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Original Article

Green Synthesis, Characterization, and Antileishmanial Activity of the Silver Nanoparticles Alone and Along with Meglumine Antimoniate against *Leishmania major* Infection

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Abstract

Background: The most commonly available drugs for leishmaniasis are pentavalent antimony compounds; whereas the recent studies showed various complications and limitations of these drugs. We aimed to green synthesized silver nanoparticles (AgNPs) and study the promising antileishmanial and synergic effects of green synthesized silver nanoparticles alone and combined with glucantime.

Methods: The precipitation technique was used to drop silver ions via an extract of *Astragalus spinosus* to AgNPs at Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University, Saudi Arabia in 2022. Then, its anti-amastigotes, caspase-3-like activity, triggering the nitric oxide (NO) as well as its cytotoxicity effects on macrophage cells as well as effects on leishmaniasis in BALB/c mice infected by *L. major* were measured.

Results: The size of the AgNPs were ranging from 30-40 nm. The IC50 value for AgNPs, AgNPs+ meglumine antimoniate (MA), and MA was 59.3, 18.6, and 51.2 µg/mL, respectively. The determined FIC value for AgNPs and MA was found to be 0.31 and 0.36, respectively; demonstrating the synergistic potency of AgNPs when combined with MA. The diameter of CL lesions treated with various doses of AgNPs and AgNPs+MA notably ($p<0.001$) decreased. AgNPs, particularly at the concentrations of ½ IC50 and IC50, considerably triggered the caspase-3 activation. The calculated CC50 of AgNPs and MA was 612.5 and 789.8 µg/mL, respectively. Green synthesized AgNPs, especially in combination with MA had synergic antileishmanial effects and displayed a promising drug candidate for treating *L. major* CL.

Conclusion: We found satisfactory findings in the parasite reduction in both in vitro and animal models. Still, more studies are expected to explain the precise action mechanisms of AgNPs and their efficacy in humans.

Introduction

Leishmaniasis is widespread in many countries in various parts of the world. It is one of the six most significant parasitic infectious diseases globally, which annually leads to many economic and human losses worldwide (1). The disease is triggered by *Leishmania* parasites, is transmitted by the bites of sandflies of the genus *Phlebotomus* and *Lotusumia*, and is a common zoonotic disease between humans and animals such as dogs and rodents (2).

The most commonly available drugs for leishmaniasis are pentavalent antimony compounds (pentostam and glucantime), other drugs used include amphotericin B, metronidazole, diamidine, pentamidine, allopurinol, ketoconazole, itroconazole, and daposin (3). Present synthetic agents always have complications and limitations, e.g., pain at the time of injection, anorexia, gastrointestinal disorders, and drug resistance (4). Given the above, researchers are interested in using some new compounds with high efficacy and minimum toxicity, such as other nanoparticle solutions.

Today, nanoparticles (NPs), particles with dimensions of 10 to 100 nanometers, have numerous benefits pharmaceutical properties (5). Among the nanoparticles, metal NPs with unique physical properties are widely used as promising agents to transport several small size drug molecules or larger ones (5). In recent years, the use of herbs and their derivatives, called "green synthesis", is considered an economical, safe, and reliable approach to synthesis of nanoparticles (6); whereas their secondary metabolites such as polyphenols and terpenoids can trigger bioremediation of ions and produce of several metal NPs (6).

Among several metal NPs (e.g., gold, iron, and selenium) for controlling CL, silver nanoparticles (AgNPs) have attracted more attention because of some of their properties, e.g., high stability, antibacterial, antifungal, and antiviral properties with low concentrations and long shelf life (7). Although several investigations have been performed on the effect of AgNPs on cutaneous leishmaniasis lesions, however, their results were dissimilar and every so often opposing, because of the synthesis method, the type of parasite, and the method of application (8-10).

This investigation was designed to synthesize the AgNPs employing the extract of *Astragalus spinosus* (EAS), a plant with various pharmacological properties in traditional and novel medicine (11) and evaluate its promising antileishmanial and synergic effects of green synthesized silver nanoparticles alone and combined with MA.

Materials and Methods

Preparing of extract

The aerial parts of *A. spinosus* were gathered in July 2022 from rural regions of Riyadh, Saudi Arabia. By percolation technique, air-dried materials (250 g) were put in water for 72 h at 21°C. After filtering and evaporating in a vacuum condition at 55°C, the extract was kept at −4°C (12, 13).

Green synthesis of AgNPs

The precipitation method was used to green synthesis of AgNPs via the reducing of Ag ions ($AgNO_3$) by EAS (14).

UV-vis spectroscopy assessment

NPs solution (300 µL) was diluted with normal saline (3 mL) and was studied by UV–vis spectrophotometer apparatus (Shimadzu UV2550, Japan) with ranging from 300 to 700 nm (15).

X-ray diffraction (XRD) study

The crystal configuration of the AgNPs was determined by measuring the Ka ray source of a copper lamp (Ka ray basis) with a wave-

length of X beams in λ= 1.54 A^0 by a XRD tool (2000 APD, Italy).

Electron microscope and dynamic light scattering

Scanning electron microscope (SEM, Mira3, with 15 kV, Czech) in amplification of 10x, and resolution of 1 nm, transmission electron microscopy (TEM, Jeol JEM-1220, JEOL, Japan), and DLS via a Zeta sizer (UK, Malvern) were applied to determine the size and shape of AgNPs.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

To assess the plant factors effective in reducing silver ions, alcohol analysis with a wave number range of 500 to 400 cm was used. For this purpose, dried and powdered Ag nanoparticles were mixed with potassium bromide to produce tablets and examined using a device (16).

Parasite and cell cultures

Promastigotes of *L. major* (MHOM/TM/82/Lev) and J774-A1 macrophage cells were cultivated in Schneider's medium with 10% heat-inactivated FBS and recovered by antibiotics (100 µg of streptomycin/ penicillin 100 µg/mL) at 37°C in 5% CO_2 (17).

Anti-amastigote Assay

For this purpose, J774-A1 cells (100,000/mL) were covered in 24-wells plate (including coverslips 1 cm^2) at 37 °C in 5% CO_2, for 24 h incubation. To induce the infection in macrophages, cells were incubated for four hours with stationary phase promastigotes at the proportion of 10: 1 parasite/macrophage at the same conditions. In the next step, medium (1 mL) containing different concentrations of AgNPs alone (5, 10, 25, 50, and 100 µg/mL) and blended with MA (Aventis, France) at the concentrations of 5, 10, 25, 50, and 100 µg/mL were added to each well and kept for 48 h. Lastly, with fixing the coverslips with absolute methanol and staining by Giemsa dye, and examined under light microscopy. Antiamastigote effects were determined by calculating the mean number of intracellular amastigotes insides 100 cells (17).

Evaluating the synergic effects of AgNPs

We calculated the fractional inhibitory concentration (FICI) index via the calculating the following ratio: the 50% inhibitory concentrations (IC50) of two combined agents/IC_{50} each agent alone; whereas, the value ≤ 0.5, 0.5-1 and 1<, displayed the synergistic, additive, and antagonistic activity, respectively (17).

Evaluation of the infection rate in macrophages

Here we experimentally studied the effect of AgNPs on inhibition of infection in cells. Promastigotes (1,000,000/mL) were exposed to AgNPs (½ IC50 and IC_{50}) for two hours at 21 °C. After washing, they were subjected to macrophage cells for four hours. As a final step, the prepared slides were dyed via Giemsa, and 100 cells were checked using a light microscope (17).

Effect on plasma membrane permeability

Once promastigotes (1,000,000/mL) were treated with AgNPs (1/4 IC_{50}, 1/3 IC_{50}, ½ IC50 and IC_{50}), Sytox green (Sigma-Aldrich, Germany) stain technique was applied according to the manufacturer's protocol (17).

Effect on provoking the nitric oxide (NO) release

Once macrophages (100,000/mL) were treated with AgNPs (1/3 IC_{50}, ½ IC50 and IC_{50}) for 48 h, the upper phase (0.1 mL) were mixed to Griess reagents (0.06 mL) according to the manufacturer's protocol in the 96-wells plate. Next, the wells were examined at 540 nm in an ELISA reader (17).

Effect on induction of apoptosis

This was performed based on the Caspase-3 Colorimetric Activity Assay Kits (Sigma-Aldrich, Germany) followed by the treatment of promastigotes (1,000,000/mL) with AgNPs (1/4 IC_{50}, 1/3 IC_{50}, ½ IC50 and IC_{50}) for 48 h. The activity was studied via the reading the OD of combination at 405 nm using an ELISA reader (18).

Cytotoxic effects

Once macrophages (100,000/mL) were treated with AgNPs (50, 100, 250, 500, and 1000 µg/mL) for 48 h, at 37°C with 5% CO2. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) analyze in 96-well plates was applied according to the previous studies to study the cytotoxicity effects of AgNPs. After determining the cytotoxic concentrations (CC_{50}), the selectivity index (SI) via the calculating the following ratio: CC_{50} /IC_{50} against amastigotes. (19).

In vivo effect of green synthesized AgNPs on CL in BALB/c mice

Totally, 48 male BALB/c mice with weight of 25-30 g with 40-50 days age were involved in this study. To induce the mice model of CL, promastigotes (0.1 ml, 2,000,000 parasites/mL) were injected subcutaneously to mice tail (19).

Ethics considerations

The research protocol was allowed by the ethical board of the Almaarefa University, Saudi Arabia (IRB06-25012022-09).

Treating infected mice

Six weeks' post-infection, when CL were watched, the mice were casually separated into six groups (eight mice/group) and were topically treated daily for 28 days including (i): normal saline; (ii) intralesional infusion MA (30 mg/kg); (iii) AgNPs 5 mg; (iv) AgNPs 10 mg; (v) AgNPs 5 mg+ MA; (vi) AgNPs 10 mg+ MA (18). To study the in vivo effect of green synthesized AgNPs on CL in BALB/c mice, the diameter of CL in mice before starting treatment and immediately after 28 days of treatment was documented via a Vernier caliper. To evaluate the parasite load, once preparing lesion smears and staining using Giemsa, they were examined by means of light microscopy (19).

Quantification of parasite load in liver

The load of *Leishmania* parasites in liver of the mice was evaluated according the limiting dilution assay (LDA) technique (17). Briefly, after collecting the liver tissues, they were mixed in Schneider's medium developed with FCS (20%), and pen/strep (100 µg/mL). By incubating the liver homogenates in 96-well plates as triplicate, at 21°C for 10 days. Then, an inverted microscope was used to detect the motile and non-motile parasites. The number of alive *Leishmania* per milligram of tissue by the maximum dilution, where growing parasites through the ELIDA software.

Assessing the proinflammatory cytokines

The evaluation of the serum amount of IFN-γ, and IL-1 as proinflammatory cytokines in the treated mice was carried out according the protocol of the diagnostic commercial ELISA kits (Abcam, USA).

Statistical analysis

The obtained data were analyzed using the SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). The tests of non-parametric tests, e.g., Mann-Whitney test were employed to assess the acquired results. $P< 0.05$ was calculated statistically meaningful.

Results

Characterization of green synthesized AgNPs

We observed the decrease of Ag^+ ions to AgNPs by shifting to dark brown color of medium. A peak at 413 nm (a wavelength of 400 to 450 nm) approved the green synthesis

of AgNPs, indicating the production of free electrons in NPs (Fig. 1A). The XRD assessment (Fig. 1B) exhibited that peaks 111 to 311 at 28.135 to 78.12º linked to nanocrystals and Ag three-dimensional formations; while, non-appearance of the remaining peaks approved the concentration of AgNPs applied in the analysis. The results showed that AgNPs size was varying from 5 to 60 nm, the maximum distribution of particles size was observed in 30-40 nm (Fig. 1C).

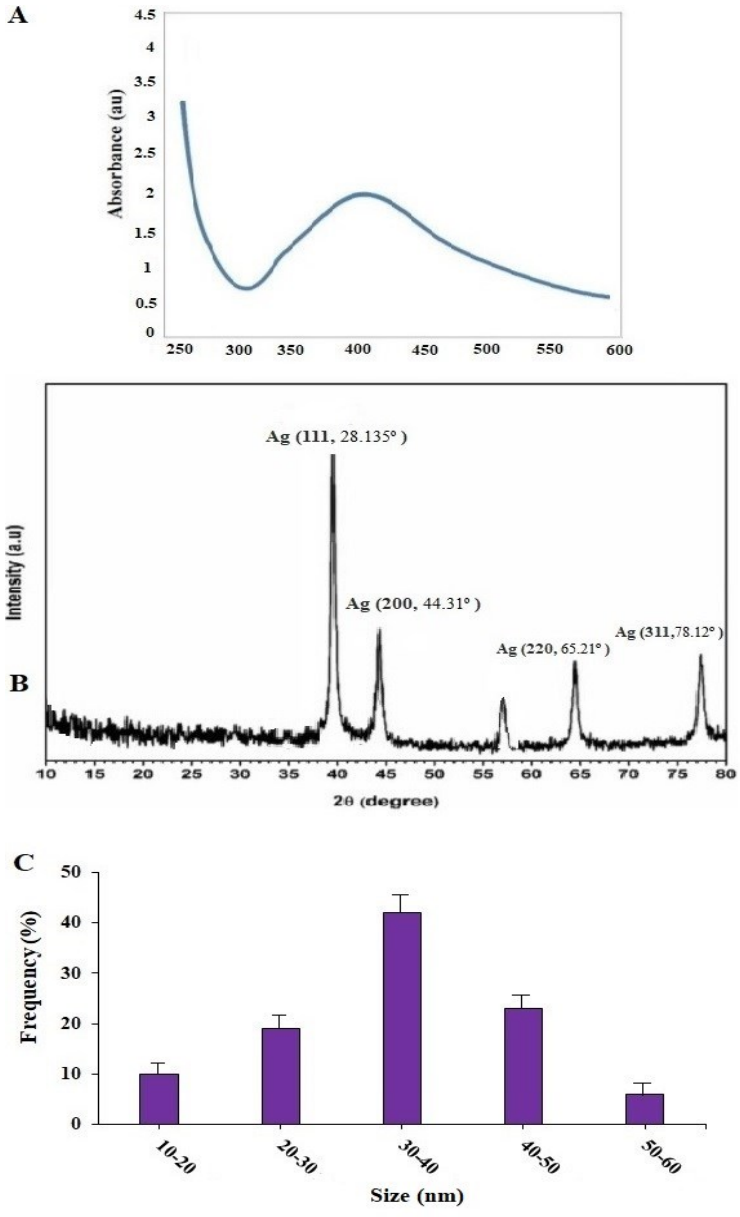

Fig. 1: (A) UV absorption; (B) X-ray diffraction (XRD) study; (C) and the size distribution of the AgNPs

SEM and TEM also displayed that AgNPs exhibited a round shape and are moderately same in size ranging from 30 to 40 nm (Figs 2A and 2B). Zeta potential analysis showed that the zeta potential of AgNPs was -14.6 mV with complete intensity; demonstrated that AgNPs had high stability due to the electrostatic revulsion with no addition of a dissimilar physical or chemical capping factor (Fig. 2C).

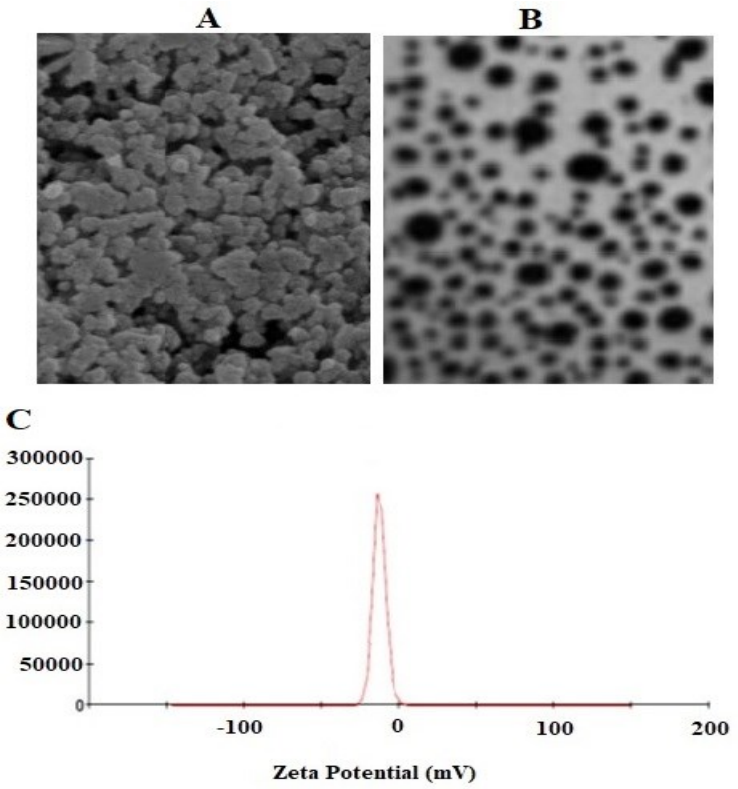

Fig. 2: Scanning electron microscope (A), transmission electron microscopy (B), and zeta potential (C) of the green synthesized AgNPs

The results showed that the 1696 cm^{-1} peak showed a carbonyl bond (C=O) and the 3241 cm^{-1} peak showed a hydroxyl bond. FTIR spectroscopy confirms that the conversion of silver ions to silver nanoparticles is due to biodegradation by plant-coated extracts of *A. spinosus* extract (Fig. 3).

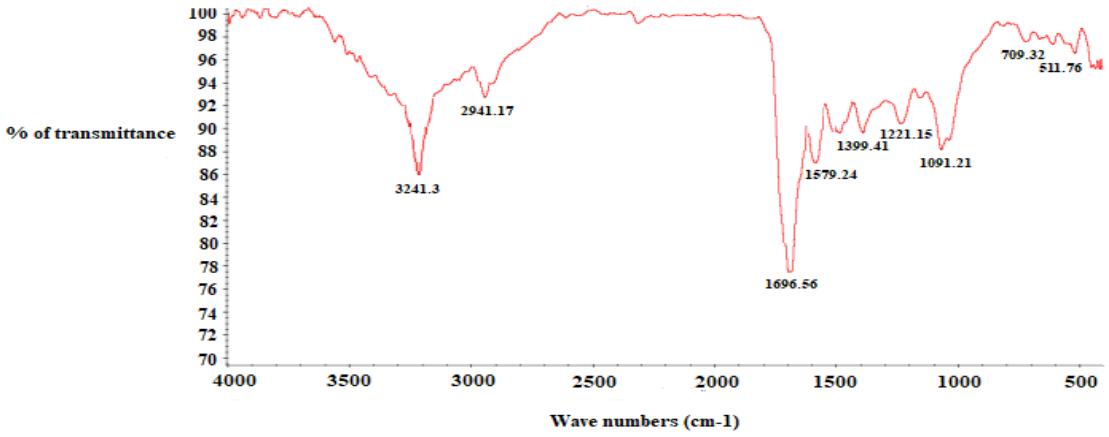

Fig. 3: FTIR spectroscopy of the green synthesized AgNPs

In vitro anti-amastigote Assay

After treating the infected macrophage cells with AgNPs alone and along with MA, the mean number of amastigotes dose-dependently (P<0.001) reduced in dose-dependent response. The IC_{50} value for AgNPs, AgNPs+MA and MA were 59.3, 18.6 ± 1.75, and 51.2 ± 2.84 µg/mL, respectively (Table 1).

Table 1: Antileishmanial and cell toxicity effects of silver nanoparticles (AgNPs), meglumine antimoniate (MA), and AgNPs+MA on *Leishmania major* amastigote and macrophage cells. (Mean ± SD). FIC: fractional inhibitory concentration

Drug	*IC_{50} (µg/mL) for Amastigote*	*CC_{50} (µg/mL) of the J774-A1 Cells*	*Selectivity index*	*FIC*
AgNPs	59.3 ± 5.125	612.5 ± 11.34	10.3	0.31
MA	51.2 ± 3.72	789.8 ± 12.1	15.4	0.36
AgNPs+MA	18.6± 2.05	-		-

Evaluating the synergic effects of AgNPs

The determined FIC value for AgNPs and MA was found 0.31 and 0.36, respectively; demonstrated the synergistic potency of AgNPs when combined with MA (Table 1).

Evaluation of the infection rate in macrophages

After pre-treating parasites with AgNPs at ½ IC50 and ½ IC50, the infectivity rate was significantly (P<0.001) reduced by 32.5 and 70.9% compared to the normal saline.

Effect on plasma membrane permeability

Concerning the effect of AgNPs on the plasma membrane permeability of parasites by the obtained relative fluorescent units, we found that AgNPs mainly at the concentration of ½ IC_{50} altered the permeability of plasma membrane (Fig. 4).

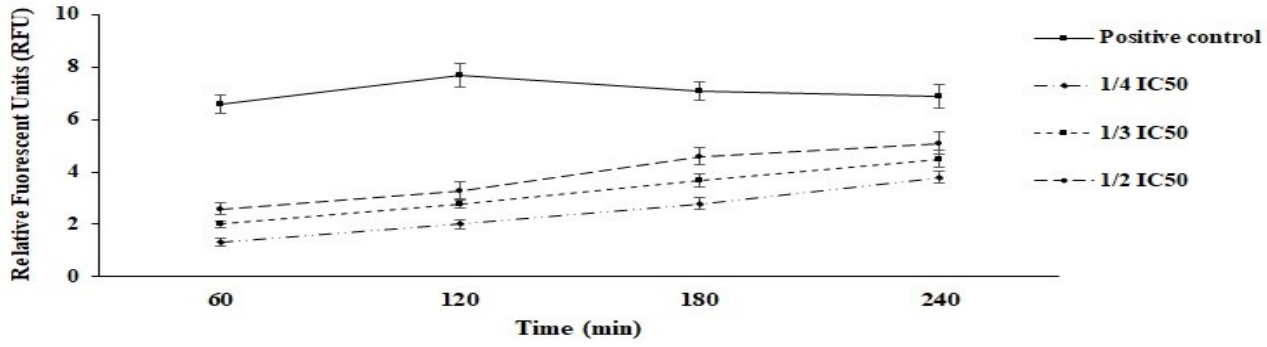

Fig. 4: The effect AgNPs on the permeability of plasma membrane of L. major promastigotes

Effect on provoking the nitric oxide (NO) release

The results showed that the green synthesized AgNPs at 1/3 IC50, ½ IC50 and IC50 triggered the production of NO by 8.3 ± 0.62, 16.2 ± 1.84, and 19.3 ± 2.15 Nm, respectively; however, a significant (P<0.001) production was observed at ½ IC_{50} and IC_{50} (P<0.001) in comparison to the normal saline (6.62 ± 0.84 nM).

Effect on induction of apoptosis

The effect of AgNPs on Caspase-3-like activity of promastigotes showed that AgNPs at the concentrations of 1/4 IC_{50}, ½ IC_{50}, and IC_{50}, considerably triggered the caspase-3 activation, by 13.2, 24.3, and 31.3%, respectively.

Cytotoxic effects

The findings of the MTT assay demonstrated that green synthesized AgNPs exhibited no significant cytotoxicity on macrophage cells. The calculated CC_{50} of AgNPs and MA was 612.5 µg/mL and 789.8 µg/mL, respectively. Consequently, the SI of >10 for AgNPs and MA exhibited no cytotoxicity on macrophages, while had specificity to parasites (Table 1).

In vivo effect of green synthesized AgNPs on CL in BALB/c mice

In mice with CL, the diameter of CL lesions treated with various doses of green synthesized AgNPs alone (5 and 10 mg/kg) and combined with MA, started to significantly (P<0.001) decrease progressively; so that at the 4th week of the treatment after infection, CL lesions had entirely vanished at the mice cured with AgNPs (5 and 10 mg/kg) along with MA (Table 2). On the other hand, in mice receipt normal saline, the mean diameter of the CL lesions increased by 8.6 mm. Treatment of the infected mice with green synthesized AgNPs alone (5 and 10 mg/kg) and combined with MA, considerably decreased (p<0.001) the mean number of parasites. The load of *Leishmania* parasites in liver of mice treated with AgNPs was evidently (P < 0.001) dropped mainly in combination with MA; whereas the serum level of IFN-γ and IL-1 was raised as dose dependent response increased (P < 0.01) (Table 2).

Table 2: Effect of silver nanoparticles (AgNPs), meglumine antimoniate (MA), and AgNPs+MA on the lesions size, the parasite load in cutaneous leishmaniasis lesions, parasite load of liver tissue, and the serum amount of IFN-γ and IL-1, in *Leishmania major* infected mice. (Mean ± SD). * P < 0.05 significant difference in comparison with control. + P<0.05 compared to MA

Drug	***Lesion size (mm)***		***Parasite number in lesion (×1000)***	***Parasite number in liver (-log)***	***Serum level of cytokines***	
	Before treatment	**After treatment**			**IFN-γ (pg/mL)**	**IL-1 (pg/mL)**
AgNPs 5 mg	10.5±0.71	2.54±0.56*	0.56±0.084*	15.1±2.2*	49.6±3.9*	26.8±2.8*
AgNPs 10 mg	9.9±1.42	1.32±0.43*	0.34±0.076*	8.4±1.14*	58.4±4.2*	34.6±3.4*
MA (30 mg/kg)	10.1±0.72	0.51±0.13*	0.089±0.013*	1.89±0.65*	65.4±5.4*	42.1±4.3*
AgNPs 5 mg+ MA	10.3±0.69	0.0±0.0*	0.0±0.0*	0.39±0.07*+	82.4±4.6*+	59.6±3.9*+
AgNPs 10 mg+ MA	10.7±0.56	0.0±0.0*	0.0±0.0*	0.1±0.02*+	92.7±5.1*+	68.2±6.5*+
Control	10.4±0.65	19.0±1.12	2.48±0.26	21.3±0.26	31.8±3.9	16.6±2.1

Discussion

We found that the size of green synthesized AgNPs was varying from 5 to 60 nm, the maximum distribution of particles size was observed in 30-40 nm; whereas, SEM and TEM also displayed that AgNPs exhibited a round shape and are moderately same in size ranging from 30 to 40 nm. We found that after treating the infected macrophage cells with AgNPs alone and along with MA, the mean number of amastigotes dose-dependently (P<0.001) reduced in dose-dependent response; whereas the obtained FIC values demonstrated the synergistic potency of AgNPs when combined with MA. By in vivo, the treatment of the infected mice with green synthesized AgNPs alone (5 and 10 mg/kg) and combined with MA, considerably decreased (P<0.001) the mean number of parasites in lesion and liver, diameter of CL lesions as well as increased the serum level of IFN-γ. Recently, nanotechnology through the nano-drug delivery systems and nanonization of the current synthetic agents provide new attitudes in leishmaniasis treatment (20). It has been proven that nano-drug delivery systems, through increasing bioavailability, effect on the target delivery, reducing toxicity, increasing the concentration, discharging agents with regulated system and prolonging systemic circulation lifetime, improved the efficacy of conventional drugs (21). Although several investigations have been performed on the effect of AgNPs on cutaneous leishmaniasis lesions, however, their results were dissimilar and every so often opposing, because of the synthesis method, the type of parasite, and the method of application (7, 21).

One of critical aspects in the study of the effect of drugs on the pathogenesis of intracellular microbes such as *Leishmania* is the ability to inhibit cell infection by these drugs (17). Our results revealed that after pre-treating parasites with AgNPs, the infectivity rate was reduced by compared to the normal saline. Disruption or destruction of cell membranes is one of the essential mechanisms of the direct action of agents on microorganisms (14, 17). Concerning the effect of AgNPs on the plasma membrane permeability of parasites by the obtained relative fluorescent units, we found that AgNPs altered the permeability of plasma membrane.

Previous studies showed the NO-related cytotoxic effects prompted by triggered macrophages against various intracellular such as *Leishmania* spp. (23). Studies on animal models of cutaneous leishmaniasis demonstrated that agents are inducing NO applied topically, displayed a relevant efficacy for treating CL lesions (23). We reported that green synthesized AgNPs triggered the production of NO as dose-dependent response in comparison with the normal saline. Caspases such as Caspase-3 is are involved in stimulation of decease protease and accordingly induces cell death in *Leishmania* (24). The effect of AgNPs on Caspase-3-like activity of promastigotes showed that AgNPs considerably triggered the caspase-3 activation. Similarly, AgNPs green synthesized by *Annona muricata* extract provoked the apoptosis through mitochondrial injury and the stimulation of p53 protein pathway in THP-1 and AMJ-13 cells (25).

Considering the cytotoxicity effects of AgNPs, the SI of >10 for AgNPs and MA exhibited no cytotoxicity on macrophages, while had specificity to parasites. Similarly, AgNPs synthesized by using the *Tuber* spp. extract displayed cytotoxicity effects on MCF-7 breast cancer cells with CC_{50} value 0.6, 0.5, and 0.48, after 24, 48 and 72 h incubation, respectively (26). Sukirtha et alhave reported cytotoxicity of AgNPs using *Melia azedarach* on HeLa cell lines with an IC_{50} value of 300 µg/mL (27). However, more studies must be performed to elucidate other aspects of cytotoxicity as well as systematic toxicity of these green synthesized AgNPs.

Conclusion

Green synthesized AgNPs, especially in combined with MA had synergic antileishmanial effects and displayed a promising drug candidate for treating *L. major* CL. We found satisfactory results in the parasite reduction in both in vitro and animal model. Induction of NO production, provoking the apoptosis, inhibition of infection in macrophages, and increasing the permeability of plasma membrane are the main mechanisms of action AgNPs against *L. major* promastigote and amastigotes. Nevertheless, more surveys are mandatory to explain the precise action mechanisms of AgNPs and their efficacy in clinical subjects.

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Conflict of interest

The author declares that they have no competing interests.

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