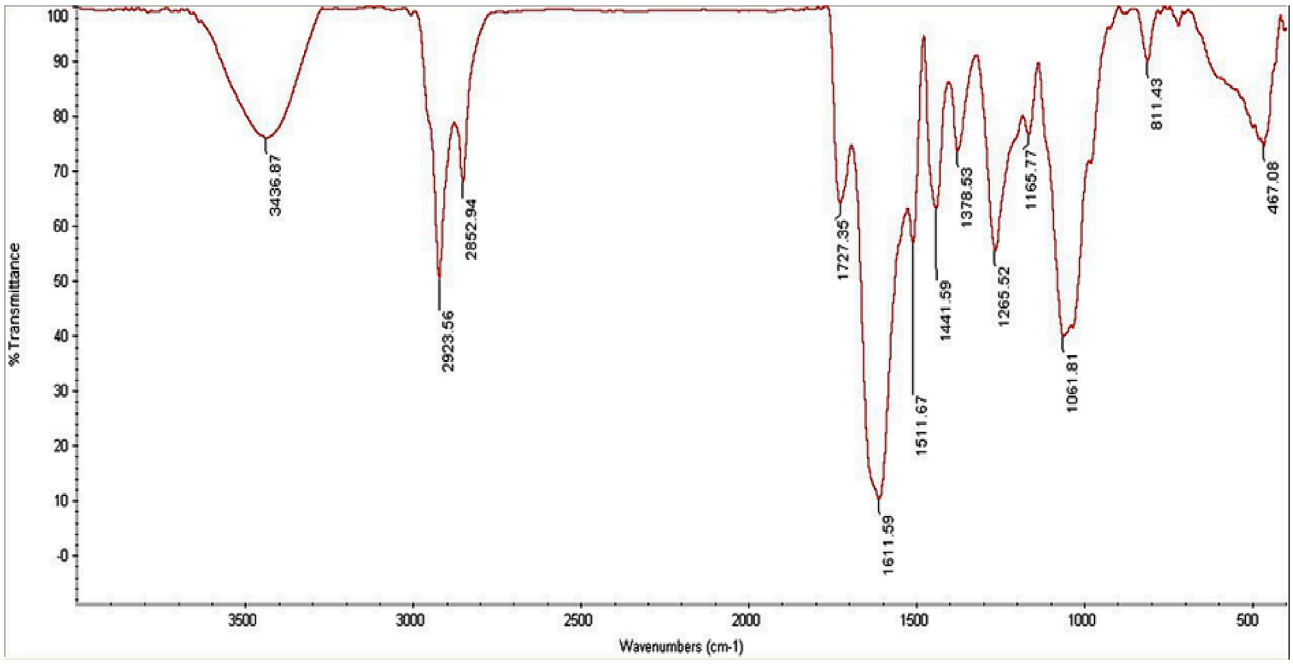

Fig. 4. FT-IR spectrum of prepared AgNPs from *S. tebesana* Bunge leaves extract.

likely attributed to the vibration of ketone group (−C = O), suggesting that the ketone group in the deterrence ester could serve as both a reducing agent and a capping agent [20,25]. The peaks reported at 1441.5, 1265.5, 1061.8, and 811.4 cm^{-1} were associated with the aromatic C-H, C-O, C-O-C, and C-H bonds present in the group of alkenes [20,26,27]. The absorption peaks observed in the FT-IR analysis of the biosynthesized nanoparticles show similarities to those of the *S. tebesana* Bunge plant extract. However, there are slight shifts and decreased intensities in the nanoparticle peaks compared to the plant extract. This suggests that the functional groups present in the plant extract play a role in the formation of the nanoparticles [14]. TEM analysis was employed to precisely examine the dimensions and morphology of the AgNPs synthesized using the *S. tebesana* Bunge extract. The TEM images (Fig. 5) reveal that the biosynthesized nanoparticles possess a uniform oval and spherical structure with a diameter ranging from 10 to 15 nm. The uniformity of these structures might be associated with the presence of biomolecules derived from the *S. tebesana* Bunge extract, which served as the surface coating for the AgNPs. Moreover, the diverse morphologies and sizes observed in AgNPs could potentially be attributed to the existence of reducing agents within the *S. tebesana* Bunge extract [28,29]. The appearance of dark shadows on the surface of silver nanoparticles in TEM images suggests the existence of biological compounds from the plant extract deposited on the surface of silver nanoparticles [23,30].

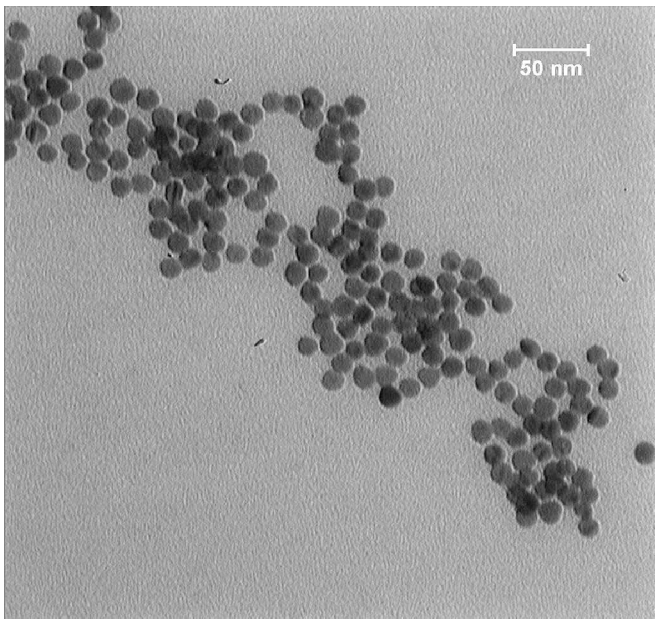

Fig. 5. TEM image of AgNPs synthesized using *S. tebesana* Bunge leaves extract.

3.3. Antibacterial activities

The antibacterial activity of AgNPs synthesized using the *S. tebesana* Bunge extract, chemical AgNPs, and *S. tebesana* Bunge extract alone against *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 9997), *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 25923), and *E. faecalis* (ATCC 29212) is summarized in Table 1. The findings of the current study indicate that both AgNPs and *S. tebesana* Bunge extract lack substantial antibacterial efficacy when used independently. However, the use of *S. tebesana*

Table 1
Antibacterial activity (MIC values in μg/mL) of *S. tebesana Bunge* extract, chemical AgNPs, biosynthesized AgNPs (The use of *S. tebesana* Bunge leaves extract in the synthesis of AgNPs), and Tetracycline.

Bacteria strains	*S. tebesana* Bunge extract	Chemical AgNPs	Biosynthesized AgNPs	Tetracycline
E. coli ATCC 25,922	> 10,000	> 2500	624	0.97
P. aeruginosa ATCC 27,853	> 10,000	2500	39.06	3.9
K. pneumonia ATCC 9997	> 10,000	> 2500	156	7.8
S. aureus ATCC 25,923	1250	2500	78.1	0.97
E. faecalis ATCC 29,212	1250	2500	156	7.8

Bunge extract in synthesizing AgNPs resulted in a noteworthy antibacterial impact, particularly against *P. aeruginosa*, with the highest reported antibacterial activity at a MIC value of 39.06 μg/mL. The study results indicated that AgNPs produced using the *S. tebesana* Bunge extract exhibit comparable antibacterial properties against both Gram-positive and Gram-negative bacteria. Previous studies have also reported a comparable outcome for AgNPs that were synthesized using different plant extracts [31–34]. According to the findings, there is no apparent variation in the antibacterial properties of the biosynthesized AgNPs when it comes to Gram-positive and Gram-negative bacteria. Furthermore, the antibacterial activities of these compounds do not seem to be significantly influenced by the composition of the bacterial cell wall. Hence, when considering the antibacterial effects of AgNPs, it is important to take into account not only their impact on the structure of the bacterial cell wall but also other mechanisms such as interference with intracellular targets (ribosomes, mitochondria, and vacuoles) and small molecules or biomacromolecules (protein, lipids, and DNA) functionality, induction of oxidative stress and cellular toxicity through the generation of free radicals and reactive oxygen species (ROS), as well as modulation of signal transduction pathways [35,36].

3.4. Cytotoxicity effects

Fig. 6 presents the outcomes of the MTT technique, illustrating the cytotoxic impacts of *S. tebesana Bunge* extract, chemical AgNPs, and biosynthesized AgNPs on human fibroblast cell line within the concentration range of 0.125 to 2 mg/mL after 24 h. At the highest concentration (2 mg/mL) and within the given timeframe, chemical AgNPs exhibited no notable alterations in the viability of fibroblast cells when compared to the control group. However, the extract of *S. tebesana Bunge* during the same time frame and at concentrations ranging from 1-2 mg/mL exhibited a noteworthy reduction in cell viability relative to the control cells ($P = 0.000$). It should be noted that AgNPs synthesized using *S. tebesana Bunge* extract demonstrated a significant decrease in fibroblast cell viability, but only when the concentration reached 2 mg/mL ($P = 0.012$). According to the findings, the highest non-toxic concentrations of chemical AgNPs, *S. tebesana* Bunge extract, and biosynthesized AgNPs were determined to be 2, 0.5, and 1 mg/mL, respectively. Despite the findings of certain studies indicating the substantial cytotoxic effects *S. tebesana Bunge* extract on cancerous cell lines [15,37], our study revealed that *S. tebesana Bunge* and AgNPs produced using its extract did not exhibit significant cytotoxic effects on fibroblast cell lines, suggesting their safety for the human normal cells. Hence, the potential significance and interest lie in the selective toxicity exhibited by these compounds.

4. Conclusion

The findings of this study demonstrate that the *S. tebesana* Bunge leaves extract enhances the antibacterial properties of AgNPs, and also represents an appropriate and biocompatible option for the synthesis of these nanoparticles. The highest antibacterial activity of AgNPs synthesized using *S. tebesana Bunge* extract was found against *P. aeruginosa* among all tested bacteria. According to the study, biosynthesized AgNPs at antibacterial activity concentrations revealed no cytotoxic effects on primary cells. Our research highlights the potential of employing biosafe and eco-friendly AgNPs synthesized in the presence of *S. tebesana* Bunge extract, which possess remarkable antibacterial properties, for various biomedical applications.

Ethical Permissions.

The Research Ethics Committee at Birjand University of Medical Sciences approved the study (IR.BUMS.REC.1400.031).

CRediT authorship contribution statement

Mohammad Mehdi Zabihi: Writing – original draft, Visualization, Validation, Investigation. **Samira Eghbaliferiz:** Formal analysis, Data curation, Methodology, Visualization, Writing – original draft. **Mohsen Khorashadizadeh:** Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. **Sobhan Mortazavi-Derazkola:** Investigation, Validation, Visualization, Writing – original draft. **Masoud Yousefi:** Writing – review & editing, Visualization, Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Supervision, Validation.

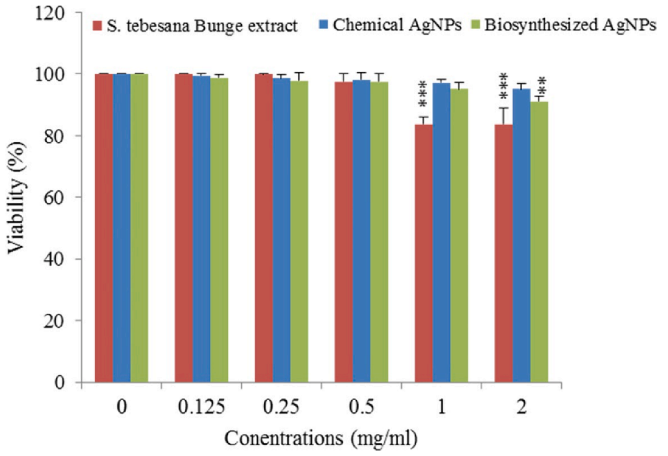

Fig. 6. Cytotoxic effects of different concentrations of *S. tebesana Bunge* extract, chemical AgNPs, and biosynthesized AgNPs on human fibroblast cell line within a 24-hour period by MTT method. One-Way ANOVA P-values indicate statistical significance compared to the control group (**: $P = 0.012$, ***: $P = 0.000$).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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