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RESEARCH PAPER

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Comparison of biosynthetic zinc oxide nanoparticle and glucantime cytotoxic effects on *Leishmania major* (MRHO/IR/75/ER)

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Abstract

Currently, zinc oxide (ZnO) particles are used in nanotechnology to destroy a wide range of microorganisms. Although pentavalent antimony compounds are used as antileishmanial drugs, they are associated with several limitations and side effects. Therefore, it is always desirable to try to find new and effective treatments. The aim of this research is to determine the antileishmanial effect of ZnO particles in comparison to the Antimoan Meglumine compound on promastigotes and amastigotes of *Leishmania major* (MRHO/IR/75/ER). After the extraction and purification of macrophages from the peritoneal cavity of C57BL/6 mice, *L. major* parasites were cultured in Roswell Park Memorial Institute-1640 culture medium containing fetal bovine serum (FBS) 10% and antibiotic. In this experimental study, the effect of different concentrations of nanoparticles was investigated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric method, in comparison to the glucantime on promastigotes, amastigotes and healthy macrophages in the culture medium. The amount of light absorption of the obtained color from the regeneration of tetrazolium salt to the product color of formazan by the parasite was measured by an enzyme-linked immunosorbent assay (ELISA) reader, and the IC_{50} value was calculated. IC_{50} after 24 h of incubation was calculated as $IC_{50} = 358.6\ \mu g/mL$. The results showed, that the efficacy of ZnO nanoparticles was favorable and dose-dependent. The concentration of 500 μg/mL of ZnO nanoparticles induced 84.67% apoptosis after 72. Also, the toxicity of nanoparticles was less than the drug. Nanoparticles exert their cytotoxic effects by inducing apoptosis. They can be suitable candidates in the pharmaceutical industry in the future.

KEYWORDS

Amastigote, *Leishmania major*, MTT assay, promastigote, ZnO nanoparticles

Abbreviations: DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; NNN, Novy-Mac Neal-Nicolle; NPs, nanoparticles.

1 | INTRODUCTION

Leishmaniasis is an infection caused by *Leishmania* parasites, which occurs in three forms: cutaneous, visceral, and mucous. Nearly 350 million individuals are affected by this parasite in more than 98 countries around the world. This parasite is a major problem in many countries. Many efforts have been made to treat this disease and even produce a vaccine, which have not been successful so far and the number of patients is still increasing. According to the latest statistics, 700,000–1,000,000 people are infected with this parasite every year, and 20,000–30,000 cases die [1, 2]. The existence of epidemiological complications in the transmission loop of various reservoirs and carriers has made this disease very important. Although cutaneous leishmaniasis is not usually associated with high mortality, the rate of infection is high and causes malformed skin lesions; in some cases, they remain for more than a year, and even with a standard treatment after recovery, the scar remains. It causes pain and suffering to the patient, so one should look for a way to control the disease [3]. During the life cycle of the genus *Leishmania* assume various morphologic and functional stages. In mammalian hosts the parasites occur as amastigote forms, which are found within the phagolysosomes of macrophages; while in sandfly hosts the parasites occur as promastigotes within the gut [4, 5]. Macrophages are considered hosts of *Leishmania*. They can eliminate *Leishmania* through toxic oxygen metabolites including superoxide anion (O^{-2}), hydrogen peroxide (H_2O_2), and nitric oxide (NO). As a result, a compound that can activate macrophages to fight this parasite will be effective in treating or controlling the disease. Parasites and their products can suppress the host's immune responses and parasite elimination mechanisms. One of these products is parasite proteins that perform various functions. Among the proteins of *Leishmania*, there are secretion-exudation proteins that are released at all stages of the life cycle and have the protective and immune-modulating role. In fact, these proteins will prevent the parasite from destruction by the immune system [6]. Promastigotes of different species of *Leishmania* produce and release various types of proteins in different stages of their growth inside the mosquito body or human macrophages, as well as in the in vitro environment. It has been seen that some secretory proteins in *Leishmania donovani* have acid phosphatases property that are involved in both invasion and resistance to the immune mechanisms of macrophages. However, these acid phosphatase proteins are not seen in *L. major* [7]. Other secreted proteins are also known in nematodes that have acetylcholinesterase property. Based on the studies conducted on the effect of nematode secretory proteins in macrophages and in vitro, it was found that these proteins reduce the expression of the toll-like receptors (TLRs), production of interleukin-12 (IL-12), and tumor necrosis factor-α (TNF-α) in macrophages [8]. Secretory proteins of the parasite are produced and secreted from both endoplasmic reticulum-dependent and nonendoplasmic reticulum pathways [9]. The first line of treatment for all types of leishmaniasis is the pentavalent antimonials—meglumine antimoniate (glucantime) and sodium stibogluconate (pentostam); they are remained as standard therapy for more than 60 years [10]. As a result of the use of this drug, local side effects such as pain during injection and general and systemic side effects such as anorexia, fever and chills, joint pain, headache, muscle pain, weakness, cough, shortness of breath, digestive disorders, facial swelling, pancreatitis, increased amylase and serum lipase, cardiovascular complications in the form of irregular heart beat and changes in the electrocardiogram, liver symptoms in the form of increased liver enzymes and anaphylactoid reactions, kidney symptoms and blood dyscrasia have been reported. Usually, it is not recommended to use this medicine in patients with liver and kidney problems. Also, reports of parasite resistance to glucantime are increasing in different regions of the world [11]. Nanoparticles have unique physicochemical properties such as small size, large surface area, and electrical charge [12]. Metal oxide nanoparticles have various usage in different sciences [13–15]. Nanoparticles are commonly used in medicine in drug delivery and cancer treatment. Various nanoparticles can be used to destroy parasites (with lethal and inhibitory effect), make effective and harmless medicinal compounds, as well as useful vaccines. According to the mentioned cases, the use of metal nanoparticles, including zinc oxide (ZnO) for the production of nontoxic, low side effects and at the same time effective drugs can be a suitable solution for the treatment of leishmaniasis. ZnO is one of the zinc compounds currently introduced by the US Food and Drug Administration as a safe substance [16, 17]. It has an antibacterial effect on Gram-positive and Gram-negative bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* [18]. The antibacterial activity of ZnO has been attributed to the production of reactive oxygen species (ROS) and disruption of bacterial DNA amplification processes. ZnO increases lipid oxidation in prokaryotic and eukaryotic cell membranes and is effective on resistant microorganisms [19, 20]. It can react with several oxidative molecules such as molecular oxygen, active oxygen species, active metals, and thiols, attacking all kinds of molecules and biological structures and causing their destruction [21]. Despite many efforts of health workers in recent decades, the control of *Leishmania* vector reservoir has not been very effective, and drug treatments including pentavalent antimony compounds such as sodium stiboglunate (pentostam), antimony meglumine (glucantime), and amphotrypsin B, and so on, faced many problems, including nonresponse and drug resistance; also, there is still no effective vaccine for

human leishmaniasis. For this reason, in recent years the World Health Organization (WHO) has proposed leishmaniasis as a forgotten tropical disease. Nanoparticles can influence the function of the immune system through the interaction with the body's innate and acquired immune system. Therefore, in this research, in addition to investigating the antiparasitic effects of ZnO nanoparticles, the antiparasitic effect of glucantime drug has been compared with nanoparticles.

2 | MATERIALS AND METHODS

2.1 | Preparation of ZnO nanoparticles

In this research, ZnO nanoparticle powder biosynthesized by *Bacillus subtilis* bacteria was used with an average diameter of 25–50 nm. To biosynthesize the nanoparticles, the bacteria *Bacillus subtilis*, with the access code of IBRC-M10742 was used; it was purchased from the Iranian Genetic Supply. Lyophilized bacteria were inoculated to the primary growth in the nutrient-broth culture medium. After about an hour of incubation, the suspension was seeded in the blood agar culture medium. The bacteria colonies were inoculated in the nutrient-broth culture medium for 24 h; the medium was diluted four times by adding 75 mL of sterile broth. The diluted medium was placed at the temperature of 37°C for 24 h under growth. After incubation, pH was regulated at 7 by 0.1 N of sodium hydroxide (NaOH). Moreover, 50 nm of zinc acetate dehydrate was infused into the bacterial suspension at the speed of 10 mL/min through a burette. The compound was stirred for 30 min, and then, 200 mL of 50 mm (sodium borohydride); $NaBH_4$ was gently added to the mixture as a reducing factor at the speed of 10 mL/min through a burette. Then the final solution was stirred for 24 h at the room temperature. After the thorough reaction of the mixture, it was centrifuged and the emergence of white precipitates indicated the initiation of the nanoparticles reduction. Subsequently, the precipitate was collected. The obtained precipitate was washed three times with deionized water. The powdered form of the nanoparticles was produced by drying the plate at 50°C in a hot oven for 6 h. Finally, the powder containing the nanoparticles was calcined at the temperature of 600°C for 2 h, to preserve the bacterial residue and the organic compounds. The salt-free medium was considered as positive control, while the salty solution without the medium was considered as the negative control [22]. A stock of ZnO colloidal nanoparticles with a concentration of 3000 μg/mL was dispersed in ultrapure water by ultrasonication at 100 W and 40 kHz for 40 min to form homogeneous suspensions. Then, different concentrations of nanoparticles were prepared to check the antiparasitic properties [2].

2.2 | Cultivation and maintenance of parasites

In this experimental study, the standard strain of *L. major* parasite (MRHO/IR/75/ER) was used, which was obtained from the Tehran University of Medical Sciences. Two modified Novy-Mac Neal-Nicolle (NNN) and Roswell Park Memorial Institute-1640 (RPMI1640) medium were used. First, the parasite was cultured in the modified NNN medium, then in RPMI1640 (GIBCO). To prevent the growth of bacteria, 100 mL/unit of penicillin, 100 μg/mL of streptomycin, and 15%–12% fetal bovine serum (FBS) were added to the cell culture media. Then, the flasks were transferred to an incubator at 24°C and examined with an inverted microscope every day. In the samples where the color of the medium had turned yellow, that means the promastigotes had entered the stationary phase the new culture medium was added. This work was done until the number of parasites reached the required amount (2×10^6). Neobar slide was used to count the number of parasites; the following formula was used to determine the number of parasites per milliliter [23]:

Average cell count from each of the sets of 16 corner squares $\times 10^4$.

2.3 | Macrophage extraction and purification

First, to prevent pain, the animal (C57BL/6 male mouse, 6–7 weeks old) was euthanized by cervical dislocation and spinal cord cutting. Using a 10-mL syringe, 5–6 mL of cold RPMI medium was injected into the peritoneal space using a 14-gauge needle, with pressure; the RPMI medium was injected into the peritoneal cavity to release the attached cells. By giving a few blows to the peritoneum and side of the mouse, the cells are suspended. The syringe containing the medium was slowly removed from the needle while the needle was in the peritoneal cavity, once again the cold RPMI medium was drawn with the same syringe and a clean needle, and the syringe was connected to the needle in the peritoneal cavity, and the medium was injected. This was done two to three times. The obtained cell suspension was kept on ice before centrifugation. Then, the cell suspension was centrifuged in a refrigerated centrifuge at 4°C for 10 min at 1500*g*. The supernatant solution was discarded and the culture medium was added to the cell sediment and

centrifuged. Finally, 1 mL of complete culture medium was added to the obtained cell sediment, which was used to count and determine the percentage of cell viability. To purify macrophages from other cell lines in the suspension obtained from peritoneal lavage, the above set was placed in a 75 cm^2 flask for cell culture. After 4–6 h of incubation in sterile and suitable culture conditions in an incubator at 37°C and 5% CO_2, the necessary conditions for the attachment of macrophages were provided; at the end of this period, and by discarding the floating nonadherent cells, which were mostly lymphocytes and other cell types, macrophages were connected to the culture medium and were prepared for the next steps. It should be noted, that the above steps were performed in 96-well plates [24].

2.4 | Examining the toxic effects on macrophages and calculating half maximal inhibitory concentration (IC_{50})

The number of 10^5 macrophages in each well was exposed to 10 different drug concentrations for 72 h. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) test was performed on the samples. The absorbance in the wells was measured by enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 570 nm [25–27]. The optical absorption values obtained were analyzed by statistical software (GraphPad prism5), and the inhibition concentration of 50% of the samples (IC_{50}) was calculated. According to the IC_{50} value, suitable concentrations for subsequent analyses were determined [28].

2.5 | Evaluation of anti-promastigote activity by MTT method

MTT is a colorimetric method that evaluates the rate of conversion of tetrazolium to formazan by mitochondrial enzymes in living cells. In short, promastigotes (5×10^4 cells/well) were poured into 96 wells of culture plates and incubated in RPMI1640 medium enriched with FBS 10%; different concentrations (125, 250, 350, 500 μg/mL) of nanoparticles were added to each well, separately. Then the plates were kept at 26 ± 1°C for 72 h. In all experiments, dimethyl sulfoxide (DMSO) 1% and culture medium alone were used as the control group. All experiments were performed in triplicate. After 3 days, parasites were counted by counting the live and motile forms in a hematocytometer slide, and MTT evaluation was also done. MTT stock solution with 5 mg/mL stock was prepared in phosphate-saline buffer, which could be stored for less than 2 weeks in the dark at 4°C. The supernatant solution was separated by centrifugation at 2000 rpm for 5 min and discarded. A total of 100 μL of RPMI medium without phenol red (RPMI-PR) and serum containing 100 μg of MTT were added to each well. The plates were incubated for 4 h in the dark at room temperature. The wells were centrifuged again at 2000 rpm for 5 min and the supernatant was discarded. A total of 100 μL of DMSO was added to each well of the plate. The amount of absorbance in the wells was measured by ELISA reader at 492 nm wavelength [29].

2.6 | Effect of nanoparticles on *L. major* amastigotes

Macrophages were examined for viability using trypan blue staining. Then they were transferred to the coverslips in the three-well plates and the plates were incubated for 5 h in a 37°C incubator containing 5% CO_2. Macrophages attach to the solid substrate (lamella) due to their specific adhesion and morphogenesis. After this period, to check the condition of cultured macrophages, a plate was checked by an inverted microscope. Twenty-four hours after macrophage culture, coverslips washed by RPMI1640, *L. major* promastigotes were added at a ratio of 10 parasites per macrophage (ratio 10:1) to the plate. No parasite was added to a group of plates, which were considered as a control group for macrophage viability. The plates were placed in a 37°C incubator containing 5% CO_2 for 2 h, then washed with RPMI1640. Then, complete RPMI1640 was added to each house plate and returned to the incubator. The next day, the used dilutions of ZnO nanoparticles and the used solvent were added to the plates, and after 72 h, the slides were evaluated in terms of counting the number of infected macrophages and amastigotes inside each infected macrophage. Three repetitions were made for each sample [28].

2.7 | Effect of glucantime on *L. major* promastigotes

MTT method was used to investigate the effect of glucantime as a common drug on *Leishmania* parasite promastigotes compared with the effect of ZnO nanoparticles. The test method was described in part 2–4, with the difference that instead of different concentrations of nanoparticles and glucantime (7.81, 15.62, 31.25, 62.5 μg/mL) were used

(according to the IC_{50} value of glucantime calculated in previous research = 14 μg/mL) [30].

2.8 | Effect of glucantime on *L. major* amastigotes

To investigate the effect of glucantime drug compared with the effect of ZnO nanoparticle on amastigote of *Leishmania* parasite, the method described in part 2–5 was done and different concentrations of glucantime were used instead of different concentrations of nanoparticle [30].

3 | RESULTS

3.1 | Cytotoxicity effect of ZnO nanoparticles and glucantime on *L. major* parasite

After macrophage cells were exposed to different concentrations of ZnO nanoparticles and glucantime, IC_{50} was calculated as 358.6 and 14 μg/mL, respectively (Figure 1).

3.2 | MTT test results of ZnO nanoparticles on *L. major* parasite

According to Table 1, MTT results show that the lowest inhibitory concentration of nanoparticles in the MTT test was 500 μg/mL, which was equal to 39.15 optical absorption.

3.3 | Effect of ZnO nanoparticles on macrophages infected with *L. major* parasite

According to Table 2, the results have a significant difference in the range ($p < 0.05$). The lowest inhibitory concentration to inhibit *Leishmania* parasites infecting macrophages was 500 μg/mL, which was the average number of cells was 20.67.

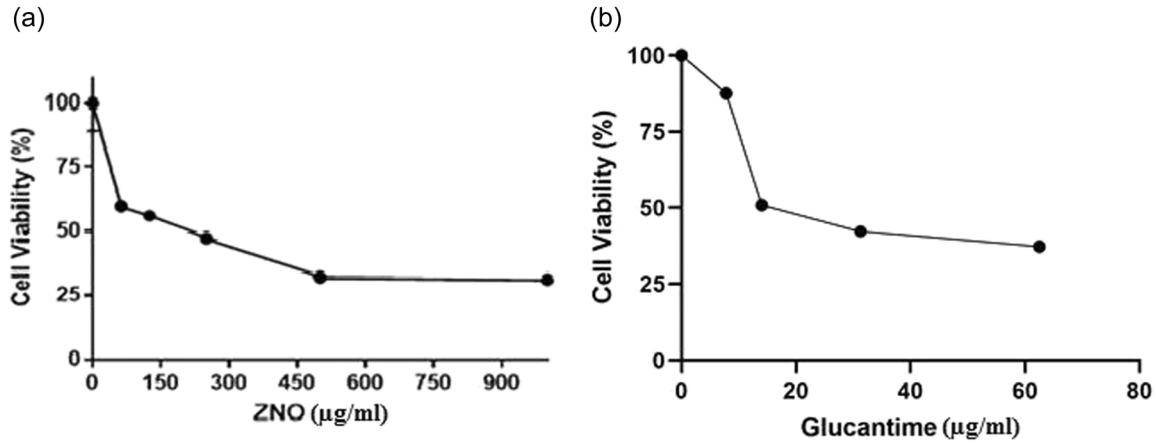

FIGURE 1 Calculation of 50% inhibitory concentration (IC_{50}). (a) Zinc oxide nanoparticle and (b) glucantime.

TABLE 1 Evaluated of MTT test of ZnO nanoparticles and glucantime on *L. major* parasite.

	Group						
Parameter	**500 μg/mL**	**350 μg/mL**	**250 μg/mL**	**125 μg/mL**	**SEM**	***p* Value**	**IC_{50}**
ZnO MTT	39.15 ± 5.37^{c}	45.728 ± 0.774^{c}	60.47 ± 2.94^{b}	80.43 ± 2.33^{a}	3.29602	<0.0001	358.6 μg/mL
	Group						
	62.5 μg/mL	**31.25 μg/mL**	**15.62 μg/mL**	**7.81 μg/mL**			
Glu MTT	46.814 ± 0.642^{d}	54.76 ± 2.92^{c}	62.605 ± 0.656^{b}	86.80 ± 2.49^{a}	1.97216	<0.0001	14 μg/mL

Note: Superscripts different letters (a–d) of rows are significantly different at $p < 0.05$.

Abbreviations: Glu, glucantime; IC50, half-maximal inhibitory concentration; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; SEM, standard error of means; ZnO, zinc oxide.

TABLE 2 Investigating the effect of ZnO nanoparticles and glucantime on macrophages infected with *Leishmania major* parasite.

Parameter ZnO	Group Control	500 μg/mL	350 μg/mL	250 μg/mL	125 μg/mL	SEM	*p* Value	IC_{50}
Macrophage count	92.00 ± 3.61^{a}	20.67 ± 2.08^{d}	49.33 ± 4.16^{c}	73.00 ± 7.21^{b}	88.00 ± 2.65^{a}	4.32820	<0.0001	358.6 μg/mL
Macrophage %	100.00 ± 0.00^{a}	22.46 ± 2.26^{d}	53.62 ± 4.53^{c}	79.35 ± 7.84^{b}	95.65 ± 2.88^{a}	4.70457	<0.0001	
Glucantime	**Group Control**	**62.5 μg/mL**	**31.25 μg/mL**	**15.62 μg/mL**	**7.81 μg/mL**			
Macrophage count	92.00 ± 3.61^{a}	28.67 ± 4.04^{d}	50.67 ± 3.06^{c}	68.00 ± 4.58^{b}	86.33 ± 4.73^{a}	4.04969	<0.0001	14 μg/mL
Macrophage %	100.00 ± 0.00^{a}	31.16 ± 4.39^{d}	55.07 ± 3.32^{c}	73.91 ± 4.98^{b}	93.84 ± 5.14^{a}	4.40184	<0.0001	

Note: Superscripts different letters (a–d) of rows are significantly different at $p < 0.05$.

Abbreviations: IC_{50}, half-maximal inhibitory concentration; SEM, standard error of means; ZnO, zinc oxide.

TABLE 3 Investigating the effect of ZnO nanoparticles and glucantime on *Leishmania major* parasite amastigotes.

Parameter ZnO	Group Control	500 μg/mL	350 μg/mL	250 μg/mL	125 μg/mL	SEM	*p* Value	IC_{50}
Amastigote count	463.00 ± 4.58^{a}	15.33 ± 3.06^{e}	134.00 ± 7.81^{d}	271.33 ± 3.06^{c}	313.00 ± 4.58^{b}	4.93288	<0.0001	358.6 μg/mL
Amastigote %	100.00 ± 0.00^{a}	3.312 ± 0.660^{e}	28.942 ± 1.687^{d}	58.603 ± 0.660^{c}	67.603 ± 0.990^{b}	4.70457	<0.0001	
Glucantime	**Group Control**	**62.5 μg/mL**	**31.25 μg/mL**	**15.62 μg/mL**	**7.81 μg/mL**			
Amastigote count	463.00 ± 4.58^{a}	35.33 ± 6.03^{e}	169.00 ± 5.00^{d}	304.33 ± 4.51^{c}	437.00 ± 5.57^{b}	5.17043	<0.0001	14 μg/mL
Amastigote %	100.00 ± 0.00^{a}	7.631 ± 1.302^{e}	36.501 ± 1.080^{d}	65.731 ± 0.974^{c}	94.384 ± 1.203^{b}	1.11672	<0.0001	

Note: Superscripts different letters (a–d) of rows are significantly different at $p < 0.05$.

Abbreviations: IC_{50}, half-maximal inhibitory concentration; SEM, standard error of means; ZnO, zinc oxide.

3.4 | Effect of ZnO nanoparticles on *L. major* parasite amastigotes

According to Table 3, the results had a significant difference in the range ($p < 0.05$). The lowest inhibitory concentration to inhibit the amastigote of *Leishmania* parasites was 500 μg/mL, the average was 15.33.

3.5 | Glucantime MTT test results on *L. major* parasite

According to Table 1, the results had a significant difference in the range ($p < 0.05$). MTT shows that the lowest inhibitory concentration of Glucantime in the MTT test was 62.5 μg/mL, which was equal to the optical absorption of 46.814.

3.6 | Effect of glucantime on macrophages infected with *L. major* parasite

According to Table 2, the results have a significant difference in the range ($p < 0.05$). The lowest inhibitory concentration to inhibit *Leishmania* parasites infecting macrophages was 62.5 μg/mL, and the average was 28.67.

3.7 | Effect of glucantime on *L. major* parasite amastigotes

According to Table 3, the results had a significant difference in the range ($p < 0.05$). The lowest inhibitory concentration to inhibit the amastigote of *Leishmania* parasites was 62.5 μg/mL, which averaged 35.33.

4 | DISCUSSION

Leishmaniasis is a significant global issue, posing a challenge for researchers due to its intracellular nature. Discovering and supplying effective medicine has been difficult, with limited progress in antiparasitic drug development. To address this, effective management strategies are needed for better drug availability. The current drugs used to treat this disease have drawbacks such as toxicity, side effects, and the occurrence of parasite resistance. Furthermore, Leishmaniasis leads to

long-term and deformed wounds, often resulting in secondary infections that require treatment. These drugs need to be repeatedly injected in high doses, leading to economic losses and limitations such as lack of effectiveness when administered orally, long treatment periods, and inadequate treatment response in a portion of patients [29–31]. Nowadays, the use of metal nanoparticles in the treatment of parasitic diseases is considered a new method. In this study, the cytotoxic effect of ZnO biosynthetic nanoparticles was investigated on the promastigote and amastigote forms of *L. major* parasite (MRHO/IR/75/ER) and macrophages. The IC_{50} value for the ZnO nanoparticles was determined to be 358.6 μg/mL using the MTT test. The lowest inhibitory concentration of the nanoparticles against promastigotes was found to be 500 μg/mL. Furthermore, the ZnO nanoparticles showed a significant inhibitory effect on infected macrophages and amastigotes at various concentrations (p-value < 0.05), with the lowest inhibitory concentration also being 500 μg/mL. To assess the efficacy of the nanoparticles, they were compared with glucantime, a commonly used drug for leishmaniasis treatment. The MTT test results revealed a significant difference in the inhibitory concentration of glucantime against *L. major* parasite (p value < 0.05), with the lowest inhibitory concentration being 62.5 μg/mL. Similarly, the lowest inhibitory concentration of glucantime on infected macrophages and *Leishmania* parasite amastigotes was also 62.5 μg/mL. Previous studies have reported the 50% inhibitory concentration of glucantime as 14 μg/mL. Comparing the results, it is evident that the cytotoxic effect of the ZnO nanoparticles is superior to that of glucantime. The 50% inhibitory concentration of glucantime, previously reported as 14 μg/mL [29], is higher than the lowest inhibitory concentration of promastigotes and amastigotes (62.5 μg/mL) observed in this study. This suggests that the concentration of glucantime needs to be increased fourfold to achieve the desired effect. In contrast, the effective concentration of the nanoparticle is lower than the appropriate dose.

Overall, these findings demonstrate the potential of ZnO nanoparticles as a more effective cytotoxic agent against *L. major* parasite compared with glucantime. The lower minimum inhibitory concentration of the nanoparticles suggests their potential for improved treatment outcomes and highlights their potential as a new approach in the treatment of parasitic diseases.

In a study conducted by Delavari et al. [32], it was found that ZnO NPs exhibited leishmanicidal activity against *L. major*. The IC_{50} value of ZnO NPs on promastigotes of *L. major* was determined to be 37.8 μg/mL. Similarly, Sumaira et al. [33] reported leishmanicidal activity of spherical ZnO NPs synthesized using a greener method. The IC_{50} value of ZnO NPs against *L. tropica* was found to be 250 μg/mL. These studies highlight the potential of ZnO NPs as effective agents against different strains of Leishmania parasites. Nazir et al. [32] investigated the toxic effect of ZnO nanoparticles in vitro and in vivo on human macrophages and rat kidney cells, and reported concentrations higher than 100 μg/mL as toxic. Gunalan et al. showed that synthesized ZnO by the green method has a stronger inhibitory effect than synthesized nanoparticles by the chemical method [34]. In another study, Khatami et al. investigated the *Leishmania*-killing effect of ZnO nanoparticles biosynthesized by *Lilium ledebourii* tuber [35]. In their study, the researchers observed that ZnO nanoparticles exhibited potent leishmanicidal activity against *L. major*, effectively inhibiting the amastigotes. The IC_{50} values of ZnO nanoparticles were found to be approximately 1 μg/mL. These findings demonstrate the remarkable efficacy of ZnO nanoparticles in combating *L. major* infection, highlighting their potential as a promising therapeutic intervention for leishmaniasis.

The results obtained from this research are consistent with previous studies, and the results show a relatively appropriate and dose-dependent inhibition of *Leishmania* amastigotes and promastigotes compared with glucantime. Mohebali et al. [13] showed that miltefosin nano drug had a favorable inhibitory effect on the Iranian strain of *Leishmania* compared with other *Leishmania* inhibitor drugs; it is a nano drug based on chitosan nanoparticle fibers. Also, the effectiveness of miltefosine in the treatment of leishmaniasis caused by *L. major* was reported to be more effective compared with glucantime in a clinical study conducted in 2007 [36]. The results of this research and previous studies indicate, that nanoparticles were relatively more effective than common drugs to inhibit the *Leishmania* parasite; therefore, there is an urgent need to find a new anti-leishmaniasis drug. However, all drugs have different toxic effects, so in this study, the toxic effect of ZnO nanoparticles on macrophages was also investigated. Comparing the results of the IC_{50} (358.6 μg/mL for nanoparticle and 14 μg/mL for glucantime), showed that glucantime had higher toxicity for macrophage cells. Overall our results indicate that nanoparticles especially ZnO have a high inhibitory potential on various microorganisms. This opens up possibilities for the development of more effective and less harmful drugs in the future. Additionally, it is hoped that this research will contribute to the development of useful vaccines for preventing and combating parasitic infections that pose a risk to human health.

ZnO nanoparticles can induce apoptosis in a dose- and time-dependent manner in vitro. With the increasing

number of leishmaniasis cases worldwide, it is important to find new and effective drugs that can treat this parasitic disease, and have the least complications and toxicity. ZnO nanoparticles with little or no side effects, which are biosynthesized in a green way can be an interesting subject for future study, and emerge as an effective therapeutic approach.

AUTHOR CONTRIBUTIONS

Fatemeh Saleh: Methodology; writing—original draft. **Farnaz Kheirandish**: Formal analysis. **Mohammad Abbasi**: Software. **Fatemeh Ahmadpour**: Software; writing—review & editing. **Saeed Veiskarami**: Software; validation. **Atefe Mirderikvand**: Writing & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

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

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REFERENCES

[1] Want MY, Yadav P, Khan R, Chouhan G, Islamuddin M, Aloyouni SY, et al. Critical antileishmanial in vitro effects of highly examined gold nanoparticles. Int J Nanomed. 2021;16: 72–85.

[2] Feizabadi E, Zavaran Hosseini A, Soudi S. Effect of intraperitoneal injection of chitosan nanoparticle loaded with *Leishmania major* excretory-secretory antigens on macrophage function in exposure to parasites in BALB/c mice. Res J Med. 2019;43:97–104.

[3] Nejati J, Mojadam M, Hanafi Bojd AA, Keyhani A, Habibi Nodeh F. An epidemiological study of cutaneous leishmaniasis in Andimeshk (2005-2010). J Ilam Uni MedSci. 2014;2:94–101.

[4] Sacks DL. Metacyclogenesis in *Leishmania* promastigotes. Exp Parasitol. 1989;69:100–3.

[5] Killick-Kendrick R. The life-cycle of *Leishmania* in the sandfly with special reference to the form infective to the vertebrate host. Ann Parasitol Hum Comp. 1990;65: 37–42.

[6] Chenik M, Lakhal S, BEN KHALEF N, Zribi L, Louzir H, Dellagi K. Approaches for the identification of potential excreted/secreted proteins of *Leishmania major* parasites. Parasitology. 2006;132:493–509.

[7] Lippert DN, Dwyer DW, Li F, Olafson RW. Phosphoglycosylation of a secreted acid phosphatase from *Leishmania donovani*. Glycobiology. 1999;9:627–36.

[8] Han C, Yu J, Zhang Z, Zhai P, Zhang Y, Meng S, et al. Immunomodulatory effects of *Trichinella spiralis* excretory-secretory antigens on macrophages. Exp Parasitol. 2019;196: 68–72.

[9] Selkirk ME, Lazari O, Matthews JB. Functional genomics of nematode acetylcholinesterases. Parasitology. 2005;131: S3–S18.

[10] Frézard F, Demicheli C, Ribeiro R. Pentavalent antimonials: new perspectives for old drugs. Molecules. 2009;14:2317–36.

[11] Ahamed M, Akhtar MJ, Raja M, Ahmad I, Siddiqui MKJ, AlSalhi MS, et al. ZnO nanorod-induced apoptosis in human alveolar adenocarcinoma cells via p53, survivin and bax/bcl-2 pathways: role of oxidative stress. Nanomed Nanotechnol Biol Med. 2011;7:904–13.

[12] Shahbazi S, Habibi M, Badmasti F, Sabzi S, Farokhi M, Asadi Karam MR. Design and fabrication of a vaccine candidate based on rOmpA from *Klebsiella pneumoniae* encapsulated in silk fibroin-sodium alginate nanoparticles against pneumonia infection. Int Immunopharmacol. 2023;125:111171.

[13] Hadighi R, Mohebali M, Boucher P, Hajjaran H, Khamesipour A, Ouellette M. Unresponsiveness to glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* parasites. PLoS Med. 2006;3:e162.

[14] Saebi E. Clinical parasitology: protozoal diseases in Iran. Tehran Aeeizh. 2011;19:601–7.

[15] Shahbazi S, Shivaee A, Nasiri M, Mirshekar M, Sabzi S, Sariani OK. Zinc oxide nanoparticles impact the expression of the genes involved in toxin–antitoxin systems in multidrug-resistant *Acinetobacter baumannii*. J Basic Microbiol. 2023;63:1007–15.

[16] Nair S, Sasidharan A, Divya Rani VV, Menon D, Nair S, Manzoor K, et al. Role of size scale of ZnO nanoparticles and microparticles on toxicity toward bacteria and osteoblast cancer cells. J Mater Sci: Mater Med. 2009;20:235–41.

[17] Premanathan M, Karthikeyan K, Jeyasubramanian K, Manivannan G. Selective toxicity of ZnO nanoparticles toward gram-positive bacteria and cancer cells by apoptosis through lipid peroxidation. Nanomed Nanotechnol Biol Med. 2011;7:184–92.

[18] Emami-Karvani Z, Chehrazi P. Antibacterial activity of ZnO nanoparticle on gram positive and gram-negative bacteria. Afr J Microbiol Res. 2011;5:1368–73.

[19] Sawai J. Quantitative evaluation of antibacterial activities of metallic oxide powders (ZnO, MgO and CaO) by conductimetric assay. J Microbiol Methods. 2003;54:177–82.

[20] Sawai J, Yoshikawa T. Quantitative evaluation of antifungal activity of metallic oxide powders (MgO, CaO and ZnO) by an indirect conductimetric assay. J Appl Microbiol. 2004;96: 803–9.

[21] Dorostkar R, Ghalavand M, Nazarizadeh A, Tat M, Hashemzadeh MS. Anthelmintic effects of zinc oxide and iron oxide nanoparticles against *Toxocara vitulorum*. Int Nano Lett. 2017;7:157–64.

[22] Saleh F, Kheirandish F, Hosseini F, Yazdian F. Evaluation the effect of ZnO nanoparticle derived *Bacillus subtilis* on the

expression of efflux pump genes (AdeB AdeRS) in *Acinetobacter baumannii*. J Environ Health Sci Eng. 2021;19:1133–41.
[23] Qiang W, Jing H, Yan-song Z, Shen-yuan F. Preparation and characterization of cellulose nanocrystal poly (vinyl alcohol) hydrogel. Fun mol wight. 2013;26(3):2383–6.
[24] Gonçalves R, Mosser DM. The isolation and characterization of murine macrophages. Curr Protoc Immunol. 2015;111: 14.1.1–14.1.16.
[25] Dutta A, Bandyopadhyay S, Mandal C, Chatterjee M. Development of a modified MTT assay for screening antimonial resistant field isolates of Indian visceral leishmaniasis. Parasitol Int. 2005;54:119–22.
[26] Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. J Immunol Methods. 1986;89:271–7.
[27] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55–63.
[28] Love DC, Esko JD, Mosser DM. A heparin-binding activity on *Leishmania* amastigotes which mediates adhesion to cellular proteoglycans. J Cell Biol. 1993;123:759–66.
[29] Bagherian A, Abbaspour H, Saeidisar S, Mirzaei M, Mirzaei HR, Mirzaei H. The effect of garlic extract on *Leishmania major* amastigotes: in vitro and in vivo studies. Res Med. 2015;38:181–6.
[30] Kheirandish F, Delfan B, Mahmoudvand H, Moradi N, Ezatpour B, Ebrahimzadeh F, et al. Antileishmanial, antioxidant, and cytotoxic activities of *Quercus infectoria* olivier extract. Biomed Pharmacother. 2016;82:208–15.
[31] Enad AT, Zghair KH. Cytotoxic effect of ZnO nanoparticles on the viability of *Leishmania donovani* promastigotes in vitro. Iraqi J. Sci. 2016;5:2811–7.
[32] Delavari M, Dalimi A, Ghaffarifar F, Sadraei J. In vitro study on cytotoxic effects of ZnO nanoparticles on promastigote and amastigote forms of *Leishmania major* (MRHO/IR/75/ER). Iranian. J Parasitol. 2014;9:6.
[33] Sumaira U, Siddique Afridi M, Salman Hashmi S, Ali GS, Zia M, Haider Abbasi B. Comparative antileishmanial efficacy of the biosynthesised ZnO NPs from genus verbena. IET Nanobiotechnol. 2018;12:1067–73.
[34] Gunalan S, Sivaraj R, Rajendran V. Green synthesized ZnO nanoparticles against bacterial and fungal pathogens. Prog Nat Sci Mater Int. 2012;22:693–700.
[35] Khatami M, Khatami S, Mosazade F, Raisi M, Haghighat M, Sabaghan M, et al. Greener synthesis of rod shaped zinc oxide nanoparticles using lilium ledebourii tuber and evaluation of their leishmanicidal activity. Iran J Biotechnol. 2020;18:2196.
[36] Esmaeili J, Mohebali M, Edrisian GH, Rezayat S, Ghazi KM, Charehdar S. Evaluation of miltefosine against *Leishmania major* (MRHO/IR/75/ER): in vitro and in vivo studies. Acta Med Iran. 2008;1;46(3):191–6.

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