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RESEARCH ARTICLE

Synthesis of silver nanoparticles using *Salvia officinalis* extract: Structural characterization, cytotoxicity, antileishmanial and antimicrobial activity

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ABSTRACT

Objective: This study aimed to investigate the cytotoxicity, antileishmanial and antibacterial potential of silver nanoparticles (AgNPs) synthesized by (*Salvia officinalis* L. (Sage)) extract.

Methods: The green synthesized nanoparticles were analyzed by using UV–vis spectroscopy, FTIR, and FESEM techniques. Cytotoxicity antileishmanial assay was evaluated by MTT.

Results: The UV–vis spectroscopy study at the band of 430 nm confirmed the formation of nanoparticles. FT-IR confirmed the presence of the ingredients in salvia leaf extract which is responsible for capping and reduction of the silver nanoparticles. FESEM report showed that the AgNPs synthesized were in the size range 30–70 nm. The AgNPs exhibited good cytotoxic activity against Hek-293, the breast cancer cell line (MCF 7) and a human glioblastoma cell line (A 172) with an IC_{50} value of 240, 50.40 and 58.60 µg/mL, respectively. The present study confirmed good antileishmanial activity against the promastigote and amastigote stages *of Leishmania major* in a dose-dependent manner. IC_{50} values of AgNPs were 62.91 and 73.89 µg/mL using promastigote and amastigotes assay respectively. AgNPs showed potent antibacterial activity against three bacteria species *S. aureus*, *B. subtilis*, and MRSA (Methicillin-resistant *Staphylococcus aureus*) at high concentrations. According to disk diffusion results, AgNPs at concentration 1000 µg/mL showed a significant inhibitory zone *against S. aureus and B. subtilis*. The AgNPs at a concentration higher than 750 µg/mL showed the valuable inhibitory zone against MRSA.

Conclusion: this study confirmed good cytotoxicity, and antileishmanial activity in the nanoparticles synthesized using *S. officinal* extract, so it can be further investigated for biomedical applications.

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INTRODUCTION

The nano-scale material has unique physical and chemical properties in dealing with the bulk structure, because of an increase in the surface area to the volume ratio of the particle [1]. Among the metals used for the formation of nanoparticles, AgNPs represent a special interesting [2]. Silver is inactive in metal form but it reacts with wound fluids and moisture in the skin and ionized [3]. In medicines, AgNPs were used to get control of burn and wound infection [4]. The special properties of

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silver convince the researcher to look attentively for biological effects like anticancer, antimicrobial, antifungal, antiviral, and anti-inflammatory activities.

Natural plant products-mediated synthesis of nano-materials has been increasingly noted due to its safety and cost-effectiveness [5]. Since the plants consist of diverse secondary metabolites *e.g.* terpenoids, flavonoids, ketones, aldehydes, amides and carboxylic acid, acts as reducing agents for the bio-reduction reaction to synthesized new metal nanoparticles.

Salvia officinalis L. is a plant in the mint family Lamiaceae. Sage contains a lot of bio-active compounds such as terpenoids, and phenolic acids (vanillic , caffeic, ferulic, and rosmarinic acid) and flavonoids (apigenin, luteolin, and quercetin) [6]. These phyto-constituents give the flexibility needed for better control over the size and shape of the nanoparticles. The present study was conducted to investigate the cytotoxicity, antileishmanial and antimicrobial activity of biogenic AgNPs using *S. officinalis*.

MATERIALS AND METHODS

Plant extraction procedure

Aerial parts of *S. officinalis* were collected from Koohpayeh (Kerman province) in July 2019. The plant was euthanatized by Fariba Sharififar and a voucher specimen was deposited in Herbarium center in the Department of Pharmacognosy, Kerman University of Medical Sciences, Iran (KF1432).

The dried plant was extracted using the maceration method with ethanol 80%. Extraction was repeated until it became clear. The extract was dried in a vacuum oven (Vacucell, Einrichtungen GmbH) at 40°C. Afterward, the methanolic extract was stored at -20°C until the experiment work.

Biosynthesis of silver nanoparticles

For the synthesis of AgNPs, AgNO3 was used as a source of metal. For this purpose, 300 µL of silver nitrate (0.1%) and 100 µg/mL of the plant extract (10 mL) were mixed. The mixture was oven-dried at 40°C and well washed by deionized water several times and dried in the oven at 50 °C.

Characterizations of silver nanoparticles

The formation of SO-AgNPs was monitored using a UV–vis spectrophotometer (PerkinElmer, Germany) at 300–600 nm. The nanoparticles of *S. officinalis* subjected to FTIR spectrometric analysis to specify the functional groups in the extract that may be responsible for reducing ions to nanoparticles. Field emission scanning electron microscopy (FESEM) (Quanta 200, USA) was used for examination of the size, morphology and distribution of synthesized samples.

Cell culture and viability assay

HEK- 293, Human glioblastoma (À172) and breast cancer (MCF-7) cell lines were obtained from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Biowest) and incubated at 37°C in humidified air with 5% CO_2. The medium was supplemented with 10% fetal bovine serum (FBS; Biowest, France), 100 mg/mL streptomycin, and 100 U/mL penicillin (Biowest, France).

Cell viability was assessed by MTT assay after exposure to SO-AgNPs. Cells were trypsinizated for 3–5 min to get the individual cells and then were counted and distributed in 96-well plate with 5,000 cells in each well. The plate was incubated for 24 h to allow the cells to form ~70–80% confluence as a monolayer. Stock solutions of SO-AgNPs were prepared in sterile distilled water and diluted to appropriate concentrations (5-640 µg/mL) with the cell culture medium. Following treatment with SO-AgNPs and 48 h incubation, amount of 20 µL of MTT solution (Atocel, Austria) (5 mg/mL) was added to each well and incubated for 3 h. The medium was discarded and insoluble purple formazan was solubilized by the addition of 150 µL DMSO and the optical density was read with a microplate reader (BioTek-ELx800, USA) at a wavelength of 570 nm. The cell viability percentage was calculated. For each cell line, the data were used to determine the concentration of the sample required to kill 50% (IC50) of the cells compared to that of the controls.

Parasite Culture

Promastigotes of *L. major* (MRHO/IR/75/ER) were provided from Leishmaniasis Research Center, Kerman University of Medical Sciences and cultured in RPMI 1640, supplemented with 100 µg/mL of streptomycin and 100 IU/mL of penicillin at room temperature and 10% v/v heat-inactivated FCS for 30 min, then kept at -20°C till later use. The proliferation rate of *Leishmania* promastigotes was detected every 24 h, and the number of parasites

was determined by counting the parasites in a drop of the medium (10 µL) using a Neubauer slide.

Anti-promastigotes assay

The effect of SO- AgNPs on promastigotes was evaluated by colorimetric cell viability using the method described by Sharifi et al.[7]. Briefly, 100 μl of the promastigotes containing 10^6 cells/mL harvested from the logarithmic growth phase, was added into a 96-well micro titer plate. Then 100µl of various concentrations (1-500 µg/mL) of each extract was added to each well and incubated at 25°C ± 1°C for 72 h. After incubation, 10 µl of MTT solution (5 mg/mL) was added into each well and plates were incubated in the same condition. Promastigotes were cultured in RPMI 1640 medium with no drug considered as an untreated control. All the experiments were done three times. Finally, absorbance was measured by an ELISA reader (BioTekELX800) at 570 nm. Fifty percent inhibitory concentrations (IC_{50} values) were also calculated by the Probit program in SPSS software (Chicago, IL USA) [8].

Anti-intramacrophage amastigotes assay

Drug susceptibility of the amastigotes on the macrophage (J774-A1 cells, ECACC 91051511) was determined by the modification method of Chang [9]. Firstly, $1cm^2$ cover slips were placed in the wells of 6-chamber slides (Lab-Tek, Nalge Nunc International, NY, USA), afterwards, the macrophages were dispensed to the plates and then, 200 μl of the cells (10^6 cells/mL) was placed in each well. Following 2 h of incubation at 37°C and 5% CO_2, promastigotes stationary phase was added to macrophages and again incubated in the same condition for 24 h. Free parasites were removed by rinsing with RPMI 1640 medium and infected macrophages treated with 100µl of various concentrations of SO- AgNPs (1-500 µg/mL) at 37°C and 5% CO_2 for 72 h. Finally, dried slides were fixed with methanol, stained by Giemsa and observed under a light microscope (Nikon, Japan). Also, macrophages containing amastigotes with no extract and no parasite were considered as an untreated control. Glucantime® was also used in a similar manner as a positive standard drug (first-line drug). Activity of anti-intramacrophage amastigotes of the extract was measured by counting the number of intra-macrophage amastigotes in each macrophage by examining 100 macrophages. All experiments were repeated in triplicates as performed for promastigotes [8].

Evaluation of AgNPs antibacterial activity

Minimum Inhibitory Concentration (MIC) determination

The antibacterial potency of produced AgNPs was evaluated by microdilution method using bacteria species *S. aureus*. *E. coli*, *B. subtilis*, and MRSA. *E. coli* and *B. subtilis* are two of the best characterized model organisms for gram negative and gram-positive bacteria, respectively. The *S. aureus* is one of the most frequent causes of nosocomial infections and MRSA are special strains with distinct microbiological and clinical features compare to other methicillin sensitive species. Amount 10 µL of overnight bacterial culture with a concentration equal to 0.5 McFarland was added to wells that contained nutrients broth medium and different concentrations of AgNPs. After incubation of well plates at 37°C for 18 h, the bacterial growth was evaluated using the spectrophotometer (OD 600 nm).

Disk diffusion assay

Four types of bacteria species including *S. aureus*. *E. coli*, *B. subtilis*, and MRSA were cultured on nutrient agar plates. Subsequently, the blank disks were immersed in test tubes containing different concentrations of AgNPs and were places at agar plates. The zones of inhibition around the disks were measured after one-night incubation at 37^0C. Antibiogram disks of Imipenem, Vancomycin, Ciprofloxacin and cefixime were used as positive controls.

Statistical analysis

All experiments were done in triplicate. All data analysis was performed using the SPSS statistical package version 17.0 (SPSS Inc., Chicago, IL, USA). Differences between means of the test and control groups were analyzed by t-test and ANOVA. Additionally, $p < 0.05$ was defined as statistically significant. IC_{50} values (the half maximal inhibitory concentration) were calculated by Probit test in SPSS.

RESULTS

Characterization of SO- AgNPs

Synthesis of AgNPS was monitored by the color change of AgNO3 from colorless to brownish-yellow and its absorption spectra appeared at 430 nm by UV visible spectrometry as shown in

Fig. 1a. Determination of functional groups and capping agents in the plant extract was done by FITR spectroscopy. Vibrational frequency of 400–4000 cm^{-1} has been shown in Fig. 1b. It showed the presence of IR absorption bands at 3386, 2921, 2851, 1718, 1608, 1358, 1069, and 834 cm^{-1}. The absorption band at 3386 may be due to hydroxyls (OH) frequencies, the appearance of a peak at 2921, 2851, 1718, 1608,1358,1069 cm^{-1} and 834 cm^{-1} are related to that C-H, C=O(NH), C=O, C-C, C-O, and C-Cl groups, respectively. The SEM images of AgNPs showed spherical shaped between 30–70 nm in size (Fig. 1c).

Cytotoxicity Assessments

Results obtained by MTT in studied cell lines exposed to 5-640 µg/mL for 48 h has been shown in Fig. 2. AgNPs were cytotoxic on cell lines with approximate IC50 values of 240, 58.60 and 50.40 µg/mL in Hek-293, A 172 and MCF 7 cell lines, respectively. These values indicate that AgNPs have a potent cytotoxic activity towards studied human cell lines.

Antibacterial effects

The biologically produced AgNPs showed potent antibacterial activity against three bacteria species *S.aureus*, *B.subtilis*, and MRSA at high concentrations. According to serial dilution study results, it was clear that concentration 1000 µg/mL reduced bacteria growth down to zero. Also according to disk diffusion results (Fig. 3), AgNPs at concentration 1000 µg/mL show significant inhibitory zone against *B.subtilis*. The AgNPs at

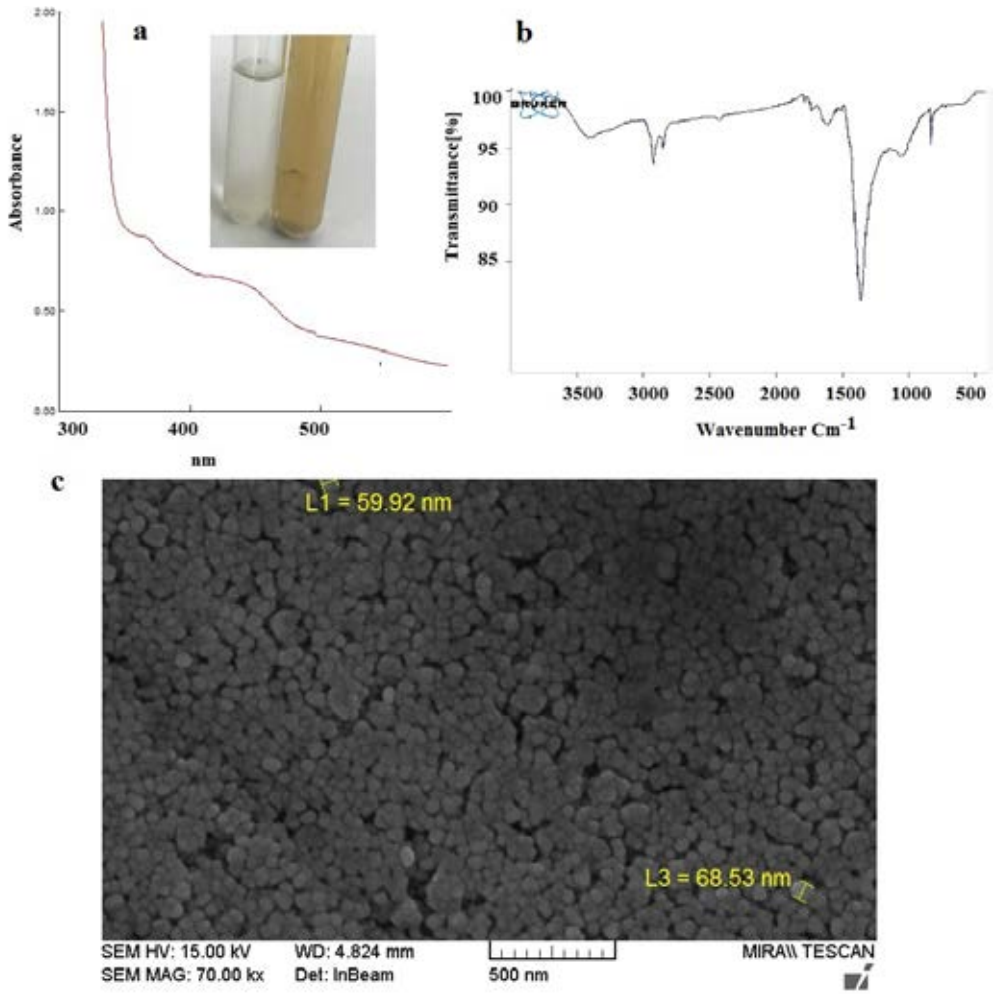

Fig. 1. Characterization of green synthesized Ag-NPs prepared using methanol extract of the *S. officinalis* plant. (a) The UV-Vis spectrum of Ag-NPs synthesized by *S. officinalis* and color change (b) Fourier-transform infrared spectra of synthesized Ag-NPs (c) FESEM image of the green synthesized Ag-NPs

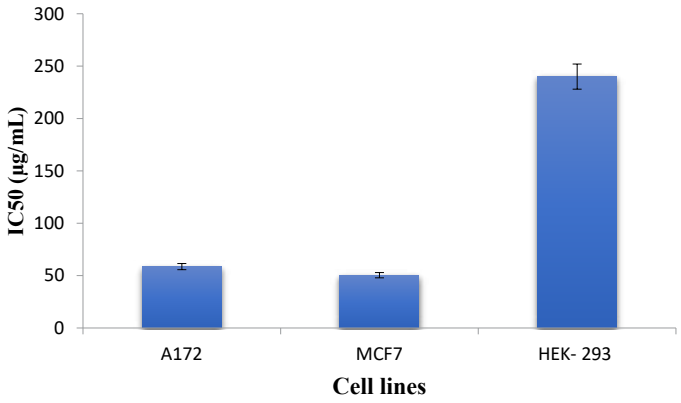

Fig. 2. Cytotoxic activity evaluation of Ag-NPs against Hek-293A172 and MCF7 cell lines. Cells were treated with various concentrations of Ag-NPs (5, 10, 20, 40, 80, 160, 320, 640 µg/mL) for 48 h and subjected to MTT assay. IC_{50} values are expressed as mean± SD of three independent experiments.

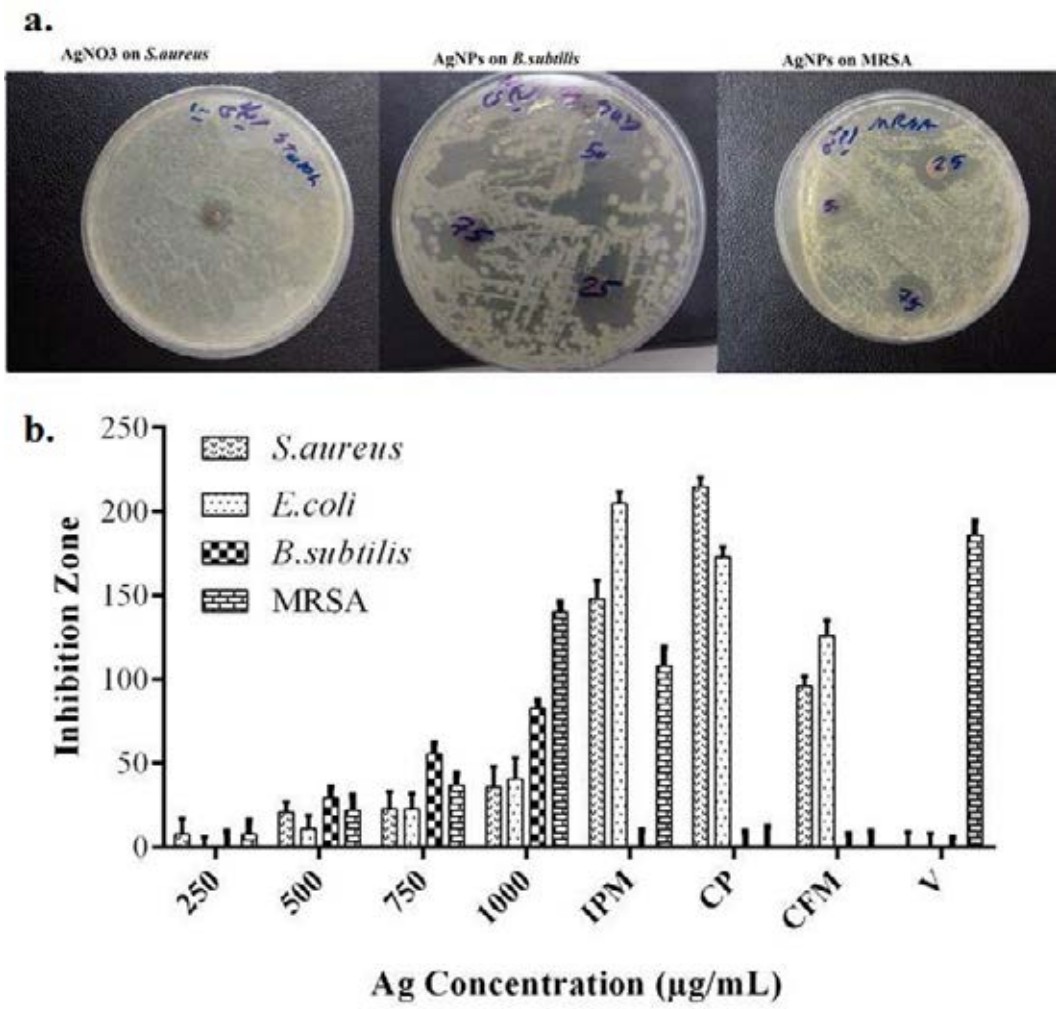

Fig. 3. a. Visual appearance of the zone of inhibition for concentration 1000 µg/ml. b. Zone of inhibition of AgNPs, IMP (Imipenem), CP (Ciprofloxacin), CFM (Cefixime) and V (Vancomycine) against *S. officinalis* extract, against *E. coli*, *S.aureus*, *B.subtilis*, and MRSA

a concentration higher than750 µg/mL showed the valuable inhibitory zone against MRSA. The zone of inhibition for 1000 µg/ml AgNPs against MRSA was significantly higher than imipenem, ciprofloxacin and cefixime. Also, as it is clear, none of the traditional antibiotics used in this study showed antibacterial activity against all four bacteria species, but AgNPs, especially at high concentration, have valuable antibacterial effects against all of the used bacteria species.

Anti-promastigotes assay

Fig. 4A shows the cell viability (%) for AgNPs and Glucantime® versus the untreated control. The IC_{50} values for AgNPs and Glucantime® were 73.89±0.85 and 398.21±2.05 µg/mL, respectively. There was a significant difference between AgNPs and Glucantime® ($p < 0.05$) (Fig. 3A).

Anti-intramacrophage amastigotes assay

The findings display AgNPs significantly ($p < 0.05$) inhibited the proliferation rate of intra-macrophage amastigotes in a dose-dependent manner (Fig. 4B). The IC_{50} values were 62.91±1.11 and 175.08±2.09 µg/mL for AgNPs and Glucantime®, respectively.

DISCUSSION

In this study, methanol extract of *S. officinalis* was used for the reduction of Ag^+ and exogenous generation of silver NPs. The synthesized NPs were further characterized using UV-Vis, FT-IR spectroscopy and FESEM. The formation of AgNPs was indicated by the observation of color change and also it was revealed by a peak observed at 430 nm in UV-Vis spectroscopy. Based on the literatures, the absorption spectrum of spherical AgNPs present a maximum between 420 and 450 nm [10]. FESEM micrograph demonstrated the presence of spherical NPs with a size range of 30–70 nm. FT-IR confirmed the presence of the ingredients in *salvia* leaf extract which is responsible for capping and reduction of the AgNPs. Hydroxyl functional groups that present in the phenols and sterols of *salvia* are likely to play an important role in the metal reduction reaction based on the previous studies [11]. The possible mechanism of Ag^+ reduction by *salvia* extract could be related to the polyphenols which are ionized and transfer an electron to Ag^+ and consequently it is reduced to Ag^0 (AgNPs). Previous studies have represented the growth dynamics of AgNPs using *Salvia officinalis* extract by varying the preparation conditions. They identified some structural modifications such as appearance of new peaks and shifting of them, due to the acid or hydroxide in the solution at different pH values [12]. In the previous studies, aqueous leaf extract of *S. officinalis* was used for synthesizing AgNPs, however in this investigation we utilized ethanolic concentrate of the extract [13, 14]. The discoveries could propose the conceivable utilization of AgNPs in mix with other chemotherapeutic operators in the clinical setting and have introduced an imaginative methodology for drug conveyance effectiveness thereby decreasing size and huge surface territory to volume proportion, which helps their capacity to enter the cell layer.

There are some reports on biological activities of green synthesized AgNPs using

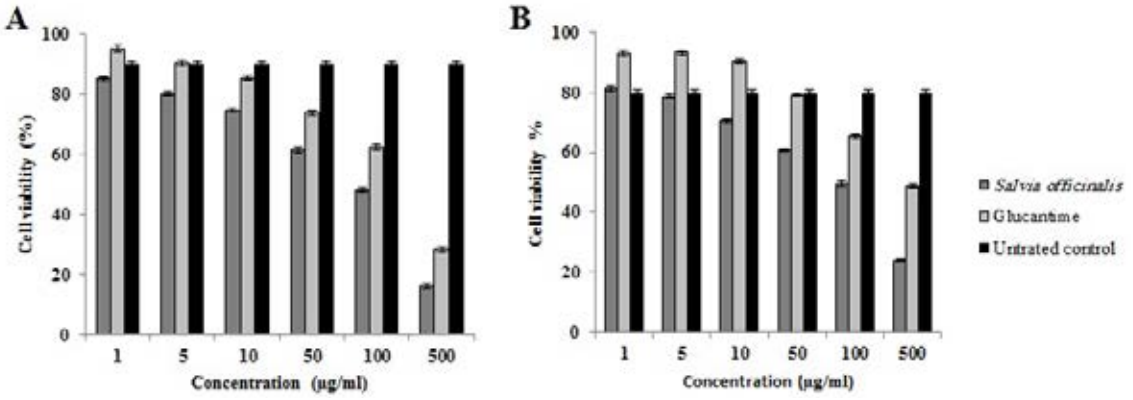

Fig. 4. Antileishmanial activity of Ag-NPs in comparison with Glucantime® as an effective positive control. Cell viability of two life stages of *L. major*: Amastigote (a) and Promastigotes (b) after treatment with different concentration of Ag-NPs and Glucantime®. $p < 0.05$ as compared to untreated control cells.

Salvia officinalis extract *e.g* antioxidant and anti-inflammatory [15], antiplasmodial [13], antibacterial activity [14, 16], protective role against harmful effect of bisphenol A in female rats [17].

In the present study, Ag-NPs were determined to be cytotoxic on the studied human cell lines. Results obtained from this study are in agreement with previous studies where *green tea* showed the highest cytotoxic activity against human keratinocyte (HaCaT) cells [18]. According to Ahn et al., the cytotoxicity of the AgNPs synthesized with the extracts of *Mucuna birdwoodiana* and *Cratoxylum formosum* makes contribution to apoptotic cell death in A549 cell lines [19].

Size, shape and charge of metallic NPs are important factors that impact the cytotoxicity of NPs by activation of essential steps of apoptosis [20]. Therefore, physicochemical properties of NPs influence their interaction with cells so, it can affect their potential toxicity in cancer and normal cells. For instance, the shape and size of NPs were shown to correlate with the production of reactive oxygen species (ROS) [21]. Moreover, various ingredients attached on the surface of NPs could be an efficient factor in inducing cytotoxic effects. As a result, Ag-NPs synthesized using different plant extracts or even different plant parts used for extraction may lead to divers cytotoxic effects [22]. Therefore, higher cytotoxicity activities of NPs synthesized using plant extracts rather than pure extracts could be related to the synergistic effects of metal ions and natural organic compounds [23]. Cytotoxic effects of biosynthesized NPs on both cancerous and normal cells are one of the main concerns in cancer treatment. There is a necessity for targeted therapies that can maximize treatment success and minimize toxicity [24].

The biogenic synthesized AgNPs mediated by *salvia* hydro-alcoholic extract showed good antileishmanial effects on promastigotes and amastigotes, which was comparable to Glucantime®. Therefore, it can be considered as a leishmanicidal agent in experimental models. There are a lot of reports about the antileishmanial effects of different metal nanoparticles [8, 25, 26].

Several mechanisms were proposed for antileishmanial activity of these nanoparticles such as binding of AgNPs to biomolecules *e.g* DNA and proteins, damaging of mitochondrial enzymes and cell cycle proteins [27].

CONCLUSION

In conclusion, in this work, *S. officinales* extract was successfully used to produce AgNPs as a reducing and stabilizing agent. AgNPs possessed cytotoxic activity in the studied cell lines and revealed notable antileishmanial activity against the promastigote and amastigote stages of *L. major* in a dose-dependent manner but showed weak bactericidal activity against Gram-negative bacterial strains. Therefore, it can be take into consideration as a promising agent for the production of the antileishmanial drug in the future.

CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

FUNDING

No funding involved.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

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