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Original Article

In Vitro Activity of Green Synthesized Silver Nanoparticles via Thymus Vulgaris Extract against *Leishmania major*

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Abstract

Background: We aimed to assess the in vitro effects of the green synthesized silver nanoparticles *(Ag NPs)* via *Thymus vulgaris (*thyme*)* against *Leishmania major* infection.

Methods: We have prepared *T. vulgalis* silver nanoparticles (TSNPs) by adding *thyme* extract to the silver nitrate aqueous solution (0.2 mM), and evaluated their antileishmanial activity. The viability of *L. major* promastigotes was assessed in the presence of various concentrations of TSNPs by direct counting after 24 h. The MTT assay was used to identify the viability of promastigotes. The same procedures were assessed in uninfected macrophage cells. The apoptotic effects of nanoparticles on *L. major* promastigotes were determined by flow cytometry assay using annexin staining. To evaluate anti-amastigotes activity of TSNPs, light microscopic observation was used to determine the number of parasites within the macrophages in each well.

Results: The effect of TSNPs on promastigotes and amastigotes of *L. major* was effective and had a reverse relationship with its concentration. TSNPs, inhibited the growth rate of *L. major* amastigotes and, the IC50 value of these nanoparticles was estimated 3.02 µg/mL (28 µM) after 72h. The results of flow cytometry showed that the toxic effects of TSNPs on promastigotes after 24 hours were statistically significant ($P<0.05$) and showed 69.51% of apoptosis.

Conclusion: TSNPs had an inhibitor effect on promastigote and amastigote forms of *L. major* in vitro. It might be considered as a candidate for the treatment of this infection.

Introduction

Despite the remarkable advances made in the field of the therapy of leishmaniasis, few effective therapeutic schemes are available for prevention and control of leishmaniasis in both humans and animals. So far, the best option to treatment the leishmaniasis diseases remains limited to chemotherapy. The recommended treatment for leishmaniasis is based on the use of pentavalent antimonials (PAs) as well as other drugs such as miltefosine, pentamidine isethionate and amphotericin B (liposomal forms) are also used as alternatives (1-3).

Nevertheless, these medications suffer from multiple limitations, including adverse effects, serious toxicity, high costs, therapeutic failure and long-term treatment, or even emergence of resistant parasites (1-5). Hence, the lack of appropriate treatment makes the development of potent anti-leishmania compounds against leishmaniasis one of the main objectives in the management of this disease (6). The development of different nanoparticles (NPs) has been one of the greatest public health achievements in history, contributing to significant improvements in treating infectious diseases and tumors (7-10).

Recently, multiple studies reported that a number of novel nanomaterial such as magnesium oxide, and iron oxide have potent antileishmanial effects (11-12). Apart from this, the therapeutic effects of many of the plants extracts like *Aloe-emodin*, *Artemisia aucheri*, *Sambucus ebulus* and *Thymus migricus* on cutaneous leishmaniasis have been conducted previously (13-15). A number of reports have disclosed that silver nanoparticles (Ag NPs) have excellent antileishmanial activities against different forms of this disease (16-18).

Considering the antimicrobial properties of silver NPs (19-20) and *Thymus vulgaris* (thyme) extract (21-22), the purpose of this study is to produce and characterize silver nanoparticles via thyme extract (TSNPs), and determine the protective effect of synthesized nanoparticles against promastigotes and intracellular amastigotes of *L. major*.

Materials and Methods

Extract preparation and synthesis of silver nanoparticles

The thyme extract and synthesis of silver NPs was carried out through the procedure reported by Mohammadi et al (21) in the Department of Materials Engineering Tarbiat Modares University, Tehran, Iran in 2021. In a brief, thyme leaves were divided to fine pieces after washing by deionized water and air dried out of direct sunlight. Then, 0.2 g of prepared thyme added to 100 ml deionized water and at 80 °C and stirred for 40 min. Right after, the solution was filtered and kept in a dark place at 4 °C. To synthesis silver nanoparticles via thyme extract (TSNPs), the prepared thyme extract was poured into the 2mM $AgNO_3$ solution in the ratio of 1 to 20. The color of solution gradually changed from colorless to brown. To remove remnant possible Ag ions, the solution was dialyzed by a dialysis bag of 12 kDa in de-ionized water and passed through a 0.22µm syringe filter.

Characterization of thyme silver nanoparticles

To evaluate preparation of TSNPs, UV-visible spectroscopy was employed through a SPUV-26 SC-Tech spectrophotometer. The UV-visible investigation of prepared NPs was carried out against water medium. Furthermore, Transmission Electron Microscopy (TEM) was used via a Leo 912 AB microscope to assess morphology and size of TSNPs. To prepare TEM samples, a few droplets of the synthesized NPs solution poured out in the device sample holder, and dried in a vacuum atmosphere.

Leishmania parasite culture

L. major promastigotes from Iranian standard strain (MRHO/IR/75/ER) were prepared

from parasitology department of Tarbiat Modares University and cultured in flasks containing RPMI-1640 medium with 10% fetal bovine serum (FBS). In order to prevent microbial growth, penicillin (100 unit ml^{-1}) and streptomycin (100 µg ml^{-1}) were added to the media. Subsequently, the medium containing the promastigotes were kept in a 25 ± 1 °C incubator and examined every day by inverted microscope.

Cell line and cultivation

RAW 264.7 Cell Line from mouse BALB/C monocyte/macrophage were provided from the Department of Medical Parasitology in Tarbiat Modares University of Tehran, Iran. Macrophage cell lines were grown and propagated in RPMI 1640 containing 10% FBS and 100 µg ml^{-1} streptomycin/ penicillin (Sigma Chemical Co.) initially, followed by incubation in a humidified incubator at 37 °C and 5% CO_2. The cell culture was sub-cultured every three days according to their propagations and turbidity of the medium.

Cytotoxicity evaluation on promastigotes

For evaluation of parasite viability, 100 µl of parasite suspension containing 2 × 10^6 cells/ml and extracts of TSNPs at eighth different concentrations (50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 µg/mL) in 96-well microplates are brought together as triplicate. Amphotericin B (1µg/mL) and Glucantime (50 µg/mL) were considered as positive controls while parasites plus RPMI-1640 (without drug) were the negative controls. After 24, 48, and 72 hours incubation in 24°C, the direct counting method in a hemocytometer (Neubauer chamber) was carried out under an inverted microscope. Results were analyzed using Graph pad Prism (version 8.0.1 software).

Anti-proliferative effects on promastigote forms

The cytotoxicity of TSNPs was determined on the promastigotes of *L. major* parasites by a well-established MTT assay (Sigma-Aldrich) with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (23-24). For that purpose, *L. major* promastigotes were divided in 96 wells plates at 1×10^5 cells/well and exposed with different concentrations of TSNPs: 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 µg/mL for 24 hours. At the end of each incubation period and for each well, 20 µl of the MTT-PBS solution (5 mg/ml) was added and further incubated for 4 hours at 24°C. Next, 100 µL DMSO (Dimethyl sulfoxide) was added to replace the culture media and was shaken at room temperature for 20 minutes to eliminate formazan crystals (23).

Cytotoxic effects on macrophages cells

The effects of TSNPs on viability of the macrophages were investigated by MTT assay. Raw 264.7 macrophages were seeded into 96-well plates at (100 µL, 1×10^6 cell mL^{-1}) in RPMI1640 supplemented with 10% FBS and antibiotics (1 % penicillin and streptomycin) and then treated with TSNPs at the concentrations of 50 to 0.19 µg/mL at 37°C in a 5% CO_2. After 24 h incubation, the rest of the stages were done as mentioned above.

Anti-intracellular amastigote activity

Raw 264.7 macrophage cells (10^5/well) were cultured in 12-well micro-plates having round glass coverslips and 2 mL of growth medium in each well. Incubation at 37°C followed in an incubator with 5% CO_2 for 4 hours. Excess cells were removed by washing twice in phosphate buffered saline (PBS). Adherent macrophages were infected using *L. major* promastigotes in a ratio of 1:10 (parasites/ cells) and incubated for a further 24 hours under those conditions. The infected cells were then washed again with PBS to remove extracellular promastigotes and were exposed to different concentrations of TSNPs (1.25 and 2.5 µg/mL) for 24 h at 37°C (23).

Flow cytometry analysis

In order to assess the drug-induced apoptosis and necrosis flow cytometry method (Bio Vision, Palo Alto, USA) was carried out by

double staining with annexin V-FLUOS and propidium iodide (PI) (36). Briefly, about 2×10^6 ml^{-1} *L. major* promastigotes were incubated with TSNPs (5 µg/mL) in 12 well plates; the inoculated plates were then incubated for 72 h at 24°C. After that, the treated and untreated samples were centrifuged (1400 *g* for 10 min), washed twice in cold PBS solution, and resuspended in 500 µL binding buffer in the presence of 5 µL of annexin V and 5 µL of propidium iodide (PI). After incubation in the dark at room temperature for 15min, the samples were analyzed by FACSCalibur flow cytometer (BD Biosciences). Analysis was performed employing FlowJo software and the percentage of viable and dead cells for each sample were estimated (24).

Statistical Analysis

SPSS software version 21 (IBM Corp., Armonk, NY, USA) and Graph Pad Prism (GraphPad Software 8, Inc., USA) were used for statistical calculations and data analysis. Group comparisons were done by one-way ANOVA and Tukey's post-hoc test. Data were expressed as mean ± standard deviation (SD), and the level of significance was assumed as (*P*) value less than 0.05 ($P < 0.05$).

Ethical approval

The research protocols used in this research were approved by the ethics committee and the research council of Qazvin University of Medical Sciences, Qazvin, Iran (The ethical cod: IR.QUMS.REC.1401.007).

Results

Characterization of thyme silver nanoparticles

The UV-visible spectroscopy of synthesized silver nanoparticles via thyme extract is shown in Fig. 1 (a). The Peak at 440 nm wavelength confirms formation of silver nanoparticles. In addition, TEM image of Ag NPs in Fig. 1 (b) shows size and spherical shape of prepared NPs. The average of NPs size is 18 nm.

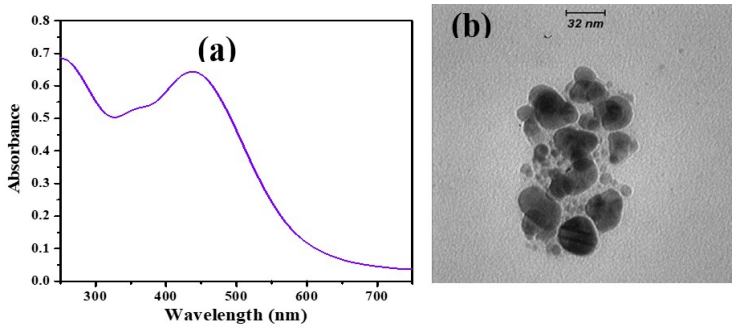

Fig. 1: UV-visible spectroscopy (a) and TEM images (b) of synthesized Ag NPs via thyme extract.

The effect of TSNPs on promastigotes growth

The results demonstrated that promastigote proliferation decreased significantly in the presence of all concentrations of TSNPs compared to those exposed to no drugs ($P < 0.05$). Overall, the growth inhibitory effects of TSNPs on parasites were concentration dependent. The maximum effect was observed in the higher concentrations of TSNPs (50, 25, 12.5, 6.25 and 3.12 µg/mL(465-29 µM)), while the least effect was seen in the lower concentrations of these nanoparticles (0.78 and 0.39). At 48, and 72 h, the higher concentrations of TSNPs (50, 25 and 12.5 µg/mL(465-116 µM)) exhibited a more profound cytotoxicity and completely killed *L. major* promastigotes. The calculated IC50 after 24 hours was 4.7 µg/mL (Fig. 2).

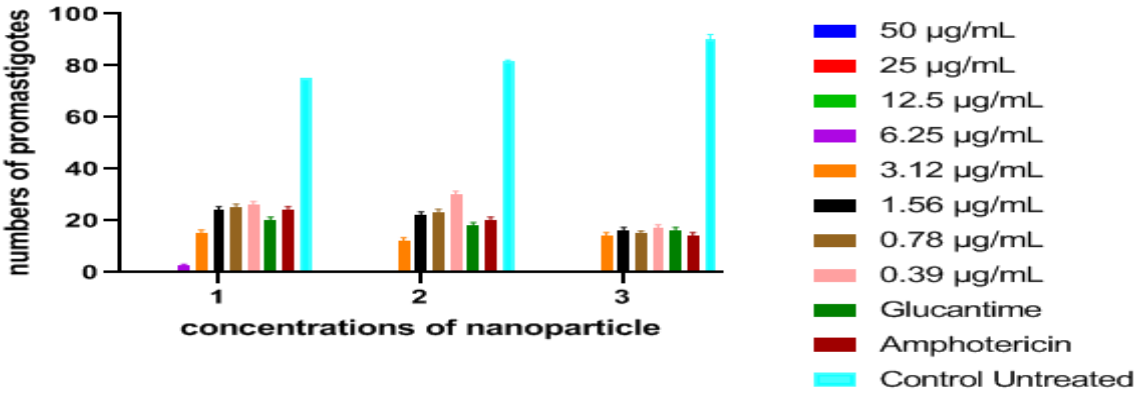

Fig. 2: Mean and standard deviation of the number of promastigotes of *L. major* ($\times 10^4$) cultured with different concentrations of TSNPs (50 to 0.39 µg/mL) for 24 (1), 48 (48), and 72 (72) h. Amphotericin B and Glucantime were applied as positive controls. Parasites plus RPMI-1640 (without drug) were the negative controls. The concentration of glucantime was 50 µg/mL and amphotericin was 1 µg/mL. The promastigotes were damaged in concentration of 50, 25 and 12.5 µg/mL

The cytotoxicity of TSNPs to the promastigotes by MTT

As Fig 3 shows, the proliferation of parasites decreased by increasing the nanoparticle concentration. In this regard, after exposure to higher concentrations of these nanoparticles from 1.56 µg/mL (14.5 µM) up to 50 µg/mL (465 µM) for 24 hours, parasite viability was significantly decreased when compared with those exposed to no drugs.

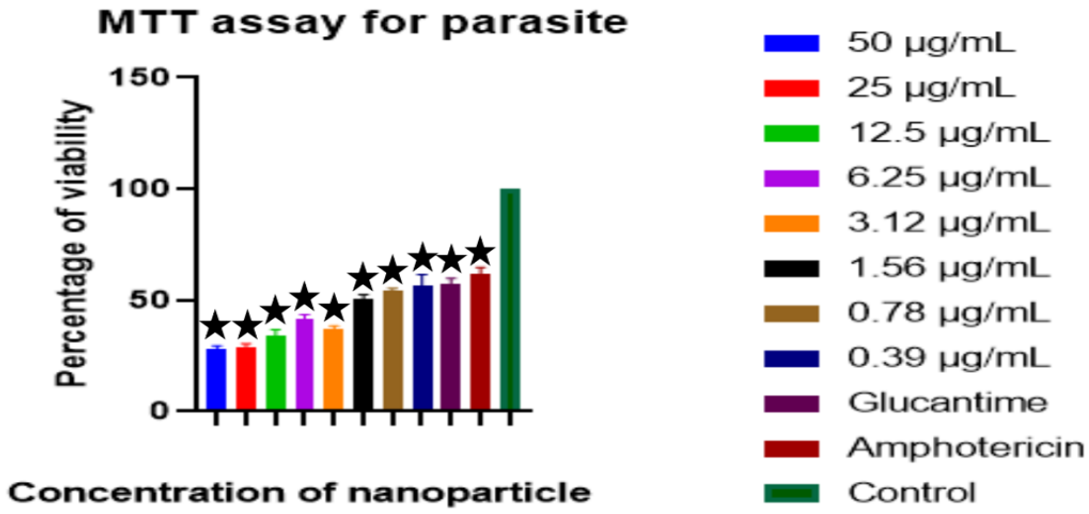

Fig: 3: The viability percent of promastigotes of *L.major* in the presence of different concentrations of TSNPs (50 to 0.39 µg/mL) for 24 h. After treatment, the viability was investigated using MTT. Amphotericin B and Glucantime were applied as positive controls. Parasites plus RPMI-1640 (without drug) were the negative controls. The concentration of glucantime was 50 µg/mL and amphotericin was 1 µg/mL. Results are displayed as the mean ± SD. * $P<0.05$

The cytotoxicity of TSNPs to macrophages by MTT

Results from the MTT assay showed that the addition of TSNPs at the doses of 50, 25, 12.5, and 6.25 µg/mL (465-58 µM) for 24 hours significantly reduced the viability of macrophage cells in tested groups when compared with the control groups. The calculated CC50 was 15.12 in this test (Fig. 4).

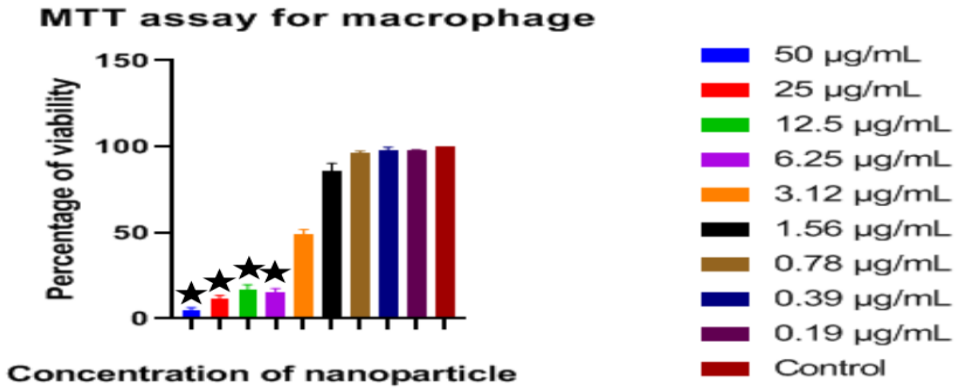

Fig. 4: The viability percent of the uninfected RAW macrophages in the presence of different concentrations of TSNPs (50 to 0.19 µg/mL) for 24 h. After treatment, the viability was investigated using MTT. Results are displayed as the mean ± SD. * $P<0.05$

Anti-amastigote effect screening

To demonstrate the efficacy of TSNPs against *L. major*, the percentage of infected macrophages and number of amastigotes per infected macrophage were determined. The results obtained from numeration of intracellular parasites by phase contrast microscopy confirmed that the percentage of infectivity in untreated infected macrophages were 30%, whereas the percent infection by amastigote upon treatment with concentrations of 2.5 and 1.25 µg/mL of these nanoparticles were 8.5 and 29, respectively (Fig. 5& 6). In addition, the half-maximal inhibitory concentration (IC50) of these nanoparticles on Raw 264.7 macrophages after 72 hours of exposure was found to be 3.02 µg/mL.

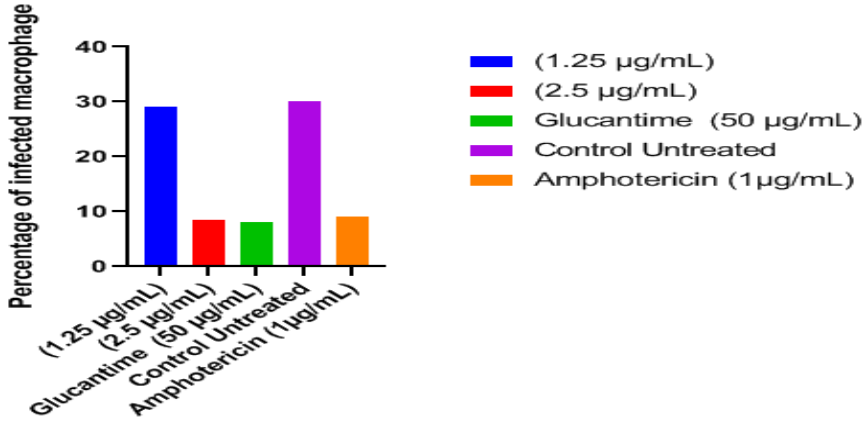

Fig. 5: Percentage of infected macrophages with amastigotes

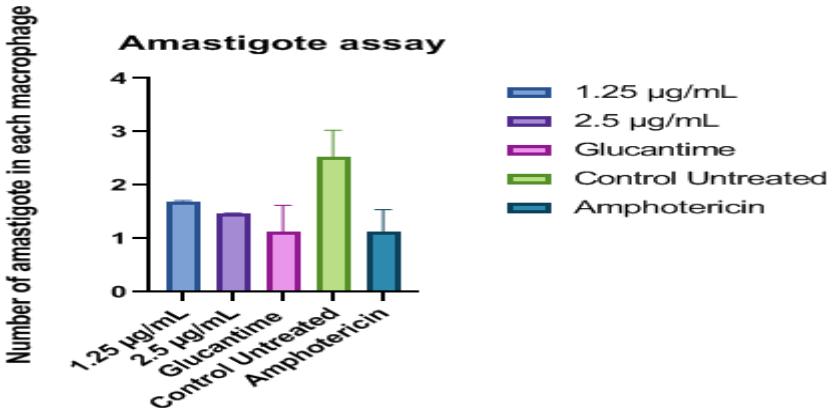

Fig. 6: Mean ± standard deviation the number of amastigotes in each macrophage

Flow cytometry assay

Double stains Annexin V-FITC/propidium iodide (PI) were used to differentiate apoptosis from necrosis in *L. major* promastigotes. The obtained findings revealed that the *L. major* promastigotes exposed to TSNPs underwent apoptosis and necrosis more than the untreated parasites or control group. The rate of normal, apoptotic and necrotic cells caused by 5 µg/mL of TSNPs in the promastigotes was 30.1%, 69.51% and 0.38%, respectively. In the control group (untreated promastigotes), however, these rates were 99.2%, 0.64% and 0.14%, respectively. The flow cytometry histograms of treated parasites and untreated parasites are shown in Fig. 7.

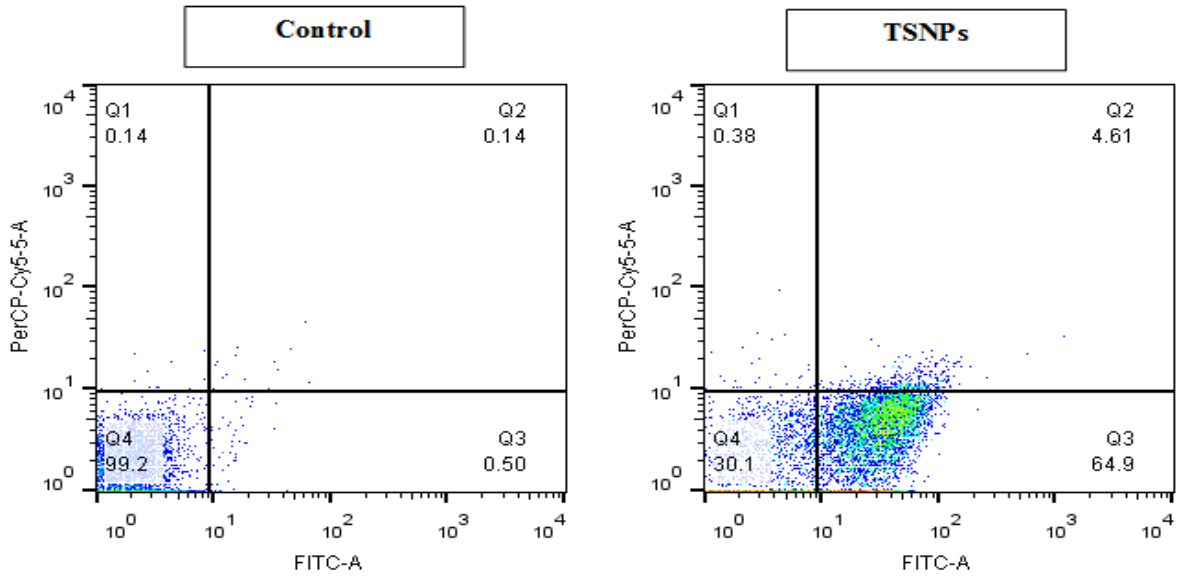

Fig. 7: Flow cytometry results of the effect of TSNPs on promastigotes of L. *major* and comparing with the control group after 72 h. Regions of quadrate show necrosis promastigotes (Propidium Iodide positive) in left top, late apoptosis in promastigotes (Annexin V and Propidium Iodide positive) in right top, right bottom region belongs to early apoptosis in promastigotes (Annexin V positive) and left bottom region belongs to live promastigotes (annexin V and propidium iodide negative)

Discussion

The appearance of drug resistance in some endemic areas is considered as a major issue due to abuse of current anti-leishmania drugs. Another problem associated with treatment of this disease using drugs is the emergence of various systemic side effects such as kidney injury, cardiomyopathy, scarring of the liver or liver failure as well as hypersensitivity reactions. Due to the heavy burden of infection worldwide in different host species, the need of effective anti-leishmanial agents with minimal toxicity is mandatory to prevent leishmania infection and reduce disease (25-26).

Accordingly, various herbal extracts, chemical compounds and nanomaterials have been investigated in vitro and *in vivo* as potential treatments (13-14). The use of nanoparticles in the current times has become popular because of their therapeutic implications in many human diseases (25). As obvious, frequently was shown that medical usage of nanomaterials can improve drug delivery, bioavailability, and combat drug resistance (25). One of the most attractive anti-leishmanial agents are silver nanoparticles, which have been recently investigated to prevent of infection in cutaneous leishmaniasis. (15-18). One study has shown that treatment with 100 µg/mL of silver nanoparticles had more toxic effects on *L. major* parasites and resulted in the destruction of more than 85 percent of infected macrophages with amastigotes of *L. major* compared to the control group (16).

The important finding is that all concentrations of green synthesized silver nanoparticles via thyme extract exhibited substantial anti-leishmanial activity against extracellular promastigotes in vitro and intracellular amastigotes in *L. major*-infected macrophages. Moreover, these nanoparticles exhibited high anti-leishmanial effects against amastigotes with a half-inhibitory concentration (IC50) value of 3.02 µg/mL. These observations highlighted the advantage of these nanoparticles in reducing infected macrophages and the inhibition of the proliferation rate of intramacrophage amastigotes. The findings of Awad et al. studies revealed that all of the concentrations of myrrh silver nanoparticles (MSNPs) had inhibitory effects on *L. major* promastigotes, but the greatest anti-leishmanial activity was related to the higher concentrations of (MSNPs) in comparison to the chemical nanoparticles (CNPs) and reference drug (27).

In another study, the effect of various concentrations of silver nanoparticles at the presence of bioresonance waves led to a decrease in the numbers of live promastigotes and proliferation rates of *L. major*. The authors concluded that the combination of bioresonance waves and silver nanoparticles as potent antileishmanial agents could be effective against cutaneous leishmaniasis (28).

Although the mechanism of antileishmanial activity of biogenic silver nanoparticles is not well-defined (29), it can stimulate the inflammatory response by helping to activate macrophage cells to produce the high level of nitric oxides (NO) which in turn causes DNA damage, cell membrane disruption, variation on protein structure and function as well as parasite destruction (30-31).

However, further investigations are necessary to understand better of the application of these nanoparticles as a new therapeutic option for the treatment of *Leishmania* infections. In the Hashemi et al. study, they synthetized silver nanoparticles using *Ferula persica* extract (Fp-NPs) and antimicrobial properties of these nanoparticles were investigated against pathogenic microorganisms. Their results revealed that Fp-NPs reduced proliferation of amastigote stages of *L. major* with the IC50 value of 26.43µg ml^{-1}. In addition, Fp-NPs exhibited remarkable anti- promastigotes activity with the IC50 value of 23.14 µg/ml (32). The demonstration of apoptosis by flow cytometry has been previously verified in different cells

with silver nanocomposites and the results showed appreciable apoptosis (33). Fanti et al. evaluated the cytotoxicity of silver nanoparticle (Ag NPs) obtained from *Fusarium oxysporium* on *leishmania amazonensis* promastigote and amastigote forms and they showed that these nanoparticles had toxic effects on these parasites. According to their results (Ag NPs) was able to induce apoptosis in promastigotes due an enhanced production of mitochondrial reactive oxygen species (ROS), phosphatidylserine exposure and destruction of parasites membrane. In addition, they showed that these nanocomposites can reduced proliferation of amastigote stages of *L. amazonensis* (33).

Our results also illustrated that TSNPs were able to induce apoptosis in promastigotes of *L. major* after exposure to 5 μg/mL nanoparticles compared to the control group. Flow cytometry results indicated a substantial increase in *L. major* promastigotes apoptosis. Hence, it found that these nanoparticles might act as an appropriate anti-leishmania agent by inducing programmed cell death (PCD) on promastigote forms.

Conclusion

Nowadays, plant-based nanoparticles as a promising substitute for the treatment of various diseases have gained importance. These results demonstrate a significant anti-leishmanial activity for TSNPs against *L. major* promastigotes and infected macrophages. Therefore, it can be inferred that this group of compounds could be used as an adjunctive therapeutic candidate for the treatment of disease due to *L. major*. In the future, further attempts should be made to evaluate the *in vivo* effects.

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Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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