Original Article

Effects of silver nanoparticle based on ginger extract on *Leishmania infantum* and *Leishmania tropica* parasites: *in vitro*

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ABSTRACT

Leishmania is the primary cause of a significant public health problem known as leishmaniasis in Iran. Pentavalent antimonial chemicals are commonly used for the treatment of leishmaniasis. However, these drugs exhibit a number of adverse effects, including drug resistance, lack of specificity, poor responsiveness, toxic effects, inconvenient injections, tissue damage, and high cost. The present study aimed to prepare and evaluate the efficacy of green-synthesized silver nanoparticles (Ag-NPs) against *Leishmania infantum* and *Leishmania tropica in vitro*. The 2, 5-Diphenyl Tetrazolium Bromide (MTT) assay was used to assess the toxicity of Ag-NPs derived from ginger extract on macrophage cells. The apoptotic potential of promastigotes caused by Ag-NPs was evaluated using the flow cytometry method. According to our findings, the proliferation of *L. infantum* and *L. tropica*, promastigotes are significantly decreased by increasing doses of NPs. The most effective doses of nanoparticle were 80 and 40 ppm after 48 and 72 hours of incubation, respectively, while doses of 0.312 and 0.156 ppm after 24 and 48 hours of incubation had the least effect on the growth and activity of *L. infantum* and *L. tropica* promastigotes. For the promastigotes of *L. infantum* and *L. tropica*, the flow cytometry test revealed that Ag-NPs-induced programmed cell death in promastigotes of *L. infantum* and *L. tropica* demonstrated 67.1% and 41.9% of apoptosis, respectively. The half-maximal inhibitory concentration for NPs against *L. infantum* and *L. tropica* were 4.54 and 4.22 ppm, respectively, based on the MTT assay. The higher concentrations of NPs (e.g., 80 ppm) led to more lethality of promastigote. In conclusion, Ag-NPs exhibited good *in vitro* anti-leishmanial activity against *L. infantum* and *L. tropica* promastigotes.

Keywords: Ginger, *Leishmania infantum*, *Leishmania tropica*, MTT, Silver nanoparticle.

1. Introduction

Leishmaniasis is caused by an intracellular parasite called *Leishmania*. This disease, transmitted by the bite of female Phlebotomine sandflies in tropical and subtropical climate zones, remains a major public health concern (1). There are at least three main clinical forms of the disease caused by *Leishmania* parasites, namely cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis, and visceral leishmaniasis (VL) (2). On average, 350 million people are believed to be at high risk of *Leishmania* infection, and 12 million people become infected with the parasite worldwide. According to World Health Organization data, between 1,000,000 and 500,000 new cases of CL and VL infections are reported each year among people in both developed and developing countries (3). *Leishmania tropica* is typically responsible for CL, while *Leishmania infantum* is generally associated with VL (4). Macrophages are immune cells with various tasks, including killing pathogens and healing injured cells. Due to these tasks, macrophages are classified into two subtypes: traditionally activated macrophages (also known as the M1 phenotype) and alternatively activated macrophages (also known as the M2 phenotype). In contrast to the M2 phenotype, which has anti-inflammatory and mending properties and prevents pathogen killing, the M1 phenotype has an inflammatory effect and kills pathogens (5). *Leishmania* spp. and other infections aim to induce the M2 phenotype and bypass the immune system (6). Despite scientific efforts, there are currently no effective leishmaniasis prevention and treatment intervention methods. For the past 70 years, chemotherapy has been the mainstay treatment for leishmaniasis, and the most efficient leishmaniasis treatments have been antimony compounds, such as sodium stibogluconate (also known as Pentostam) and meglumine antimoniate (commonly known as Glucantime). It has also been discovered that these drugs can prevent the production of adenosine triphosphate by obstructing the activity of the phosphokinase enzyme (7). The drugs used to treat leishmaniasis infection have not been proven to be completely effective and exhibit several negative side effects, including drug resistance, non-specificity, poor responsiveness, toxic effects, inconvenient injections, long-term use, tissue damage, and high cost (4). As antiparasitic medicines have unfavorable side effects or could result in serious complications, proper treatment methods, and effective anti-leishmania ingredients must be identified and developed (8). Nanoparticles (NPs) are known to have biomedical and pharmaceutical applications (9). The use of NPs in medicine is a recent and innovative development that supports the efforts of the international health community to eradicate leishmaniasis endemics (10). It may be relatively hopeful to utilize silver nanoparticles (Ag-NPs) to combat parasites, which are pathogenic organisms as well. The antiparasitic effects of this NP on *leishmania* and malaria parasites have been previously investigated in this regard, and the results are quite encouraging (11). Low quantities of the NP should be examined and used to address this problem because it should be highlighted that using this material in high doses is hazardous to host cells and is not approved. Given the preceding rationale, the goal of this study was to examine the impact of Ag-NPs at various concentrations on *L. tropica* and *L. infantum in vitro.*

2. Materials and Methods

2.1. Synthesis of Silver Nanoparticles

Nano-sized silver particles were produced by the Pharmaceutical Biotechnology and Pharmaceutical Sciences Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences. In this process, the ginger rhizomes were washed in deionized (DI) water and then cut into small pieces before thoroughly dried for 4 days in the shade at 27°C. Subsequently, 0.2 g of finely chopped ginger was added to 100 mL of DI water and stirred for 40 min at 80°C. The extract was preserved at 4°C away from the light after being filtered using Whatman No. 1 paper before being centrifuged for 5 min at 4000 rpm. The 0.2 mM AgNO3 solution was prepared for the production of Ag-NPs, and the ginger extract was added to the AgNO3 aqueous solution (1:20). Initially, the solution was essentially colorless; however, as the reaction progressed, it changed from light yellow to dark brown, serving as a visual indicator of the presence of NPs. Next, using a 12 kD dialysis bag, the Ag-NPs were dialyzed in water for 24 h before being filtered using 0.22 m syringe filters (12).

2.2. Cultivation of Parasites

Promastigotes of *L. infantum* (MHOM/TN/80/IPT1) and *L. tropica* (MHOM/IR/02/Mash10) were obtained from the Department of Parasitology of Tarbiat Modares University, Tehran. For the growth and replication of the *L. infantum* and *L. tropica* promastigotes, the nutritional RPMI fetal calf serum (Gibco, German) (10% v/v) was employed, followed by incubation at 25 ± 1°C. This medium was enhanced with penicillin (100 µg/mL streptomycin and 100 IU/mL penicillin) and fetal calf serum (10% v/v) (13).

2.3. Promastigote Assay

For this test, 100 µL of promastigotes (2×10^6 cells/mL) were seeded in a 96-well plate with 100 µL of RPMI1640 + 15% fetal bovine serum in the presence of various concentrations of the Ag-NPs solution (80, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312, and 0.156 ppm) for 24, 48, and 72 h. Glucantime (Sanofi-Aventis France) (50 µg/mL) was used as a positive control group. Finally, the anti-

leishmanial effects of Ag-NPs on promastigotes of *L. tropica and L. infantum* were assessed using the direct counting method in a Neubauer chamber. The results were compared with control groups and analyzed using Graph Pad Prism 5.0 (12).

2.4. Assessment of Nanoparticle Cytotoxicity on Macrophages

To assess the cytotoxicity of nanoparticles on macrophages, the 2, 5-Diphenyl Tetrazolium Bromide (MTT) test was used. The MTT test was performed using a murine macrophage cell line (RAW 264.7). To evaluate the effect of the nanoparticle on macrophage cells, the MTT (Sigma Aldrich) test was applied. MTT solution was prepared by mixing 1 mL of phosphate-buffered saline (PBS) and 5 mg of MTT powder (Sigma Chemical Company, Germany). After trypsinization, RAW 264.7 macrophage cells were implanted in 96-well microplates with 100 L each well at 5×10^5 cells/well in Dulbecco's Modified Eagle medium containing 10% fetal calf serum. This plate was incubated for 72 h at 37°C and 5% CO_2. After adding 20 µL MTT reagent to each well following 72 h, in order to allow the cells to convert the tetrazolium to an insoluble formazan, the plate was incubated at 37°C for 5 h in the dark. Subsequently, 100 µL of the nanoparticle at various concentrations (40, 20, 10, 5, 2.5, 1.25, 0.625, 0.32, 0.156, 0.078 ppm), along with the RPMI culture medium, were separately added to the wells. Each well received 100 µL of dimethyl sulfoxide (DMSO) after draining the supernatant. An enzyme-linked immunosorbent assay (ELISA) plate reader instrument (Stat Fax, USA) with a 540 nm setting was then used to measure the optical density. The following formula was used to determine the cell viability rates as a percentage in the exposed and control groups (14).

Viable (Live) macrophages (percentage) = (AT-AB) / (AC-AB) × 100

AT: Macrophage absorbance when exposed

AC: Untouched macrophage absorption

AB: Absorbency of the blank

2.5. MTT assay to assess cytotoxicity of nanoparticles on promastigotes

The MTT solution was created in a darkened space by dissolving 5 mg of MTT powder (tetrazolium salt) in 1 mL of the PBS solution. Additionally, 96-well culture plates with various nanoparticle doses (80, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312, and 0.156 ppm) had 5×10^5 promastigotes per mL added. Twenty microliters of the prepared MTT solution were added to each well after a 72-hour dark incubation period. Following centrifugation, the cells' supernatant was collected. After a second 5-hour incubation period at room temperature, 100 µL of DMSO was added to each plate well, and the plate was then put into an ELISA reader with a 540 nm wavelength to measure the absorbance of each well (15).

2.6. Flow Cytometry for Cell Apoptotic Status Determination

The annexin V-FITC apoptosis detection kit (BioVision, Palo Alto, USA) was utilized to distinguish between necrotic and apoptotic cells. The testing process was performed thoroughly following the manufacturer's instructions. In summary, 4 ppm of nanoparticle was exposed to 2×10^6 *L. infantum* and *L. tropica* promastigotes separately and incubated for 72 h. In order to remove any excess nanoparticles, the parasites were washed with cold PBS and collected by centrifugation at 1400 g for 10 min. The samples were then added with 500 mL of buffer, 500 mL of propidium iodide (PI), and 5 mL of annexin V (annexin-V kit, IQ Products BV, Groningen, Netherlands) after incubation on ice for an additional 15 min. This kit could distinguish between necrotic (PI positive/upper left) and normal, live (both annexin-V and PI negative/lower right) cells, as well as apoptotic (only annexin-V positive as primary apoptosis/lower right) cells. A flow cytometer (BD FACSCanto II, USA) was then used to analyze the samples. Graphs and percentage charts were used to display the output of the instrument. Data was examined using FlowJo software (version 10) (16).

2.7. Statistical Analysis

IBM SPSS (version 21) was used for statistical analysis. The Kolmogorov-Smirnov test was used to ensure normal distribution of the data, and one-way ANOVA and LSD were used to assess mean differences. Finally, graphs were produced using GraphPad Prism (version 8.0.1) (17).

3. Results

3.1. Effect of Nanoparticle on Promastigote Growth Inhibition

After incubation for 24, 48, and 72 h, figures 1 and 2 illustrate the effects of different doses of NP on *L. infantum* and *L. tropica* promastigotes. According to the findings, the proliferation of *L. infantum* and *L. tropica*, promastigotes were significantly decreased with increasing doses of NPs ($P<0.05$). The most effective doses of NPs were 80 and 40 ppm after 48 and 72 h of incubation. In contrast, the promastigotes growth and activity of *L. infantum* and *L. tropica* were not significantly affected by dosages of 0.312 and 0.156 ppm after 24 and 48 h of incubation ($P>0.05$).

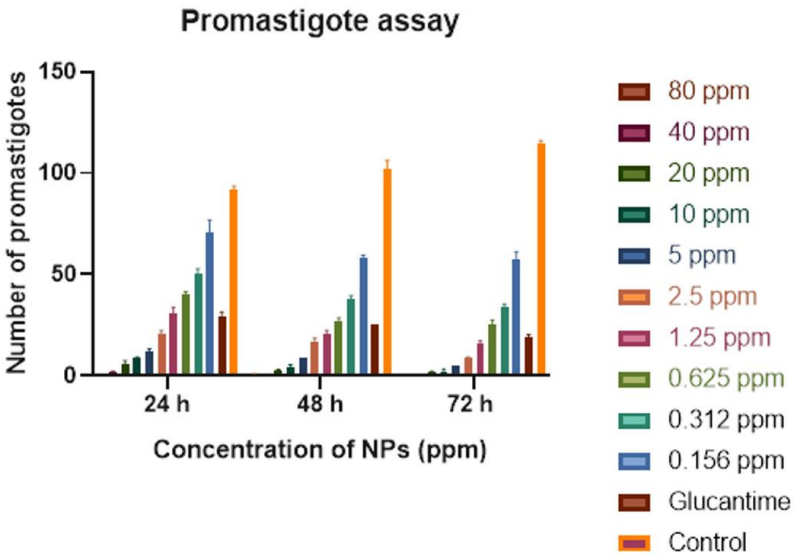


Fig 1. Mean and standard deviation of the number of promastigotes of *L. infantum* (×104) cultured with different concentrations of silver NPs based on ginger extract compared with control groups at 24, 48 and 72 hours.

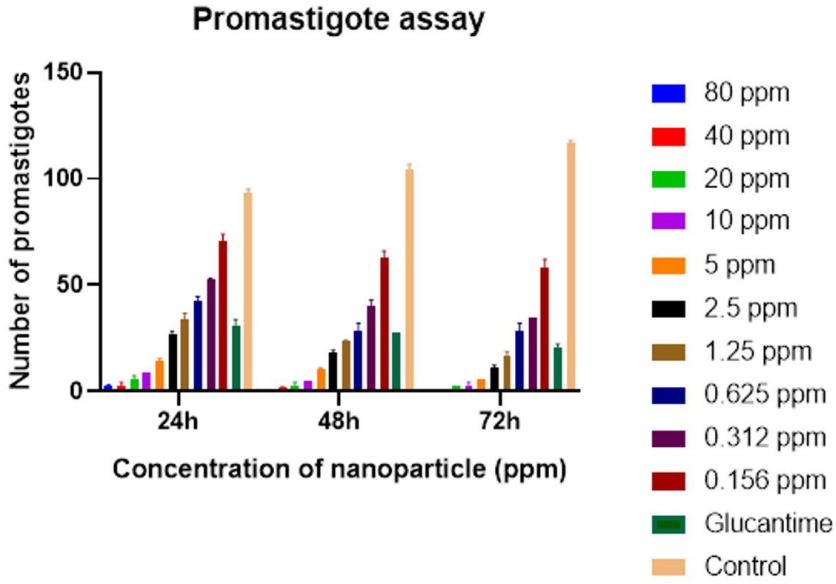


Fig 2. Mean and standard deviation of the number of promastigotes of *L. tropica* (×104) cultured with different concentrations of silver NPs based on ginger extract compared with control groups after 24, 48 and 72 hours.

3.2. Determination of the Half-Maximal Inhibitory Concentration

As per the MTT assay, the half-maximal inhibitory concentration (IC50) value for the NP after 72 h was 4.54 ppm for *L. infantum* and 4.22 ppm for *L. tropica*. Additionally, NP anti-leishmanial capability was correlated with exposure time and dose dose (Figures 3 and 4).

3.3. MTT Assay for Promastigotes

Following the MTT test, optical density was utilized to measure the cytotoxicity of NP on *L. infantum* and *L. tropica* promastigotes. Figures 5 and 6 demonstrate a dose-response relationship that affects the survivability of parasites. The concentrations of 80 and 40 ppm had the most destructive effect on promastigote, leading to a decrease in parasite survival with an increase in NP dosage.

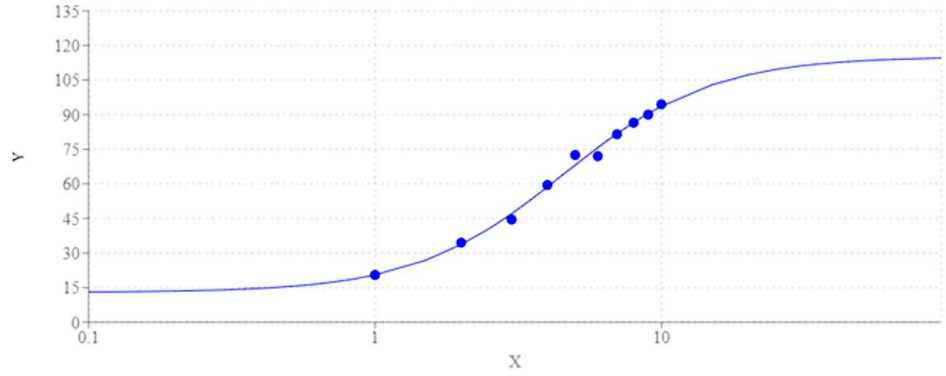


Fig 3. Determination of IC50 for *Leishmania infantum*. Note. IC50: Half-maximal inhibitory concentration. According to this chart, the IC50 for *L. infantum* was 4.54 ppm.

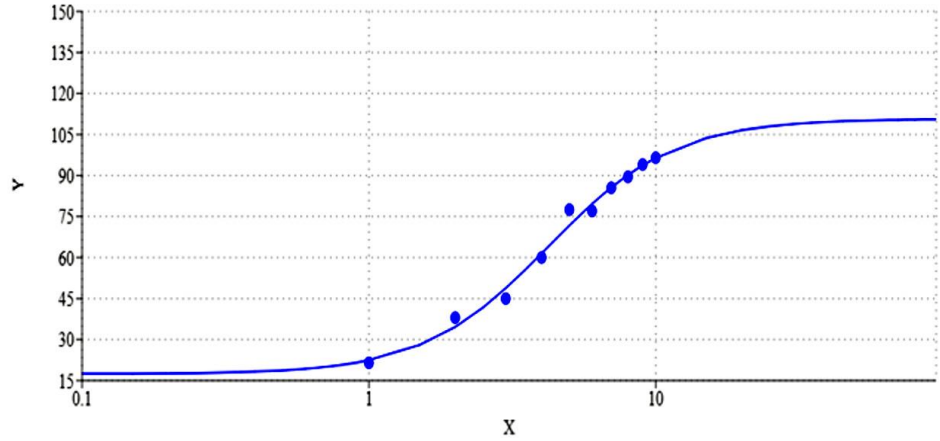


Fig 4. Determination of IC50 for *Leishmania tropica*. Note. IC50: Half-maximal inhibitory concentration. According to this chart, the IC50 for *L. tropica* was 4.22 ppm.

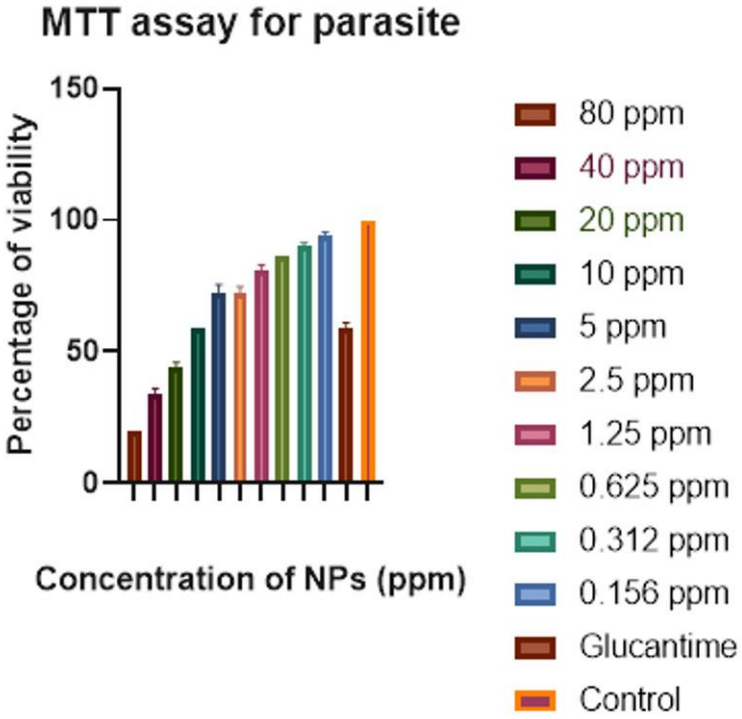


Fig 5. The viability of *L. infantum* promastigotes in the presence of different concentrations of the NPs based on ginger extract, after 72 hours incubation in compare with control group.

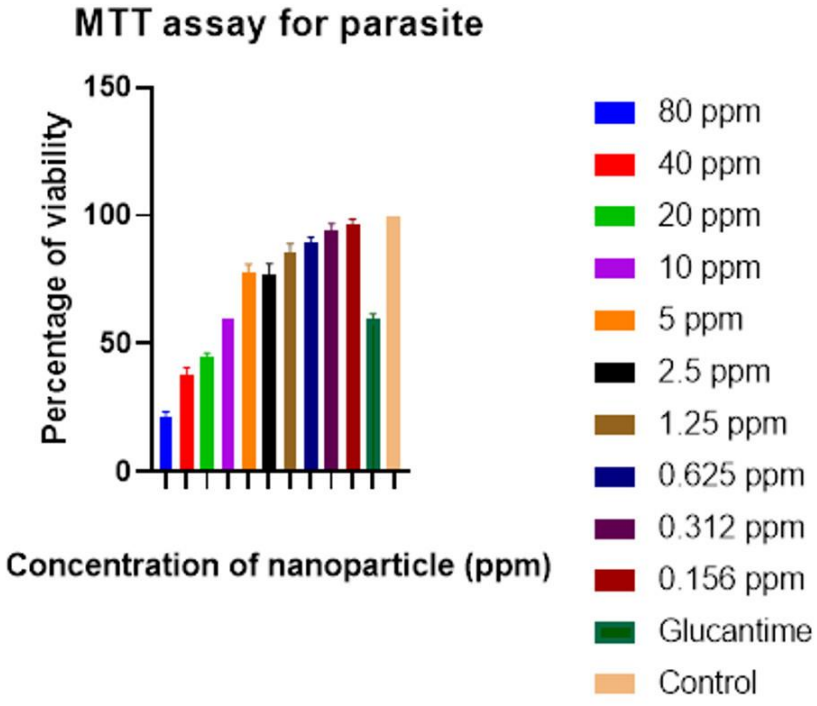


Fig 6. The viability of *L. tropica* promastigotes in the presence of different concentrations of the NPs based on ginger extract, after 72 hours incubation, in compare with the control groups.

3.4. Flow Cytometry Analysis

Flow cytometry was used to assess the percentages of necrotic, living, and apoptotic cells in *L. infantum* and *L. tropica* promastigote populations after labeling with Annexin-V and PI. Following 72-hour incubation, the percentages of apoptotic and necrotic promastigote cells in contact with 4 ppm concentrations of nanoparticle were 67.1 % and 41.9 % for *L. infantum* and *L. tropica*, respectively. Furthermore, for *L. infantum* and *L. tropica*, the percentage of viable cells in the control group (no treatment) was 95.7% and 97.3%, respectively (Figures 7 and 8).

3.5. Nanoparticle Cytotoxicity on Macrophage Cells

The MTT results for assessing NP cytotoxicity on macrophages revealed that high dosages of Ag-NPs (e.g., 40 and 20 ppm) had higher harmful effects on macrophage cells than lower levels, compared to the control group. Additional information is illustrated in figure 9.

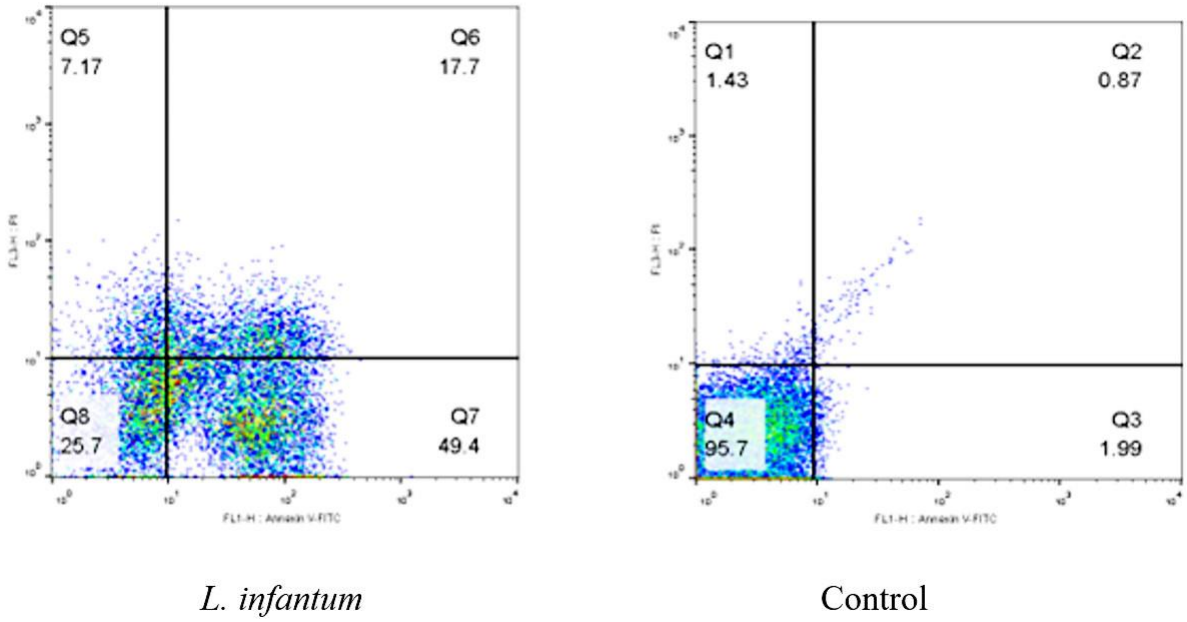


Fig 7. Flow cytometry analysis of the effect of NPs based on ginger extract on promastigotes of *Leishmania infantum* compared with the control group (untreated) after 72 hours. Quadrant regions depict late apoptosis in right-top, necrosis promastigotes in left-top, live promastigotes in the left-bottom, and apoptotic promastigotes in the right-bottom.

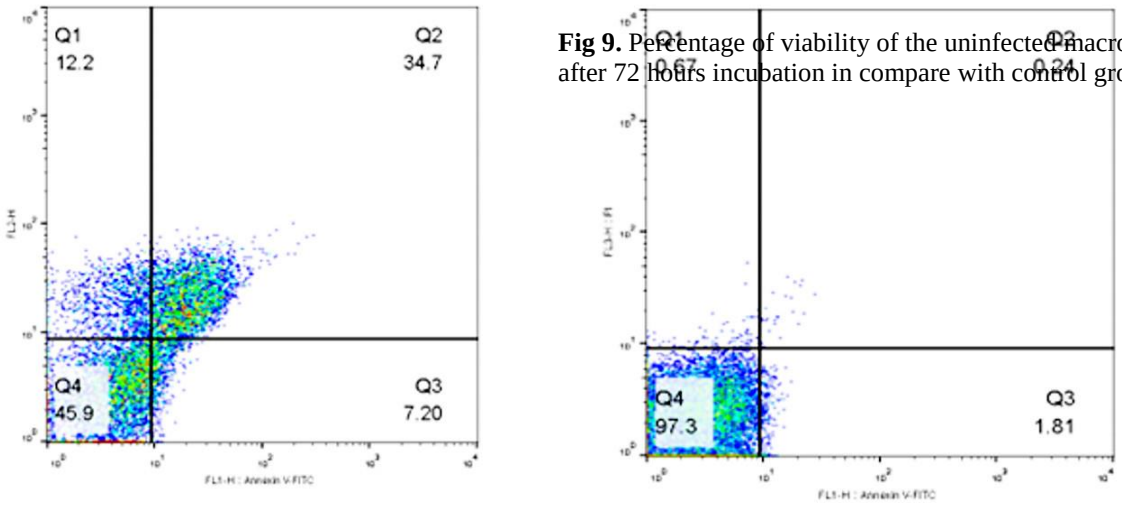
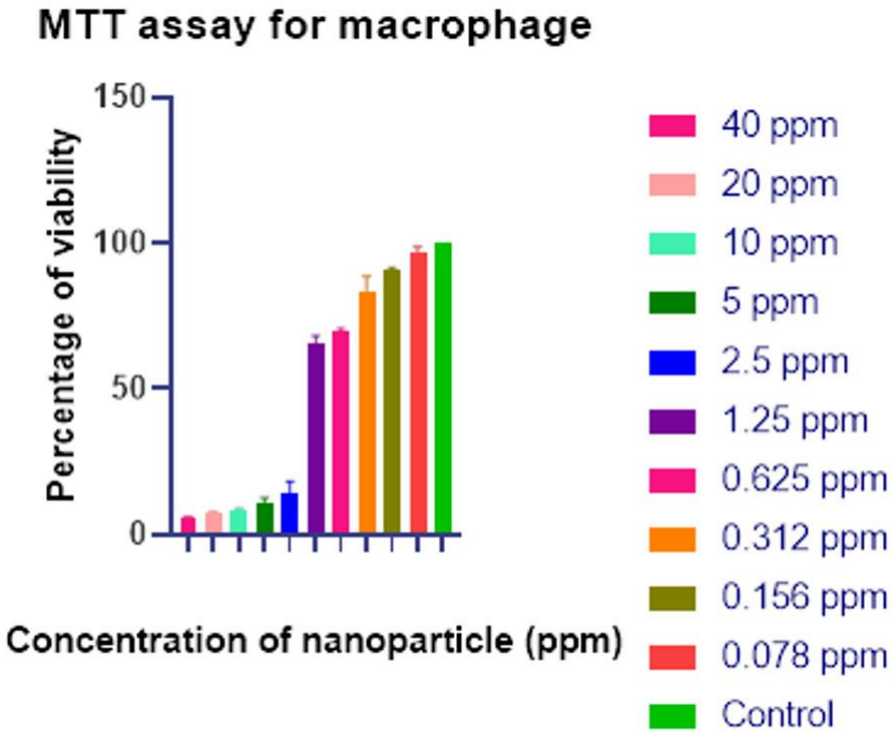


Fig 9. Percentage of viability of the uninfected macrophages with di
after 72 hours incubation in compare with control group.

Fig 8. Flow cytometry analysis of the effect of NPs based on ginger extract on promastigotes of *Leishmania tropica* compared with the control group (untreated) after 72 hours. Quadrant regions show late apoptosis in right-top, necrosis promastigotes in left-top, live promastigotes in the left-bottom, and apoptotic promastigotes in the right-bottom.



4. Discussion

Leishmaniasis is a significant health concern in the tropical and subtropical regions of the world (18). The currently used medications (Glucantime and Pentostam) for leishmaniasis have been reported to be unsuccessful due to their severe side effects, high costs, high toxicity, painful injections, and the evolution of drug resistance in some endemic areas (19). As a result, research is now focused on discovering more affordable and effective medications with fewer side effects (20). Scientists are constantly working on developing efficient anti-leishmanial drugs that might achieve therapeutic objectives. Results from previous research showed the anti-leishmanial activity of Ag-NPs (19). Earlier studies showed that 100 ppm of Nano-silver could damage 85% of infected macrophages with amastigotes of *L. major* (20). In the present study, *L. infantum* and *L. tropica* promastigotes have exhibited significant toxicity in the presence of the NPs. Compared with typical medications, such as Glucantim, our results demonstrated a significant reduction in the proliferation of *L. infantum* and *L. tropica* promastigotes with increasing the Ag-NPs concentration and exposure time. Notably, large concentrations of Ag NPs (80, 40, 20, 10 ppm fully prevented the proliferation of *L. infantum* and *L. tropica* promastigotes) after 24, 48, and 72 h. The current MTT assay results indicated that there was a concentration-dependent decrease in viability for macrophage cells and *L. infantum* and *L. tropica* promastigotes exposed to NPs, with viability percentages of 5.5%, 20%, and 21.5% for macrophages and *L. infantum* and *L. tropica* promastigotes treated with the highest NPs concentration. Additionally, the IC50 concentration after 72 h of exposure for *L. infantum* and *L. tropica* was 4.54 and 4.22 ppm, respectively, demonstrating an effective reduction in promastigotes and growth inhibition. Previous research suggested that Ag-NPs could induce specific cells to undergo programmed cell death (21). Our results demonstrated a higher likelihood of apoptosis induction in promastigotes of *L. infantum* and *L. tropica* after exposure to 4 ppm NPs, compared with the control group (promastigotes without treatment), indicating noticeable apoptotic effects. This suggests that Ag-NPs may act against *L. infantum* and *L. tropica* by inducing apoptosis. A study evaluated the effectiveness of various concentrations of green synthesized Ag-NPs via ginger rhizome extract against *L. major* promastigotes and amastigotes, the results of which showed the effectiveness of Ag-NPs on promastigotes and amastigotes of *L. major* and that it had a reverse relationship with its concentration (22). In a review of the effect of silver nanoparticles, it showed the benefits of silver nanocomposites on various diseases. The review have evaluated the cytotoxic effects of these nanocomposites against parasites, viruses, fungi, bacteria, and various types of cancer. Their findings showed that silver nanocomposites had a satisfactory cytotoxic effect against these microorganisms and cancer cells (23). Mohebali et al. investigated the antileishmanial properties of Ag-NPs on *L. major in vitro* and *in vivo*. Their findings showed that Ag-NPs suppressed the proliferation of *L. major* amastigote stages similar to reference medication. Furthermore, they concluded that nano-silver could prevent severe infection in cutaneous leishmaniasis caused by *L. major* (19). According to a study by Karimipour et al. on NPs and *Toxoplasma gondii* tachyzoites, IC50 was estimated at 2 ppm, with the 80 ppm concentration exhibiting the most damaging effect, inducing apoptosis in approximately 55.22% of tachyzoites (24). This supports the effectiveness of Ag-NPs as effective antileishmanial agents against *Leishmania* infections. In conclusion, owing to the distinct structural properties of NPs, they are now used more frequently to treat various ailments. Our study demonstrated the adequate *in vitro* activity of Ag-NPs against *L. infantum* and *L. tropica* promastigotes. Flow cytometry results indicated substantial apoptosis (67.1% and 41.9%) in *L. infantum* and *L. tropica*. However, further investigations are essential to understand the *in vivo* antileishmanial effects of NPs.

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Authors' Contribution

AK (first author), methodologist/principal researcher ; HS and ZM researcher; AD (third author), supervisor, manuscript writer/methodologist/principal researcher/statistical analyst/discussion writer; MP (third author), advisor and methodologist/principal researcher.

Ethics

This study was confirmed by the Medical Ethics Committee of the Faculty of Medical Sciences of Tarbiat Modares University.

Conflict of Interest

The authors do not have any conflict of interest.

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Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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