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Comparative *in vitro* anti-leishmanial activity of *Thymus vulgaris*-mediated silver nanoparticles against *Leishmania tropica* and *L. infantum*

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ABSTRACT

Background: Leishmaniasis remains a significant global health concern, with current treatments often limited by toxicity, cost, and emerging drug resistance. We aimed to evaluate the antileishmanial potential of eco-friendly silver nanoparticles synthesized using *Thymus vulgaris* extract (TSNPs) against *Leishmania tropica* and *L. infantum in vitro*.

Methods: The antileishmanial effects were evaluated against the promastigote stages of *L. tropica* and *L. infantum* using cell counting, MTT assay, and flow cytometry techniques. Cytotoxic effects on RAW 264.7 macrophages were determined by the MTT assay, and the half-maximal inhibitory concentration (IC_{50}) values were calculated for both species.

Results: TSNPs exhibited a characteristic plasmon resonance peak at 440 nm and spherical morphology with an average size of 18 nm. Treatment with TSNPs significantly reduced promastigote viability in a dose- and time-dependent manner. The IC_{50} values after 72 h were 2.27 ppm for *L. tropica* and 2.04 ppm for *L. infantum*. Flow cytometry revealed induction of apoptosis in promastigotes, with 56.78% and 59.27% apoptotic cells for *L. tropica* and *L. infantum*, respectively, at 25 ppm. Macrophage cytotoxicity was observed only at higher concentrations (≥25 ppm).

Conclusion: Thyme-derived silver nanoparticles demonstrate potent and selective antileishmanial activity against both *L. tropica* and *L. infantum*, primarily through the induction of apoptosis. These findings highlight TSNPs as a promising green therapeutic candidate for leishmaniasis, warranting further in vivo investigation.

Keywords: Silver nanoparticles, *Thymus vulgaris*, *Leishmania tropica*, *Leishmania infantum*, Antileishmanial activity.

Introduction

Leishmaniasis is an infectious parasitic disease between humans and animals with worldwide spread caused by *Leishmania* parasites (1, 2). This disease imposes a considerable burden on patients and families, with the poorest populations facing the highest risk of morbidity and mortality across five continents (3). The clinical presentation of *Leishmania* infection can be classified into four forms, including cutaneous (CL), mucocutaneous (MCL), visceral (VL), and Post-Kala-Azar Dermal (PKDL) leishmaniasis (3, 4).

Visceral leishmaniasis (VL), also known as kala-azar, is caused by *L. infantum* and is widely distributed across the Mediterranean basin, the Middle East, Central Asia, parts of Latin America, and North Africa, where it represents a significant public health challenge (4). It is considered a major public health concern and is spread through the bite of an infected female phlebotomine sandfly, mainly species belonging to the genus *Phlebotomus* in the Old World. Dogs serve as the principal reservoir host of *L. infantum*, contributing significantly to its maintenance and transmission cycle in endemic regions (4,5). Although, the majority of infections are asymptomatic, human infection can lead to systemic disease characterized by prolonged fever, hepatosplenomegaly, weight loss, anemia, and, if left untreated, high mortality rates (5,6).

L. tropica is implicated in CL, a disease that typically produces chronic skin lesions which heal slowly and may leave permanent scars. Transmission in urban environments is driven by *Phlebotomus sergenti* sand flies feeding on uninfected hosts, whereas in non-urban regions, animal reservoirs are thought to maintain the parasite. Endemic transmission has been reported in countries such as Syria, Iraq, Iran, Afghanistan, Algeria, and Turkey, as well as in parts of southern Europe. Its distribution closely follows the habitat range of *Phlebotomus* sand flies, particularly *P. sergenti*, enabling transmission in both urban and peri-urban environments (7).

Due to treatment limitations and emerging drug resistance, *L. tropica* and *L. infantum* remain important public health concerns; therefore, understanding recent advances in leishmaniasis therapy is crucial for public health policy and practice. A range of drugs and chemical agents are employed as for the treatment of leishmaniasis. CL caused by *L. tropica* is commonly treated with pentavalent antimonials, such as sodium stibogluconate and meglumine antimonate, as well as alternative agents including paromomycin, pentamidine, and amphotericin B. In contrast, *L. infantum*, the causative agent of visceral leishmaniasis, is primarily managed using drugs such as liposomal amphotericin B, miltefosine, and paromomycin (8-10).

Although these drugs are employed in clinical practice, they suffer from several drawbacks, such as adverse effects, significant toxicity, elevated expenses, insufficient therapeutic efficacy, prolonged treatment regimens, and growing resistance among parasites. These shortcomings of existing therapies underscore the urgent need for safer, more effective, and lower-cost treatment alternatives (9,10). In this context, nanoparticle (NP)-based substances have emerged as promising platforms to overcome these challenges in infectious diseases and cancer (11-13). In intracellular parasitic infections such as leishmaniasis, nanoparticle (NP)-based systems enable targeted delivery of antiparasitic agents to infected macrophages, thereby enhancing therapeutic efficacy and reducing systemic toxicity. In addition, these agents can modulate host immune responses by inducing apoptosis in infected macrophages or eliminating intracellular parasites, thereby reducing the parasite reservoir (11,12).

Recent studies have demonstrated the potent anti-leishmanial activity of various nanomateri-

als, including magnesium oxide, zinc oxide, titanium dioxide, iron oxide, selenium, gold, and silver nanoparticles (12-15). Among these, silver nanoparticles (AgNPs) are the most extensively studied for anti-leishmanial effects (2,12). AgNPs have also demonstrated synergistic effects when combined with conventional drugs, while exhibiting reduced toxicity to host cells at optimized doses (2,12). Although *Thymus vulgaris* and other herbal extracts play critical roles as antioxidant, anti-inflammatory, and antitumor agents, their antimicrobial activity is also an important property (12,16).

We aimed to conduct an *in vitro* investigation to evaluate the efficacy of *T. vulgaris* based green silver nanoparticles against the promastigote and amastigote forms of *L. tropica* and *L. infantum*.

Materials and Methods

Preparation of silver nanoparticles via thyme extract (TSNPs)

The preparation of thyme extract and the synthesis of silver nanoparticles were performed according to a previously reported procedure (17). Briefly, thyme leaves were washed several times with deionised (DI) water, allowed to air-dry, and finely chopped. To prepare the extract, 0.2 g of the material was mixed with 100 mL of deionized water, heated at 80 °C for 40 min with stirring, then filtered and stored at 4 °C in the dark. Silver nanoparticles via thyme extract were synthesized by adding 1 mL of the prepared extract to 20 mL of a 2 mM aqueous $AgNO_3$ solution at a 1:20 (v/v) ratio. After one hour of incubation, the solution gradually changed in color from light yellow to brown, indicating nanoparticle formation. The final suspension was dialyzed against water for 24 h using a 12 kDa dialysis bag and then filtered through a 0.22 µm syringe filter for particle characterization and antiparasitic assessment.

Characterization of thyme-derived silver nanoparticles (TSNPs)

The morphology and size of TSNPs were determined by transmission electron microscopy (TEM; Leo 912 AB). Following appropriate dilution with deionized water and sonication, the TSNPs dispersion was uniformly deposited onto a carbon-coated copper grid, air-dried at room temperature, and subsequently examined at 160 kV. UV–visible spectroscopy was employed to detect and verify the formation of AgNPs synthesized using the aqueous extract of *thyme* at room temperature with an SPUV-26 SC-Tech spectrophotometer.

Leishmania parasite culture

The promastigote forms of *L. infantum* (MHOM/TN/80/IPT1) and *L. tropica* (MHOM/IR/02/Mash10) were obtained from the Department of Parasitology, Tarbiat Modares University, and maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum (Gibco, Germany). The culture medium was further supplemented with penicillin (100 IU mL^{-1}) and streptomycin (100 µg mL^{-1}) to prevent microbial contamination. Promastigotes were incubated at 25 ± 1 °C and examined daily using an inverted microscope.

RAW 264.7 macrophage cell culture

The murine macrophage cell line RAW 264.7 was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin (100 U/mL) in a humidified atmosphere at 37 °C with 5% CO_2. These cells were used for cytotoxicity assays and for the establishment of amastigote–macrophage cultures. Cell growth was monitored daily using an inverted microscope, and cultures were subcultured every three days.

Determination of cytotoxic effects on promastigotes

Parasite viability was evaluated by incubating 100 µL of *L. tropica* and *L. infantum* pro-

mastigote suspensions (2×10^6 cells/mL) with 100 µL of TSNPs at ten different concentrations (50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.195, and 0.097 ppm) in 96-well plates in triplicate. Following incubation at 24 °C for 24, 48, and 72 h, promastigotes were assessed using a Neubauer hemocytometer to determine cell counts and morphological characteristics, and the results were compared with control groups, including untreated parasites and those treated with glucantime (50 µg/mL). The IC_{50} value was calculated from the promastigote assay results using the Quest Graph™ IC_{50} Calculator (AAT Bioquest) (12).

MTT assay for L. tropica and L. infantum promastigotes

A cell inhibitory MTT assay was employed to investigate the effects of thyme-derived silver nanoparticles (TSNPs) on *L. tropica* and *L. infantum* promastigotes. Briefly, promastigotes (1×10^5 cells/well) were seeded into 96-well plates and treated with TSNPs at concentrations ranging from 50 to 0.39 ppm for 24 h. Subsequently, 20 µL of MTT reagent (Sigma-Aldrich, Hamburg, Germany) was added, and the plates were incubated again under the same conditions. Following incubation, dimethyl sulfoxide (DMSO) was added to stop the reaction, and absorbance was measured at 570 nm using an ELISA reader (LX800; Biotec, Winooski, VT, USA) (12).

The activity of thyme-derived silver nanoparticles on macrophages cells

Macrophages were exposed to various concentrations of thyme-derived silver nanoparticles (TSNPs) (50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.195, and 0.097 ppm) to assess cytotoxicity. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 72 h, after which the MTT assay was performed.

Flow cytometry analysis

Apoptotic and necrotic cell populations induced by drug treatment were analyzed by flow cytometry (BioVision, USA) using annexin V-FLUOS and propidium iodide (PI) dual staining (36). Approximately 2×10^6 mL^{-1} *L. tropica* and *L. infantum* promastigotes were cultured in 12-well plates in the presence of TSNPs (25 ppm) and incubated at 24 °C for 72 h. Following centrifugation at 1400 × g for 10 min, both treated and untreated samples were washed twice with cold PBS and resuspended in 500 µL binding buffer containing annexin V (5 µL) and propidium iodide (PI; 5 µL). Samples were incubated in the dark at room temperature for 15 min and subsequently analyzed using a FACSCalibur flow cytometer (BD Biosciences). Data were displayed as dot plots and percentage distributions and analyzed using FlowJo software (version 10). This assay enabled discrimination of viable cells (annexin V^-/PI^-; lower left), early apoptotic cells (annexin V^+/PI^-; lower right), late apoptotic cells (annexin V^+/PI^+; upper right), and necrotic cells (annexin V^-/PI^+; upper left (18).

Statistical Analysis

Data analysis was carried out using SPSS v25 (IBM Corp., Armonk, NY, USA) and GraphPad Prism v8. Comparisons among groups were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Data are presented as mean ± standard deviation (SD), and results were deemed statistically significant at a P value ≤ 0.05 with a 95% confidence interval (CI).

Results

Characterization of biosynthesized silver nanoparticles using thyme

The UV–visible spectroscopy of silver nanoparticles synthesized via thyme extract exhibits a prominent surface plasmon resonance peak at 440 nm, confirming successful nanoparticle

formation. TEM micrographs indicate that the nanoparticles are nearly spherical with an average diameter of 18 nm.

Promastigote Assay

The impact of various concentrations of TSNPs on the number of *L. tropica* and *L. infantum* promastigotes was assessed after 24, 48, and 72 h of incubation. Significant differences were observed between all nanoparticle-treated groups and the control group ($P < 0.05$). The findings indicated that increasing TSNP concentrations markedly inhibited the proliferation of promastigotes. Furthermore, increasing TSNP concentration and prolonging the exposure period significantly intensified the toxic effects on *L. tropica* and *L. infantum* promastigotes. Therefore, the antipromastigote activity of TSNPs was dose- and time-dependent (Figures 1 and 2).

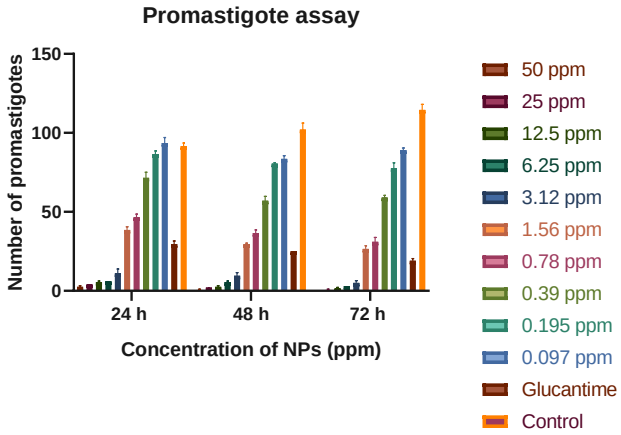

Figure 1: Effects of thyme-derived silver nanoparticles on *Leishmania infantum* promastigote growth. Note: TSNPs, *Thymus vulgaris* silver nanoparticles. The results demonstrated a dose-dependent reduction in the number of *L. infantum* promastigotes following treatment with TSNPs. Higher concentrations of TSNPs (50 and 25 ppm) exerted the greatest inhibitory effects on promastigote viability compared with the conventional antileishmanial agent, glucantime. In contrast, lower concentrations of TSNPs showed limited activity. Moreover, the antipromastigote effects of TSNPs were time-dependent, increasing with prolonged exposure

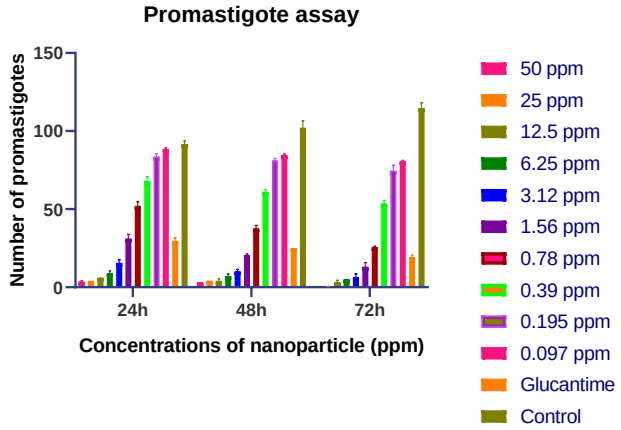

Figure 2: Effects of thyme-derived silver nanoparticles on *Leishmania tropica* promastigote growth. Note: TSNPs, *Thymus vulgaris* silver nanoparticles. The results demonstrated a dose-dependent reduction in the number of *L. tropica* promastigotes following treatment with TSNPs. Higher concentrations of TSNPs (50 and 25 ppm) exerted the greatest inhibitory effects on promastigote viability compared with the conventional antileishmanial agent, glucantime. In contrast, lower concentrations of TSNPs showed limited activity. Moreover, the antipromastigote effects of TSNPs were time-dependent, increasing with prolonged exposure

Promastigotes cytotoxicity using MTT analysis

The cytotoxic effects of TSNPs on the viability of *L. tropica* and *L. infantum* promastigotes were investigated by measuring optical density (OD) following the MTT assay. As shown in Figure 3, parasite proliferation decreased with increasing nanoparticle concentration. Notably, lower concentrations of these nanoparticles (e.g., 0.097 and 0.195 ppm) were associated with higher viability after 24 h.

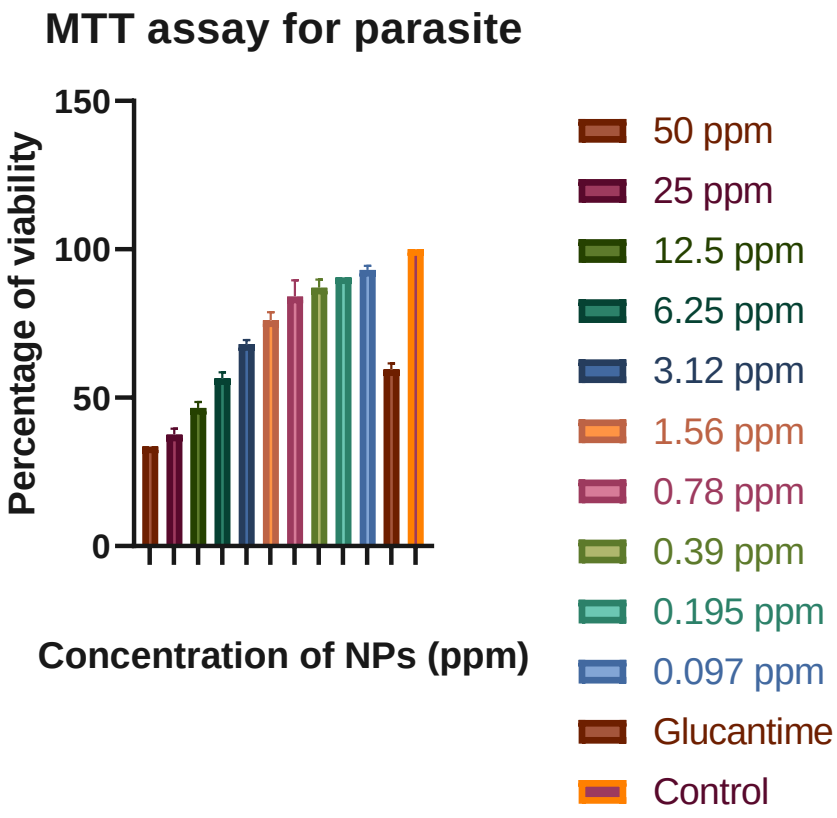

Figure 3: Evaluation of *Leishmania infantum* Viability Using the MTT Assay. Note. MTT: 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide; TSNPs, *Thymus vulgaris* silver nanoparticles. The viability of *L.infantum* was assessed using this assay. Higher parasite survival was observed at lower TSNP concentrations, whereas increasing nanoparticle concentrations resulted in reduced viability. A dose-dependent decrease in viability was observed compared with the control group. Notably, TSNPs at 50 and 25 ppm exhibited greater inhibitory effects than the conventional antileishmanial agent, glucantime.

Cytotoxic effect of thyme-derived silver nanoparticles on macrophages

The MTT assay was also used to evaluate the cytotoxic effects of TSNPs on RAW 264.7 macrophage cells. Cell viability decreased with increasing nanoparticle concentrations. Importantly, concentrations of 50 and 25 ppm of these synthesized nanoparticles exhibited the highest cytotoxic effects on macrophage cells, whereas lower concentrations had only minor effects and showed no statistically significant difference compared with the untreated control group (Figure 4).

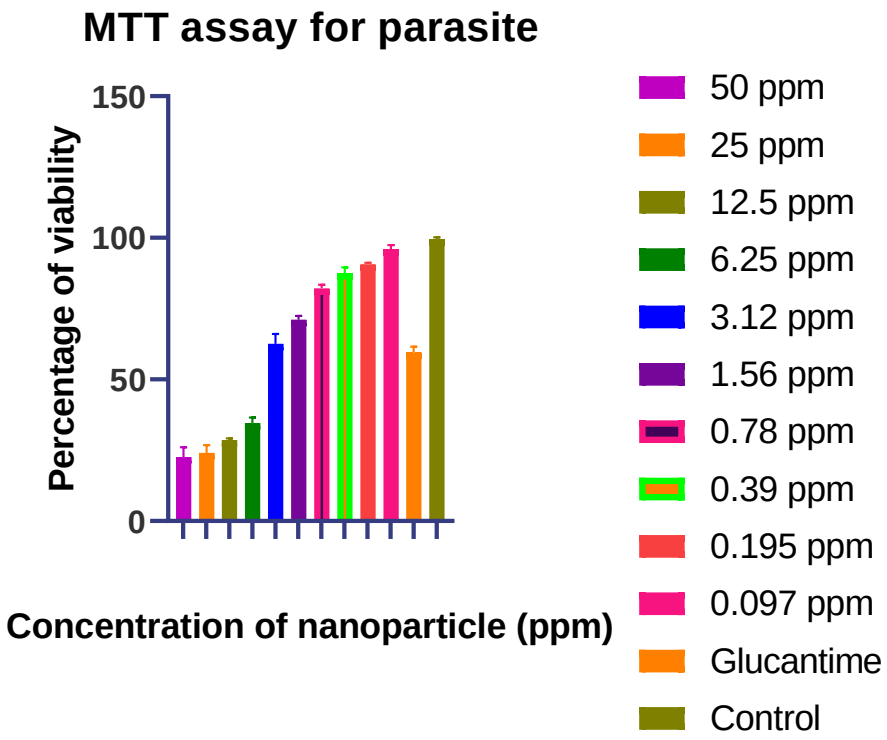

Figure 4: Evaluation of *Leishmania tropica* Viability Using the MTT Assay. Note. MTT: 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide; TSNPs, *Thymus vulgaris* silver nanoparticles. The viability of *L.tropica* was assessed using this assay. Higher parasite survival was observed at lower TSNP concentrations, whereas increasing nanoparticle concentrations resulted in reduced viability. A dose-dependent decrease in viability was observed compared with the control group. Notably, TSNPs at 50 and 25 ppm exhibited greater inhibitory effects than the conventional antileishmanial agent, glucantime.

Calculation of the half-maximal inhibitory concentration (IC_{50})

The promastigote assay revealed IC_{50} values of 2.27 ppm and 2.04 ppm for *L. tropica* and *L. infantum*, respectively, after 72 h of exposure to TSNPs.

Flow Cytometry Analysis

Annexin V–FITC/propidium iodide (PI) dual staining was employed to distinguish apoptotic from necrotic cells in *L. tropica* and *L. infantum* parasites. Exposure to TSNPs significantly increased apoptosis and necrosis in *L. tropica* and *L. infantum* promastigotes compared with untreated parasites. After 72 h of incubation with TSNPs (25 ppm), the proportions of normal, necrotic, and apoptotic promastigotes were 42.8%, 0.43%, and 56.78% for *L. tropica* and 40.6%, 0.15%, and 59.27% for *L. infantum*, respectively. In contrast, the control groups of both *L. tropica* and *L. infantum* exhibited 98.9% normal cells, 0.2% necrotic cells, and 0.93% apoptotic cells. Flow cytometry histograms for both exposed and unexposed parasites are shown in Figures 5-7.

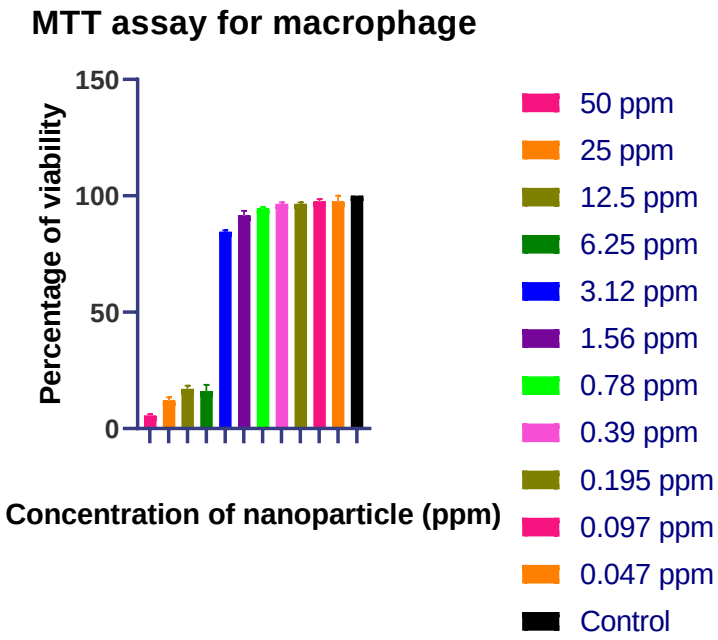

Figure 5: Evaluation of Macrophage Viability Using the MTT Assay. Note. MTT: 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide; TSNPs, *Thymus vulgaris* silver nanoparticles. The viability of RAW 264.7 macrophage cells was assessed using this assay. Higher cell survival was observed at lower TSNP concentrations, whereas increasing nanoparticle concentrations resulted in reduced viability. A dose-dependent decrease in viability was observed compared with the control group. Notably, TSNPs at 50 and 25 ppm exhibited greater inhibitory effects than the conventional antileishmanial agent, glucantime.

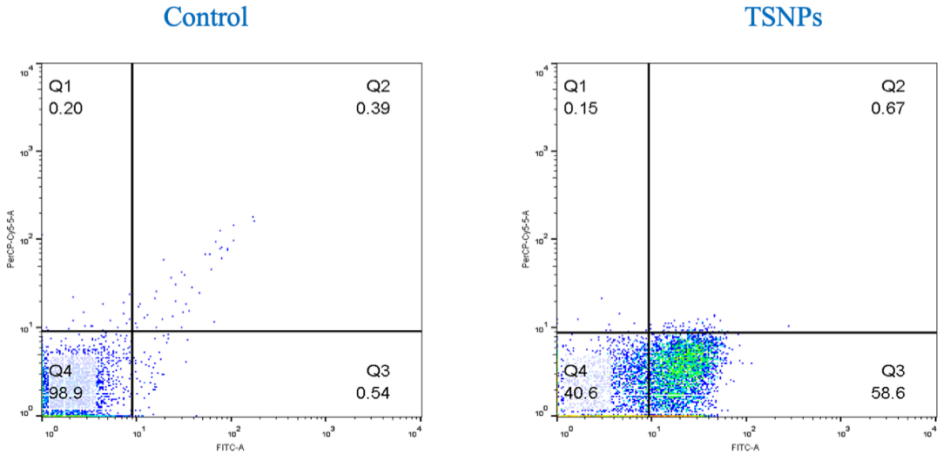

Figure 6: Flow cytometry analysis of *Leishmania infantum* promastigotes exposed to TSNPs (25 ppm) compared with the control group. Note: TSNPs, *Thymus vulgaris* silver nanoparticles. This assay was performed to evaluate parasite viability and apoptosis. After 72 h of incubation, 59.27% of *L. infantum* promastigotes underwent apoptosis, while 40.6% remained viable. In contrast, the untreated control group exhibited a viability rate of 98.9%. Quadrants represent necrotic (upper left), late apoptotic (upper right), apoptotic (lower right), and viable promastigotes (lower left).

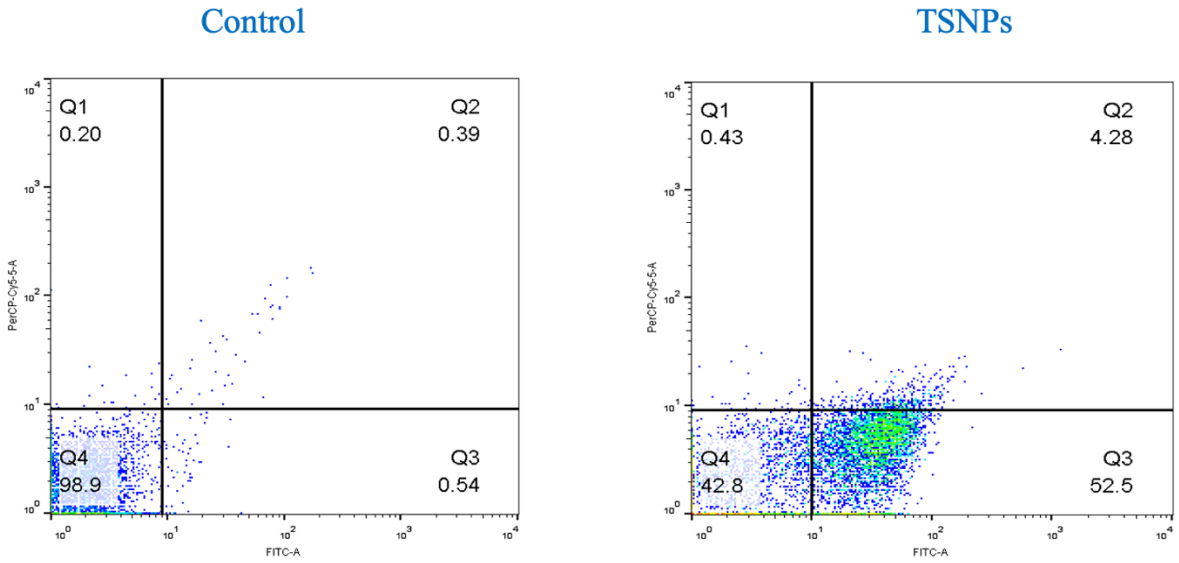

Figure 7: Flow cytometry analysis of *Leishmania tropica* promastigotes exposed to TSNPs (25 ppm) compared with the control group. Note: TSNPs, *Thymus vulgaris* silver nanoparticles. This assay was performed to evaluate parasite viability and apoptosis. After 72 h of incubation, 56.78% of *L.tropica* promastigotes underwent apoptosis, while 42.8% remained viable. In contrast, the untreated control group exhibited a viability rate of 98.9%. Quadrants represent necrotic (upper left), late apoptotic (upper right), apoptotic (lower right), and viable promastigotes (lower left).

Discussion

Leishmania infections represent a major global public health challenge (1-3). The emergence of drug-resistant strains due to inappropriate use of existing drugs is a serious concern; therefore, understanding the prevalence and burden of drug use disorders is essential for informing public health policy and practice. Another limitation of pharmacological treatment for this disease is the occurrence of multiple systemic adverse effects, including renal injury, cardiomyopathy, hepatic scarring or failure, and allergic reactions (8-11).

A variety of agents including chemicals (8–10), plant extracts (16, 19), and nanomaterials (15) have been tested in vitro and in vivo as potential therapeutics. Among these, nanoparticles (NPs) have emerged as a particularly attractive candidate (12, 15).

Metallic nanoparticles, such as AgNPs, exhibit stable antimicrobial activity, multifunctional properties, and favorable biocompatibility with humans and other organisms (2,12). Accordingly, given their reduced toxicity and fewer side effects compared with chemical drugs, we investigated the effectiveness of TSNPs against *L. infantum* and *L. tropica*. Earlier investigations have emphasized the antiparasitic properties of *Thymus* species, particularly against *Leishmania* parasites (12,16). Nilforoushzadeh et al. (16) reported that herbal extracts of thyme, propolis, and yarrow significantly reduced lesion size in BALB/c mice with cutaneous leishmaniasis, showing greater efficacy and good tolerability compared to glucantime. Similarly, Agha and Baaj (20) demonstrated strong anti-*L. infantum* activity of *T. vulgaris* volatile oil, suggesting thyme-derived compounds as promising natural alternatives for the treatment of cutaneous and visceral leishmaniasis. Mohebali et al. (21) assessed the efficacy of AgNPs against *L. major* under *in vitro* and *in vivo* conditions. The results indicated a marked reduction in amastigote proliferation comparable to conventional treatment and highlighted the potential role of nano silver in preventing secondary infections in cutaneous

leishmaniasis. In another study, ginger-mediated, green-synthesized silver nanoparticles exhibit potent antileishmanial activity against both promastigote and amastigote forms of *L. major* (2). These nanoparticles displayed dose- and time-dependent inhibitory effects, with higher concentrations completely inhibiting promastigote proliferation and markedly reducing the intracellular amastigote load, alongside decreased macrophage and parasite viability. While our prior work demonstrated the efficacy of TSNPs against *L. major*, the present study provides the first comparative *in vitro* evaluation of these green-synthesized nanoparticles against both *L. tropica* and *L. infantum*, highlighting species-specific apoptotic responses and IC_{50} values. These synthesized nanoparticles exhibited significant inhibitory effects against both promastigote and amastigote forms of *L. major* in a concentration-dependent manner. These nanoparticles effectively suppressed amastigote proliferation, with an IC_{50} value of 3.02 µg/mL after 72 h of exposure (12). An important outcome of the present study was that TSNPs exhibited strong *in vitro* antileishmanial activity against extracellular promastigotes across all tested concentrations. Moreover, TSNPs showed dose-dependent antileishmanial effects. A significant reduction in the proliferation of *L. tropica* and *L. infantum* promastigotes was observed with increasing TSNPs concentrations and longer exposure times, demonstrating greater efficacy than the standard drug Glucantime. Notably, higher nanoparticle concentrations (50 and 25 ppm) markedly inhibited promastigote growth after 24, 48, and 72 h, particularly against *L. tropica*. Based on the MTT assay results, a concentration-dependent reduction in the viability of *Leishmania* promastigotes and macrophage cells was observed following nanoparticle treatment compared with the control group. Furthermore, these nanoparticles demonstrated potent antileishmanial activity against parasites, with a half-maximal inhibitory concentration (IC_{50}) of 2.27 ppm and 2.04 ppm for *L. tropica* and *L. infantum*, respectively. Under *in vitro* conditions, our experiments demonstrated the induction of programmed cell death in promastigotes of *L. infantum* and *L. tropica* following exposure to nanoparticles; however, the apoptotic effects were more pronounced in *L. infantum* than in *L. tropica*. Therefore, these nanoparticles might have therapeutic potential against leishmaniasis by inducing programmed cell death. TSNPs have previously been shown to induce apoptosis in *L. major* promastigotes, with significant cytotoxic effects observed after 24 h and apoptosis detected in 69.51% of treated cells (12). Thus, these nanoparticles appear to be viable anti-*Leishmania* candidates, mediating their effects via the induction of programmed cell death in promastigotes.

Our study had several limitations as follows: 1) the study was performed only under *in vitro* conditions, 2) the molecular mechanisms of TSNP-induced parasite death were not fully investigated, and 3) the long-term safety and biocompatibility of TSNPs were not evaluated.

Conclusion

TSNPs demonstrated considerable in vitro antileishmanial activity against both *L. tropica* and *L. infantum* in a dose- and time-dependent manner. The synthesized nanoparticles significantly reduced promastigote viability and induced apoptosis, while showing limited cytotoxicity toward macrophages at lower concentrations. In addition, TSNPs exhibited slightly greater activity against *L. infantum* compared with *L. tropica*. These findings highlight the potential of green-synthesized silver nanoparticles as alternative candidates for leishmaniasis treatment. Nevertheless, further *in vivo* investigations and detailed safety evaluations are required to validate their therapeutic applicability.

Ethical Approval

This research was reviewed and approved by the Ethics Committee and Research Council of

Qazvin University of Medical Sciences, Iran, under ethical code IR.QUMS.REC.1401.007.

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Conflicts of Interest

The authors declare no conflicts of interest.

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