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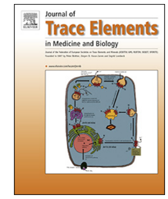

Efficacy of manganese oxide (Mn_2O_3) nanoparticles against *Leishmania major in vitro* and *in vivo*

Pooya Tavakoli[a], Fatemeh Ghaffarifar[a,*], Hamid Delavari[b], Nima Shahpari[c]

[a] *Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*
[b] *Nanomaterials Group, Department of Materials Engineering, Tarbiat Modares University, Tehran, Iran*
[c] *Department of Electrical Engineering, University of Isfahan, Isfahan, Iran*

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ABSTRACT

Background: The pentavalent antimonial compounds are the first drug of choice for leishmania infection, but have several side effects that cause some restriction for use. Extension of nanoparticle use in biological research and proven effectiveness of manganese nanoparticles on fungi and bacteria, along with the lack of information about its antileishmanial effects, have motivated this study. Manganese can induce cell apoptosis by increasing FOXO3a-Bim/PUMA mRNA activation and activating of caspase-3 pathway.

Methods: This study was aimed to examine the efficacy of manganese oxide nanoparticles against*Leishmania major* (MRHO/IR/75/ER) *in vitro* and *in vivo*. To evaluate the antileishmanial activity of NPs, light microscopic observation was used to determine the number of remaining parasites in each well. The MTT test was used to determine the cytotoxicity effects of Mn_2O_3 NPs against *L. major* promastigotes and macrophage cells. The effect of nanoparticles on cultured amastigotes under *in vitro* conditions was also investigated. The possible apoptosis of *L. major* by Mn_2O_3 NPs was evaluated with flow cytometry assay. Additionally, the preventive and therapeutic effects of Mn_2O_3 NPs in BALB/c mice following cutaneous *L. major* infection was tested. The effect of Mn_2O_3 NPs on promastigotes and amastigotes were proven by MTT assay and amastigote assay, respectively.

Results: The IC_{50} value of Mn_2O_3 NPs against *L. major* promastigotes and macrophages was 15 and 40 μg ml^{-1} respectively. The results of flow cytometry showed about 57% of the promastigotes were induced to apoptosis with Mn_2O_3 NPs. In *in vivo* studies, the size of the ulcers were significantly reduced, and the survival rate of the mice, in comparison with the control group, was increased.

Conclusion: Mn_2O_3 NPs has a beneficial effect on *L. major* promastigotes *in vitro* and *in vivo* and could be considered as a candidate for the treatment of this infection.

1. Introduction

Leishmaniasis is a vector-borne infection caused by an obligate intracellular parasite belonging to the *Leishmania* spp. [1]. The annual incidence rate of cutaneous leishmaniasis (CL) is 0.7–1.2 million cases among which, 70–75 % occur in 10 countries including Algeria, Ethiopia, North Sudan, Afghanistan, Iran, Syria, Colombia, Brazil, Costa Rica, and Peru [2].

Nowadays chemotherapy is an efficient way to cure leishmaniasis. The pentavalent antimonial compounds, such as sodium stibogluconate and meglumine antimoniate are the first drugs of choice for Leishmania infection [3,4], but these drugs have several weaknesses that cause some restrictions. Being expensive, high toxicity, numerous systemic side effects, drug resistance, long-term of remedy, and painful injection are some factors which decreases their acceptability by patients. Therefore, there is an immediate need for their replacement by other drugs or compounds for CL treatment [5].

During recent years, because of World Health Organization's (WHO) emphasis on finding new antileishmanial drugs derived from natural products, numerous studies have been performed universally [6]. Right this way use of natural compounds and nanoparticle based compounds are in use and antileishmanial activity of several substances have been proved [7,8]. One of these substances, silver NPs, which had a direct effect on *L. amazonensis* and the immunomodulative capabilities of infected macrophages, reduced the infection without inducing synthesis of inflammatory mediators [9]. In addition, some information is available on the antileishmanial effects of certain nanoparticles such as silver, gold, titanium dioxide, zinc oxide, and magnesium oxide

* Corresponding author at: Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, P.O. Box 14115-111, Iran.
E-mail addresses: pooya.tavakoli@modares.ac.ir (P. Tavakoli), ghafarif@modares.ac.ir (F. Ghaffarifar), hamid.delavari@modares.ac.ir (H. Delavari), n.shahpari@eng.ui.ac.ir (N. Shahpari).

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nanoparticles on *L. major* [10]. In view of these issues, some investigations noted that manganese oxide nanoparticles can effectively kill the fungi and bacteria and its antimicrobial properties have been investigated [11]. Because of these investigations and the lack of data about the antileishmanial effects of manganese nanoparticles, the authors were persuaded to design the current study. In the present study, the efficacy of manganese oxide nanoparticles against an Iranian standard strain of *Leishmania major* (MRHO/IR/75/ER) (*L. major*) *in vitro* and *in vivo* was screened.

2. Materials and methods

2.1. Manganese oxide NPs

Spherical manganese oxide NPs with purity of 99.2% and size of 30 nm were purchased from US Research Nanomaterial (Houston, USA). To perform all treatment tests, manganese NPs were dispersed in 10 ml sterile PBS with sonication in order to have a solution with a concentration of 800 μg ml^{-1} of manganese. Also, to increase the stability of the solution, 0.5 g sodium citrate was added to prevent their precipitation by creating a charge on the surface of the particles.

2.2. Characterization

The crystal structure of the particles was analyzed using X-ray powder diffraction (XRD, model Philips XPERT MPD) with a cobalt Kα radiation, λ = 1.79 Å. Scherrer's formula $L = 0.9\lambda/\beta\cos(\theta)$ was used to calculate the average crystallite size (L) of particles, where λ is the wavelength of the X-ray radiation, θ and β are the Bragg angle and the full width at half maximum (FWHM) of the corresponding peak, respectively. The morphology of the particles is determined by field emission scanning electron microscopy (FE-SEM model MIRA-III-TESCAN). Moreover, the FE-SEM was equipped with energy-dispersive X-ray spectroscopy (EDS) which is used to confirm the presence of manganese in dispersed solution.

2.3. L. major promastigotes culture

The *L. major* promastigotes were cultured in RPMI 1640 (Gibco, New York, NY, USA) at 26 °C and supplemented by 10% heat-inactivated FBS (Gibco, New York, NY, USA) to better feed the parasites and increase their proliferation and 500 μl penstrep (Gibco, New York, NY, USA) with concentration of 100 IU ml^{-1} of Penicilin and 100 μg ml^{-1} of Streptomycine to prevent the growth of bacteria [12].

2.4. Promastigotes assay

The *L. major* promastigotes in the logarithmic growth phase at 26 °C for 24, 48, and 72 h in the presence of several concentrations (400, 200, 100, 50, 25, 12.5, and 6.25 μg ml^{-1}) (2500, 1250, 625, 312, 156, 78, and 39 μM) of the Mn_2O_3 NPs solution in the 96-well plate (SPL Life Science) were incubated. Before using the NPs solution, it was sonicated. Promastigotes were directly counted in the Neubauer chamber with light microscope for the assessment of the anti-promastigote effects of NPs after 24, 48 and 72 h of incubation. A negative control without any treatment and positive controls with glucantime (Sanofi-Aventis France) (100 μg ml^{-1}) and amphotericin B (GILEAD UK) (1 μg ml^{-1}) were established in this study [13].

2.5. Amastigotes assay

At first, the macrophage cells in 8-well culture chamber slides with RPMI 1640 in a CO_2 incubator (37 °C, 5% CO_2, and 95% relative humidity) for 24 h were cultured. Then the medium culture was removed and the stationary phase of promastigotes was added to a ratio of 1:10. The promastigotes were incubated for 24 h to have sufficient time to phagocyte by macrophages. Excess parasites were removed by washing. After this time, the medium culture was removed and the cells were cultured in fresh medium that contained Mn_2O_3 NPs with concentrations of 25 and 50 μg ml^{-1} (156 and 312 μM) Glucantime (100 μg ml^{-1}) for positive control was used. After 72 h the slides were fixed with methanol and stained with Giemsa. The number of amastigotes per 100 macrophages should be determined with the aid of light microscope [12].

2.6. Promastigote viability measurements by MTT assay

The *L. major* promastigotes ($2 \times 10^6\ ml^{-1}$ cells) in the logarithmic phase were seeded in 96-well microplate and suspended in RPMI 1640 and 10% FBS and they were treated with several concentrations of Mn_2O_3 NPs. The microplate was incubated at 26 °C for 72 h and after that 20 μl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] with 5 mg ml^{-1} concentration was added to each well and they were incubated for 4 h. The medium was removed by centrifugation and 100 μl of DMSO (Sigma, USA) was added to each well. MTT evaluation was performed using an ELISA reader at 540 nm. All assays were repeated in triplicate [14].

2.7. Macrophage viability measurements by MTT assay

The macrophage cells (10^5 cells ml^{-1}) were cultured in 96-well plate and after adherence of the cells, they were exposed to the different concentrations of NPs. After 72 h of incubation The MTT assay (as briefly said above) was used to assess the viability of macrophages [12].

2.8. Flow cytometry analysis

In this study, flow cytometry method was used by double staining with annexin V-FLUOS and propidium iodide (PI) to assess the probable apoptosis (IQ Products BV, Groningen, Netherlands). As previously described, in this technique, annexin-V can be used for differentiating between necrotic (PI positive / upper left), apoptotic (only annexin-V positive as early apoptosis / lower right - both annexin-V and PI positive as late apoptosis / upper right) and alive cells (both annexin-V and PI negative / lower left). Briefly, $2 \times 10^6\ ml^{-1}$ promastigotes treated with Mn_2O_3 NPs (25 μg ml^{-1} or 156 μM) and untreated *L. major* promastigotes were washed with cold PBS solution twice and then centrifuged for ten minutes at 1400 *g*. Afterwards, the promastigotes were incubated at 25 °C and in the dark site for 15–20 minutes with 5 μl of annexin-V-FLUOS in the presence of 5 μl PI plus 500 μl buffer. Ultimately, the samples were analyzed by FACSCalibur flow cytometer (FACSCanto II). The results were analyzed with the FlowJo software and for each sample, the percentage of necrotic, apoptotic and normal cells were estimated [15].

2.9. In vivo assessment of Mn_2O_3 NPs against L. major

Female BALB/c mice were purchased from the Razi Institute of Iran (Karaj, Iran) and kept in colony room at a controlled temperature (22 ± 1 °C) and humidity (50 ± 10%) under a 12/12 h light/dark cycle and free access of food and water. The protocol was confirmed by the Tarbiat Modares University of Medical Sciences Ethical Laws Committee's Institutional Animal Care and Use Committee (Grant No. 78172). The *L. major* (MRHO/IR/75/ER) used for infection was in stationary phase to have the ability to invade the mice and induce the lesions ($2 \times 10^6\ ml^{-1}$). The mice were divided into three groups and each group contained 5 mice. At first, mice were subcutaneously infected with 100 μl of stationary phase of promastigotes and after the appearance of lesions, all groups except the group 1 were treated for 28 days, the group 1 is a negative control group. The mice in group 2 and group 3 were received glucantime (60 mg/Kg) *via* injection and 50 μl NPs (with concentration of 200 μg ml^{-1} or 1250 μM per day by

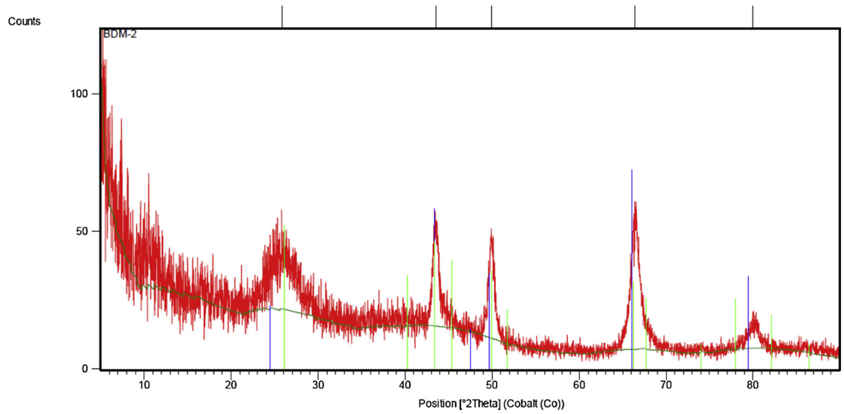

Fig. 1. The XRD pattern for Mn_2O_3 NPs.

spraying on the lesions), respectively. After 28 days of the treatment, the mice were followed up for 8 weeks; at this time, the diameter of the wound, the weight and the survival rate were examined, but the mice did not received medication [12]. For measurement of survival rate, the mortality of mice in all groups were assessed weekly during 18 weeks after challenge and the results were reported as percentages of live mice for each group in the end of each week.

2.10. Statistical analysis

Data were analyzed by one-way ANOVA and Mann–Whitney tests, using SPSS 24 for Windows. A p-value less than 0.05 was considered significant (SPSS Inc., Chicago, IL, USA).

3. Result

3.1. Characterization of nanoparticle

Fig. 1 shows the hexagonal structure of crystal of manganese oxide (Mn_2O_3) which is in good agreement with the JCPDS 00-030-0820 standard card. The size of the crystallites of the Mn_2O_3 NPs is 7 nm by applying Scherrer's formula on peak of (002) at $2\theta = 43.60°$. Fig. 2 show a typical SEM image of Mn_2O_3 NPs. Mn_2O_3 NPs with diameter about 30 nm can be observed. The lower crystalline size from XRD can be attributed to the formation of polycrystalline Mn_2O_3 NPs. Fig. 3 shows the EDS test to confirm the presence of manganese in dispersed solution.

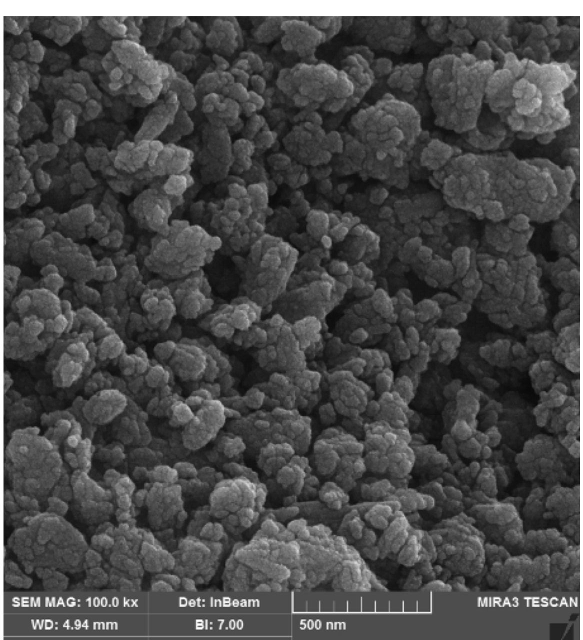

Fig. 2. The morphology of Mn_2O_3 NPs by FE-SEM image.

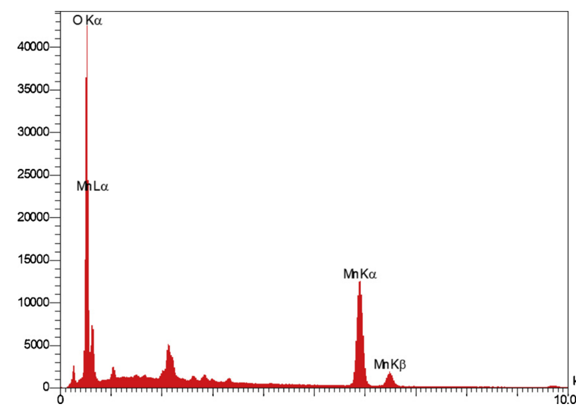

Fig. 3. EDS analysis of the Mn_2O_3 NPs.

3.2. Promastigote assay by microscopic observation

The light microscopic examination determines the number of remaining parasites in each well to assess the antileishmanial activity of Mn_2O_3 NPs (Table 1). We cultured $6 \times 10^5\ ml^{-1}$ promastigotes at the beginning of incubation.

3.3. Amastogite assay

At first, the rate of infected macrophages were determined. To do this, the prepared slides as controls were examined with the microscope. It was detected that 29% of macrophages in control group were infected. After reviewing the treated slides by 25 and 50 $\mu g\ ml^{-1}$ (156 and 312 μM) concentration of NPs, it was found that 12% and 9% of the macrophages were infected, respectively. In comparison, in positive control 17% of the macrophages were infected. The results demonstrated that Mn_2O_3 NPs reduces the number of *L. major* amastigotes in macrophages and the IC_{50} value was determined 42 $\mu g\ ml^{-1}$ or 262 μM (Table 2)

3.4. The cytotoxicity of Mn_2O_3 NPs to the promastigotes by MTT

Mn_2O_3 NPs was assayed against *L. major* promastigotes and the IC_{50} value of NPs at 72 h was obtained 15 $\mu g\ ml^{-1}$ or 93.6 μM (Fig. 4).

Table 1
Mean and standard deviation (SD) of the number of *Leishmania major* promastigotes treated with different concentrations of Mn_2O_3 NPs in comparison to groups treated with glucantime and amphotericin B and negative control group. (Number of promastigotes counted in each well × 10^4).

concentrations	24 h (Mean ± SD)	48 h (Mean ± SD)	72 h (Mean ± SD)
400 μg ml^{-1} NPs (2500 μM)	22 ± 2.8*	15 ± 2.8*	15 ± 1.4*
200 μg ml^{-1} NPs (1250 μM)	24 ± 1.4*	17 ± 2.8*	17 ± 1.4*
100 μg ml^{-1} NPs (625 μM)	27 ± 0*	25 ± 1.4*	22 ± 2.8*
50 μg ml^{-1} NPs (312 μM)	31 ± 1.4*	30 ± 0*	25 ± 2.8*
25 μg ml^{-1} NPs (156 μM)	34 ± 1.4*	32 ± 2.8*	28 ± 1.4*
12.5 μg ml^{-1} NPs (78 μM)	34 ± 2.8*	33 ± 2.8*	28 ± 2.8*
6.25 μg ml^{-1} NPs (39 μM)	37 ± 4.2*	35 ± 2.8*	30 ± 4.2*
Negative control	65 ± 3.5*	68 ± 2.8*	75 ± 2.8*
100 μg ml^{-1} Glucantime	22 ± 2.8*	14 ± 1.4*	10 ± 2.8*
100 μg ml^{-1} Amphotericin 1 μg ml^{-1}	23 ± 1.4*	20 ± 3.5*	16 ± 2.8*

* Indicates a significant difference with control group in all concentrations and all times ($p < 0.05$).

3.5. The cytotoxicity of Mn_2O_3 NPs to macrophages by MTT

When macrophages were incubated for 72 h in the presence of different concentrations of Mn_2O_3 NPs, the highest cytotoxicity was observed after 72 h at higher concentrations (Fig. 5). The IC_{50} value of the Mn_2O_3 NPs on macrophage cells was 40 μg ml^{-1} or 249.6 μM.

3.6. Flow cytometry assay

In the current investigation, the flow cytometry assay was employed to calculate the percentage of necrotic, apoptotic, and normal cells. For this purpose, after 72 h incubation of treated *L. major* promastigotes with 25 μg ml^{-1} or 156 μM of the NPs, the percentage of normal, necrotic, and apoptotic cells were estimated as 42.6%, 0.27%, and 57.07%, respectively. However, the status in the control group was determined as 99.2%, 0.02%, and 0.78%, respectively. The outputs of flow cytometry analysis are illustrated in Fig. 6.

3.7. In vivo study

Five weeks after injection of promastigotes, the lesions were observed in all the mice and the mice in group 2 (glucantime group) and group 3 (Mn_2O_3 NPs group) started getting treated. The mean diameters of lesions in group 2 and 3 were significantly smaller than the control group ($p < 0.05$) and there was not significant differentiation between group 2 and 3 ($p > 0.05$). After 4 weeks of follow up, no significant differentiation in size of the lesions was observed in the treated groups ($p > 0.05$) (Table 3. and Figs. 7, 8). The survival rate for treated and control groups is shown in Fig. 9.

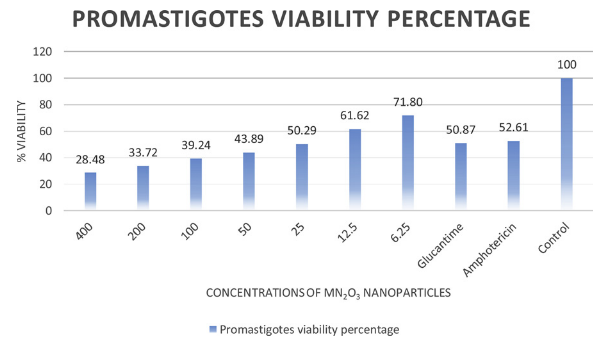

Fig. 4. The viability of *L.major* promastigotes in the presence of various concentrations of the Mn_2O_3 NPs (400-6.25 μg ml^{-1}), after 72 h incubation. There is significant differences between all treated groups and control group ($p < 0.05$).

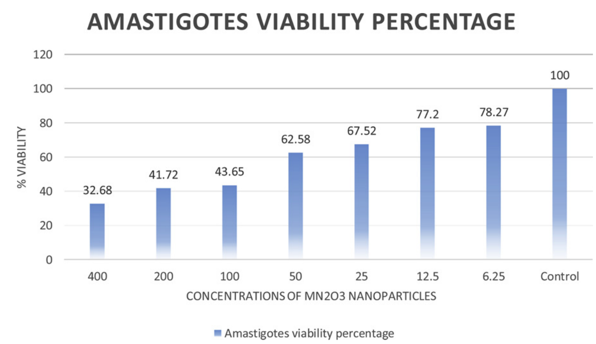

Fig. 5. The viability effects of the Mn_2O_3 NPs on macrophages after 72 h incubation in comparison to control group.

4. Discussion

Today, Leishmania is considered as one of the main health problems in the world. Drugs such as glucantime and pentostam are used to treat leishmaniasis; however, they include restrictions on consumption. Expensive cost, failure of treatment, long course of remedy, painful administration, and various systemic side effects have severely restricted its use. In this study, the antileishmanial effect of Mn_2O_3 NPs against *L. major* promastigotes with IC_{50} value of 15 μg ml^{-1} (93.6 μM), by exposing NPs with them and MTT assay test were studied. MTT assay was also used to study the effect of probable toxicity of NPs on macrophage cells, the IC_{50} value was 40 μg ml^{-1} (249.6 μM)

This study is the first study in this field, no similar study has been conducted using manganese oxide NPs in CL. The results indicate that *L. major* promastigotes and amastigotes were sensitive to Mn_2O_3 NPs at different concentrations. Based on research into different microorganisms in the past, special properties are considered for manganese nanoparticles. One of these studies is a study conducted on species of bacteria and nanoparticles which showed promising antimicrobial effects against *Staphylococcus aureus* and *Escherichia coli* in comparison to streptomycin [16]. In the other study, the antifungal action of curcumin stabilized Mn NPs were contemplated with diffusion technique against

Table 2
Effect of different concentrations of Mn_2O_3 NPs on the rate of *Leishmania major* amastigotes in comparison to control and glucantime groups.

Groups	Amastigote rate (per macrophage) M ± SD	% Infected Macrophage
Mn_2O_3 NPs 50 μg ml^{-1} (312 μM)	1.11 ± 0.04 *	9%
Mn_2O_3 NPs 25 μg ml^{-1} (156 μM)	1.41 ± 0.09 *	12%
Glucantime (100 μg ml^{-1})	1.76 ± 0.07*	17%
Control	2.34 ± 0.09	29%

* Indicates a significant difference with control group ($p < 0.05$).

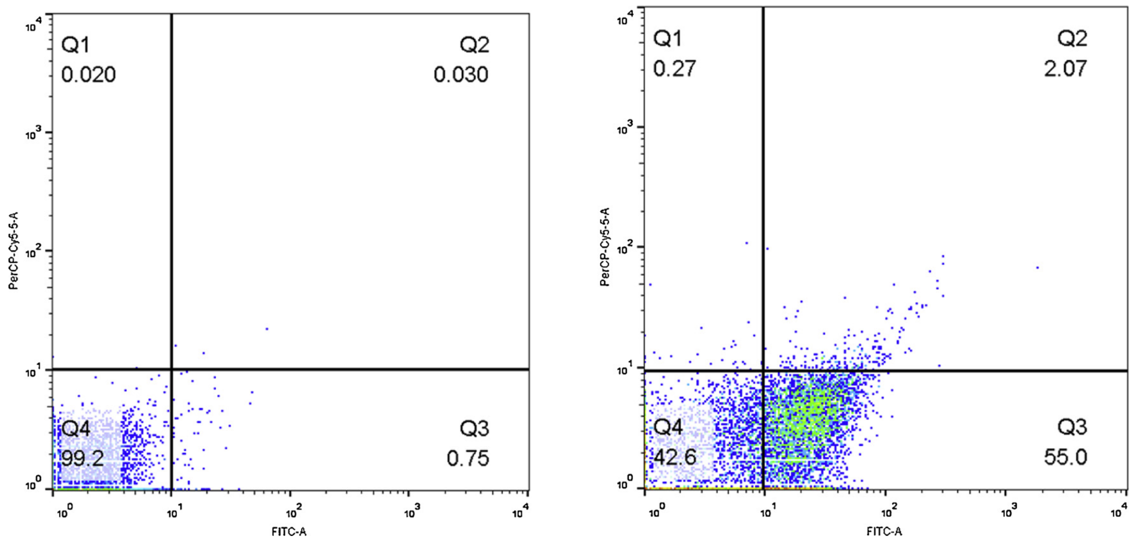

Fig. 6. Flow cytometry analysis: Apoptosis occurrence in *L. major* promastigotes control (left Fig.) and 72 h after treatment with Mn_2O_3 NPs (25 μg ml^{-1}) (156 μM) (Right Fig.).

four fungal strains, *Candida albicans*, *Aspergillus niger*, *Curvularia lunata*, and *Trichophyton simii* and they were contrasted with Fluconazole as a standard medication. The outcomes uncovered that the antifungal activity of curcumin stabilized Mn NPs were better than the curcumin and standard medication [17]. Therefore, according to the studies presented and the results of this research, the antibacterial, and antifungal properties of manganese nanoparticles can be confirmed.

For amastigotes assay, concentrations of 25 and 50 μg ml^{-1} (156 and 312 μM) were used to evaluate the antiamastigotes effect of NPs. The results of this study about treated amastigotes in comparison of the amastigotes in control group, showed the effect of Mn_2O_3 NPs, and that Mn_2O_3 NPs reduces the number of parasites with increasing concentration of NPs. In addition, no significant toxicity has been observed for macrophages exposed to NPs at 25 and 50 μg ml^{-1} (156 and 312 μM) concentrations [12,18].

Flow cytometry was used to investigate the probable apoptosis in promastigotes of *L. major* as briefly said. After 72 h incubation of promastigotes in 26 °C and exposure to 15 μg ml^{-1} (93.6 μM) nanoparticles, flow cytometric results were evaluated. The results of the study showed an acceptable level of apoptosis in promastigotes. There are other studies that confirm manganese apoptosis in other cells. One of these studies has shown that, Mn induced cell apoptosis in a dose-dependent manner both *in vitro* and *in vivo* by dramatically increased Bim and PUMA mRNA and protein expression [19]. Other studies have shown that Mn treatment could apparently increase the expression of active caspase-3 and initiate *in vitro* and *in vivo* neuronal apoptosis [20]. In addition, the immunofluorescence test, consistent with Western blot results, revealed that active caspase-3-positive neurons were significantly increased after exposure to Mn [21]. Now, for the first time, apoptotic effects of the Mn_2O_3 NPs on L. *major* propmastigotes are reported.

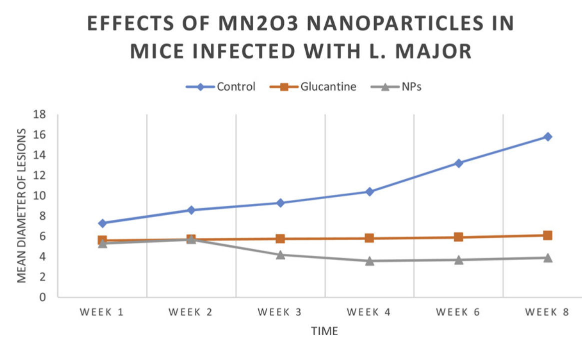

Fig. 7. Therapeutic effects of Mn_2O_3 NPs on the lesion sizes (mm) of localized cutaneous leishmaniasis induced by *Leishmania major* in BALB/c mice.

Based on the evidence and the results of the in-vitro study, it has been shown that manganese can be a favorable drug. Therefore, *in vivo* studies were also conducted for further studies. The *in vivo* study revealed that Mn_2O_3 NPs have a positive effect on the cure of wound in leishmania infected mice (group3). This wound healing was result of induction of apoptosis by manganese nanoparticles. Mn NPs have many effects on various microorganisms, such as viruses, fungi and bacteria, based on previous studies [22], and now, according to the results of this study, the antiparasitic property of manganese nanoparticles is known to be present in both *in vitro* and *in vitro* conditions. More biochemical analysis and pathological research is necessary to investigate the adverse effects of Mn NPs. The use of Mn NPs with other skin repair compounds can make a significant contribution to the healing of the wounds.

Table 3
The mean ± SD lesion size (mm) in the test and control groups after treating the mice with Mn_2O_3 NPs and glucantime for 4 weeks and 4 weeks of follow up.

Groups	Week 1	Week 2	Week 3	Week 4	Week 6	Week 8
Control	7.3 ± 0.2	8.6 ± 0.1	9.3 ± 0.4	10.4 ± 0.4	13.2 ± 0.2	15.8 ± 0.4
Glucantime	5.6 ± 0.1 *	5.7 ± 0 *	5.7 ± 5 0.07 *	5.8 ± 0 *	5.9 ± 0.1 *	6.1 ± 0.1 *
Mn_2O_3 NPs	5.3 ± 0.4 *	5.7 ± 0.2 *	4.2 ± 0.1 * / **	3.6 ± 0.4 * / **	3.7 ± 0.2 * / **	3.9 ± 0.07 * / **

* Indicates a significant difference with control group in all weeks after treatment ($p < 0.05$).
** Indicates a significant difference with Glucantime group ($p < 0.05$).

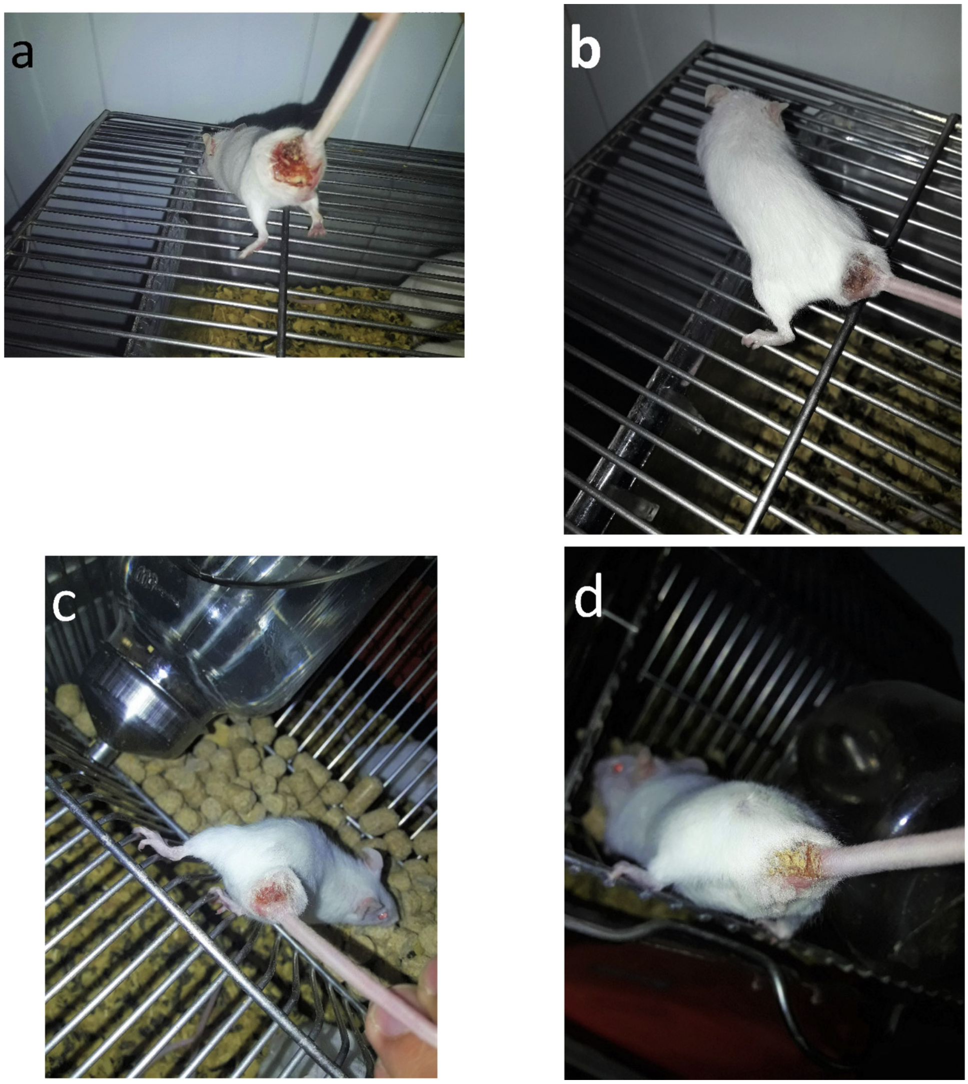

Fig. 8. Mice infected by *L. major* in the base of the tail with 2×10^6 promastigotes as a control mouce (a). Glucantime treated mouse (b). Effect of Mn_2O_3 NPs spraying after 3 week (c). Lesion at the end of 4 weeks follow up in Mn_2O_3 NPs treated group (d).

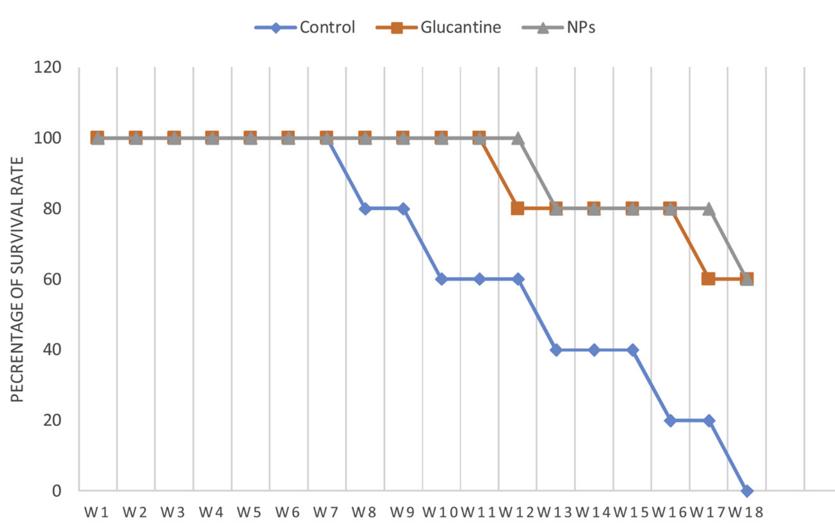

Fig. 9. Percentage of survival rate of infected BALB/c mice with 2×10^6 in treated groups with Mn_2O_3 NPs and control groups during 18 weeks after challenge. The number of mice in each group is 5.

5. Conclusion

Recently, nanoparticles have been considered in the treatment of various diseases. The results in this study indicated that IC_{50} of Mn_2O_3 NPs against *L. major* promastigotes was calculated 15 μg ml^{-1} (93.6μM) whereas IC_{50} for macrophage was 42 μg ml^{-1} (262 μM).

The results of flow cytometry showed that Mn_2O_3 NPs causes Programmed Cell Death (PCD) and 57% of apoptosis in the exposed group of promastigotes. Also, in the *in vivo* study, reduction of size and wound healing and increasing in survival rate were observed too. Based on the results obtained, favorable antileishmanial effect was observed and therefore, manganese oxide nanoparticles can be considered as a candidate for treatment in future studies.

Authors' contributions

P. Tavakoli and F. Ghaffarifar conceived the study; F. Ghaffarifar designed the study protocol; F. Ghaffarifar was supervisor of this research; H. Delavari was advisor of the research; H. Delavari analyze the nanoparticle parts; P. Tavakoli drafted the manuscript; P. Tavakoli, H. Delavari, N. Shahpari and F. Ghaffarifar critically revised the manuscript.

Ethics approval

This research approved by ethical committee of Tarbiat Modares University with number IRMODARES.REC 1397-177; 5-Jan-2019.

Declaration of Competing 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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