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Original Article

Antileishmanial Activity of Manganese (III) Oxide Nanoparticles (Mn2O3 NPs) Alone and in Combination with Shark Cartilage Extract against *Leishmania infantum*: *in Vitro* and *in Vivo* Approaches

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Abstract

Background: The recent increase in unresponsive cases to current treatments poses a significant challenge in the effort to discover new antileishmanial drugs. We aimed to investigate the effects of Mn_2O_3 NPs alone or in combination with shark cartilage extract (ShCE) on *L. infantum* (*MCAN/ES198/LIM-877*) both *in vitro* and *in vivo*.

***Methods*:** The susceptibility of promastigotes and amastigotes was evaluated using MTT and microscopy assays with different concentrations of Mn_2O_3 NPs alone or in combination with ShCE (Mn_2O_3-ShCE). The combination index (CI) was used to assess potential synergy. Cytotoxicity against J774.A1 macrophages was measured, and the selectivity index (SI) was calculated. Also, apoptosis was analyzed in promastigotes. The therapeutic and prophylactic effects were tested in BALB/c mice, and survival rates were monitored.

***Results*:** Mn_2O_3 NPs caused a dose-dependent decrease in proliferation of promastigotes (IC_{50}=19.8±1.1 µg/mL) and amastigotes (IC_{50}=29.9±0.05 µg/mL). Co-treatment with ShCE significantly enhanced the antileishmanial activity, reducing the IC_{50} values of promastigotes and amastigotes. The CC_{50} values were 159.9±4.2 µg/mL for Mn_2O_3 NPs and 276.5±7.9 µg/mL for Mn_2O_3-ShCE. Apoptosis rates in promastigotes treated with Mn_2O_3 and Mn_2O_3-ShCE were 80.7% and 86.3%, respectively. Parasite loads in the tissues significantly decreased (P<0.001) in BALB/c mice treated with Mn_2O_3 NPs, and survival rates increased significantly (P<0.05). The combination with ShCE further improved outcomes, with 100% survival observed in the Mn_2O_3-ShCE group (P<0.01).

***Conclusion*:** These findings demonstrated a synergistic effect between Mn_2O_3 NPs and ShCE against *L. infantum*. Therefore, it may serve as a promising candidate for future development of leishmanicidal drugs.

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Introduction

Neglected tropical diseases (NTDs) are major infectious diseases that cause serious health issues in developing countries. Leishmaniasis is one of the most significant NTDs, with estimated annual cases ranging from 600,000 to 1 million for cutaneous leishmaniasis (CL) and 50,000 to 90,000 for visceral leishmaniasis (VL), resulting in considerable morbidity and mortality in 100 endemic countries (1). Two main clinical forms of leishmaniasis are recognized: VL and CL (2). The severe form, VL, is associated with malnutrition, poor hygiene, and immunodeficiency conditions. If left untreated, VL is fatal in many cases (3). Current treatment predominantly relies on chemotherapy; however, available drugs are limited by toxicity, high cost, and resistance. This highlights the urgent need for safer and more effective therapies (4). Nanotechnology has emerged as a promising strategy in antileishmanial research, functioning either as a drug delivery system or by exerting the intrinsic antiparasitic activity of nanomaterials (5).

Metal oxide nanoparticles can generate reactive oxygen species (ROS), thereby interfering with essential metabolic and antioxidant pathways in *Leishmania* spp. (6). The antileishmanial activity of some metal oxide NPs has been investigated (5,6). Mn_2O_3 nanoparticles have been reported to exhibit activity against *L. major* (7).

In addition to direct parasiticidal strategies, leishmaniasis is associated with immune dysfunction, particularly T cells. Research using murine models of leishmaniasis has demonstrated that IL-12 significantly enhances host defense by promoting Th1 cytokine production, including IFN-γ. This activation enables macrophages to effectively eliminate parasites through enhanced nitric oxide production (8-10). Therefore, therapeutic strategies that combine direct antiparasitic activity with immune modulation may provide superior outcomes.

ShCE delivers impressive therapeutic benefits for some diseases and shows strong immunomodulatory properties. It has been reported to stimulate Th1-associated cytokines while minimally inducing Th2 cytokines, thereby favoring a Th1-biased immune profile. In murine models of leishmaniasis, immunomodulatory interventions that enhance Th1 responses have been associated with improved parasite control (9,11).

Given the enhancement effect of ShCE and Fe_3O_4 NPs on the Leishmania parasite *in vitro* (12) along with its immunomodulatory properties in murine leishmaniasis shown in our previous study (9), this study aimed to assess whether Mn_2O_3 NPs, alone or in combination with ShCE, exhibit synergistic antileishmanial activity against *L. infantum in vitro* and *in vivo*.

Method and Materials

Mn_2O_3 NPs

Mn_2O_3 NPs with a purity of 99.2% were purchased from US Research Nanomaterial (Houston, USA) and were characterized as described in our previous study. In brief, the structural information of NPs and the crystallite size were determined using X-ray powder diffraction (XRD, Model Philips XPERT MPD) and Scherrer's equation, respectively. In addition, a field emission scanning electron microscope (FE-SEM) equipped with energy–dispersive X-ray spectroscopy (EDS) was used to determine the morphology and the presence of manganese in the sample. The size of the crystallites in the Mn_2O_3 NPs was approximately 7 nm, as determined using Scherrer's formula applied to the (002) peak at $2\theta = 43.60$ degrees (7).

Shark cartilage extract preparation

Shark cartilage extract (Bushehr Port, Persian Gulf, Iran) was prepared using the method

described by Hassan et al (9,11). It contains several proteins with various therapeutic effects. As an immunomodulator, the extract stimulates Th1 inflammatory cytokines through two low-molecular-weight proteins of approximately 14 and 15 kDa (9).

Drug preparation

For *in vitro* experiments, a Mn_2O_3 NPs work solution was prepared at a concentration of 1000 µg/mL in cold, sterile PBS and sonicated under optimized conditions (7). Serial concentrations of 1.56-100 µg/mL of Mn_2O_3 and ShCE were prepared separately in sterile RPMI 1640 and used for *in vitro* testing. In the combination treatment, a mixture of Mn_2O_3 NPs and ShCE was used at concentrations of 1.56+1.56, 6.25+6.25, 12.5+12.5, 25+25, 50+50, and 100+100 µg/mL, respectively. The animals were treated with either ShCE (oral, 20 mg/kg), Mn_2O_3 (i.p, 50 µg/mL), or a combination of both drugs twice daily (at 12 h intervals) for 10 consecutive days (7).

In vitro assay: Cultivation of L. infantum

The standard strain of *L. infantum* (MCAN/ES198/LIM-877) was first cultured in Novy–MacNeal–Nicolle (NNN) medium, then transferred to RPMI-1640 (Gibco, NY, USA) supplemented with 20% FBS and Pen-Strep antibiotics, including 100 U/mL penicillin and 100 µg/mL streptomycin, for mass production of the parasites at 18-24 °C (7,9).

J774A.1Macrophage Cell culture

The murine macrophage cell line, J774.A1, was obtained from the Iranian Pasteur Institute. Mass production of the cells was achieved by cultivating in fresh serum-free RPMI-1640 containing 10% FBS and 1% Pen-Strep at 37 °C and 5% CO_2 (7).

MTT assay

The viability of promastigotes exposed to different concentrations (1.56-100 µg/mL) of Mn_2O_3 NPs alone or in combination with ShCE was evaluated using MTT assay as described in our previous studies (7,9). GraphPad Prism software was used to determine the 50% inhibitory concentration (IC_{50}) value. Since metal oxide nanoparticles can interfere with colorimetric assays, a control test was performed using a cell-free system with growth media, assay reagents, or PBS to prevent this interference (13).

Amastigote assay

To assess the effects of the Mn_2O_3 NPs, ShCE, and their combination on *L. infantum* amastigotes, J774.A1 macrophages (1×10^5 cells/mL) were cultured in 24-well microplates with rounded coverslips at the bottom and incubated at 37 °C in 5% CO_2. Macrophage infection was performed by adding the metacyclic promastigote form of *L. infantum* at a parasite-to-cell ratio of 10:1, followed by incubation for an additional 24 h. After this period, infected macrophages were further incubated for 72 h in the presence or absence of the test compounds at various concentrations (1.56-100 µg/mL). The number of intracellular amastigotes per 100 infected macrophages in treated and untreated wells was counted on Giemsa-stained slides, and IC_{50} was subsequently calculated (7,14).

Macrophage cytotoxic activity

The cytotoxic activity of Mn_2O_3 NPs and their combination with ShCE was evaluated on uninfected J774.A1 macrophages using the MTT assay in the presence or absence of different drug concentrations (1.56-100 µg/mL), and 50% cytotoxicity concentration (CC_{50}) was determined. The SI was also calculated as the ratio of CC_{50} to the IC_{50} of amastigotes (12,14).

The combination index (CI) was calculated to assess the potential synergy of Mn_2O_3 and ShCE. The formula used was: CI = (C)/(A) + (C)/(B), where (A) and (B) represent the

individual concentrations of Mn_2O_3 and ShCE, respectively, and (C) represents the combined amount of both substances. The CI value is used to classify the type of interaction as synergy (CI < 1), additivity (CI = 1), or antagonism (CI > 1). Additionally, the theoretical IC_{50} was determined to evaluate the synergistic effect of the combination therapy. The calculation for the theoretical IC_{50} was based the equation: theoretical IC_{50} = (IC_{50} Mn_2O_3/2) + (IC_{50} ShCE/2) (15,16).

Flow cytometry analysis

The PI Annexin V-fluorescein isothiocyanate (FITC) (Mab Tag, Germany) Apoptosis Detection Kit was used to determine the percentage of apoptotic promastigotes of *L. infantum*. The logarithmic-phase promastigotes (1×10^5) were treated with IC_{50} concentrations of Mn2O3 NPs, ShCE, and their combination, and apoptosis was assessed according to the kit's instructions. Finally, the samples were analyzed using a CyFlow flow cytometer (Sysmex-Partec, USA) and FlowJo™ software (12, 14).

In vivo assay

Eighty female inbred BALB/c mice older than eight weeks (16-18 g) were obtained from the Pasteur Institute of Iran and housed at standard conditions in the Animal House. Sixty mice were infected intraperitoneally (i.p.) with 100 µL PBS suspension containing stationary-phase promastigotes (9) and randomly divided into two main groups: control (CTRL) and experimental groups. Thirty infected mice were considered as the infected control groups: infected-untreated: CTRL (-), infected-PBS treated: CTRL (-)-PBS, infected-GLU treated (20 mg/kg/day/i.p.): CTRL (+). The remaining thirty experimental mice were treated with 0.1 ml Mn_2O_3 NPs (50 µg/mL, i.p.) and ShCE (20 mg/kg, orally) (9,11), or their combination.

Twenty mice were assigned to the prophylactic group and received ShCE orally and Mn_2O_3 NPs via i.p. injection at the specified doses daily for 15 days before infection. Treatment began three weeks post-infection, when the VL model was established in mice, and continued for 10 consecutive days. After the end of the treatment period, five mice from each group were euthanized, and the parasite was quantified in 30 mg of homogenized spleen and liver tissues using a limiting dilution assay (9). The tissues were serially diluted in 96-well microplates and incubated at 25 °C for two weeks to determine the final dilution at which at least one parasite was detected in the well.

Survival time was monitored for 15 weeks post- infection and analyzed using the Kaplan-Meier method with the log-rank test for five mice in each group (15).

Data analysis

At least three independent experiments were performed, and the results were expressed as mean ± SD. An unpaired t-test and ANOVA were used to normally distributed variables, verified using the Shapiro-Wilk test. For non-normally distributed variables, the Mann-Whitney and Friedman's tests. IC_{50} values for promastigotes and amastigotes, and CC_{50} values for J774.A1 macrophages were determined using GraphPad Prism software. Survival time was analyzed using the Kaplan–Meier method with the log-rank test, based on five mice per group.

Ethics approval

This study was approved by the Ardabil University of Medical Sciences with ethical approval number IR.ARUMS.AEC.1401.016.

Results

Promastigote assay

The average viability percentage of promastigotes at prearranged concentrations of Mn_2O_3 NPs, ShCE, and their combinations is shown in Fig. 1. All three treatment modalities demonstrated significant anti-leishmanial activity in a dose-dependent manner over 72 h of

incubation (P<0.05). The combined inhibitory effect on cell viability was greater than that of each treatment alone (P<0.01). The IC_{50} values of Mn_2O_3 NPs, ShCE, and Mn_2O_3-ShCE combination were 19.8± 1.1, 22.8± 1.5, and 5.9± 2.3 µg/mL, respectively (Table 1).

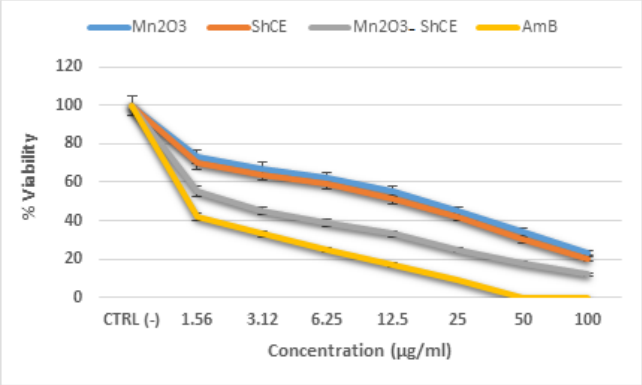

Fig. 1: The mean viability values of *L. infantum* promastigotes treated with Mn_2O_3 NPs, ShCE alone, and their combination (Mn_2O_3-ShCE) compared with the negative control (CTRL (−)) and the positive control, Amphotericin B (AmB)

Table 1: Estimation of IC_{50} values for Mn_2O_3 NPs and ShCE alone and their mixture (Mn_2O_3-ShCE) against promastigotes and amastigotes of *L. infantum*, and CC_{50} values of drugs on J774.A1 macrophages

Mn_2O_3 NPs and combinations	Promastigote		Amastigote		J774 Macrophage cells	SI
	IC50± SD (µg/mL)	*P-value VS. AmB*	IC50± SD (µg/mL)	*P-value VS. GLU*	CC50± SD (µg/mL)	
Mn_2O_3	19.8± 1.1	$P > 0.05$	29.9± 0.05	$P < 0.001$	159.9± 4.2	5.34
Mn_2O_3-ShCE	5.9± 2.3	$P > 0.05$	9.8± 0.08	$P < 0.0001$	276.5± 7.9	28.2
ShCE	22.8± 1.5	$P > 0.05$	>100	ND	>500	>10
AmB	1.38± 0.95	NR	15± 0.05	NR	12± 1.3	8
GLU	278± 2.9	NR	95± 0.08	NR	522± 1.9	5.5

IC_{50}: 50% inhibitory concentration on promastigotes and amastigotes growth.
CC_{50}: 50% cytotoxic concentration on macrophage.
SI: Selectivity index (CC_{50}/IC_{50} of amastigotes).
NR: Not related, AmB: amphotericin B, GLU: glucantim

Intracellular amastigote assay

The study demonstrated significant differences in the number of intracellular *L. infantum* amastigotes compared with the negative control group (P<0.001). Interestingly, all concentrations of Mn_2O_3 alone and Mn_2O_3-ShCE showed a significant effect on intracellular amastigotes compared to GLU, the positive control (P<0.001) (Table 2). The lowest IC_{50} values were observed for the Mn_2O_3–ShCE (IC_{50}=9.8± 0.08 µg/mL) (Table 1). The CI index was calculated to be 0.43, indicating a synergistic effect (CI<1). Our analysis revealed a

theoretical IC_{50} value of 64.95 µg/mL, which was significantly higher than the experimental IC_{50} value of 9.8 µg/mL (P<0.0001), further confirming the synergistic effect of the combination treatment (Fig. 2).

Table 2: Susceptibility of *L. infantum* amastigotes to different concentrations of Mn_2O_3 NPs, ShCE, and Mn_2O_3-ShCE after 72 h of incubation

Concentration (µg/mL)	Mn_2O_3 NPs		ShCE		Mn_2O_3- ShCE	
	Mean±SD	*P-value VS GLU*	Mean±SD	*P-value VS GLU*	Mean±SD	*P-value VS GLU*
100	1.7±0.02	*P*<0.001	5.25±0.02	*P*>0.05	0.9±0.05	*P*<0.001
50	2.8±0.01	*P*<0.001	5.45±0.02	*P*<0.05	1.1±0.02	*P*<0.001
25	3.4±0.05	*P*<0.01	6.65±0.05	*P*<0.05	1.8±0.03	*P*<0.001
12.5	3.6±0.09	*P*<0.05	6.80±0.02	*P*>0.05	2.5±0.01	*P*<0.001
6.25	3.8±0.01	*P*<0.05	6.85±0.02	*P*>0.05	3.2±0.02	*P*<0.001
3.12	3.9±0.03	*P*<0.05	6.85±0.05	*P*>0.05	3.8±0.06	*P*<0.05
1.56	3.9±0.04	*P*<0.05	6.85±0.08	*P*>0.05	3.9±0.05	*P*<0.05
GLU 100 µg/mL	3.95±0.05	NR	3.95±0.05	NR	3.95±0.05	NR
CTRL (-)	6.85±0.09	NR	6.85±0.09	NR	6.85±0.09	NR

NR: Not related, CTRL (-): negative control, GLU: glucantim, positive control

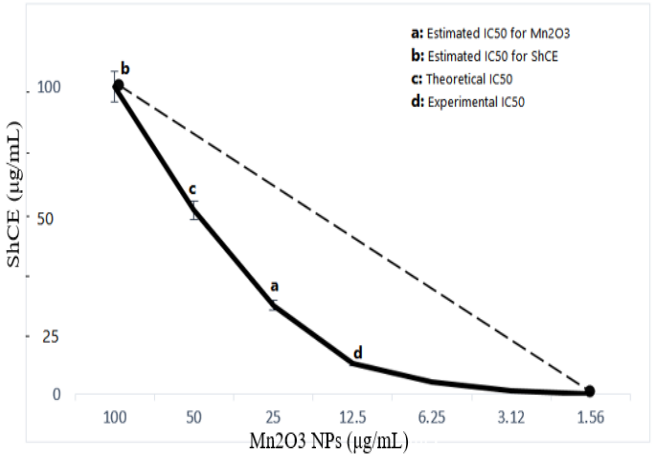

Fig. 2: The isobologram analysis of the antiamastigote activity of Mn_2O_3 and ShCE combination. The theoretical IC_{50} was 64.95 µg/mL, whereas the experimental IC_{50} value was 9.8 µg/mL

Cytotoxicity assay on J774.A1 macrophages

The highest cytotoxicity was observed with Mn_2O_3 NPs (CC_{50}=159.9±4.2 µg/mL), which decreased when used in combination, resulting in a CC_{50} of 276.5±7.9 µg/mL. Mn_2O_3-ShCE exhibited an acceptable selectivity index (SI), indicating a favorable cytotoxicity profile (Fig. 3 and Table 1).

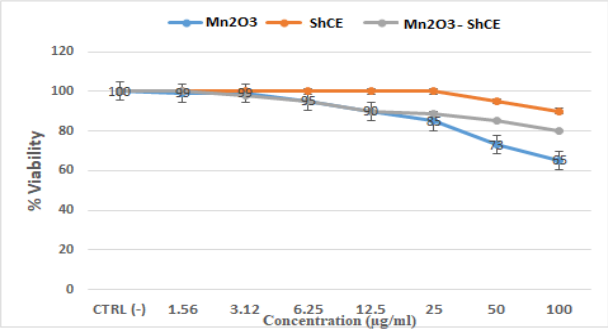

Fig. 3: The mean viability values of the J774.A1 macrophage cell line treated with different concentrations of combinations compared with the negative control, CTRL (−)

Flow cytometry analysis

The results showed that Mn_2O_3 NPs at IC_{50} concentration (19.8 µg/mL), whether used alone or in combination with ShCE, significantly induced apoptosis in *L. infantum* promastigotes (Fig. 4). Mn_2O_3-ShCE exhibited an even higher percentage of apoptosis (86.3%) compared with Mn_2O_3 NPs or ShCE alone.

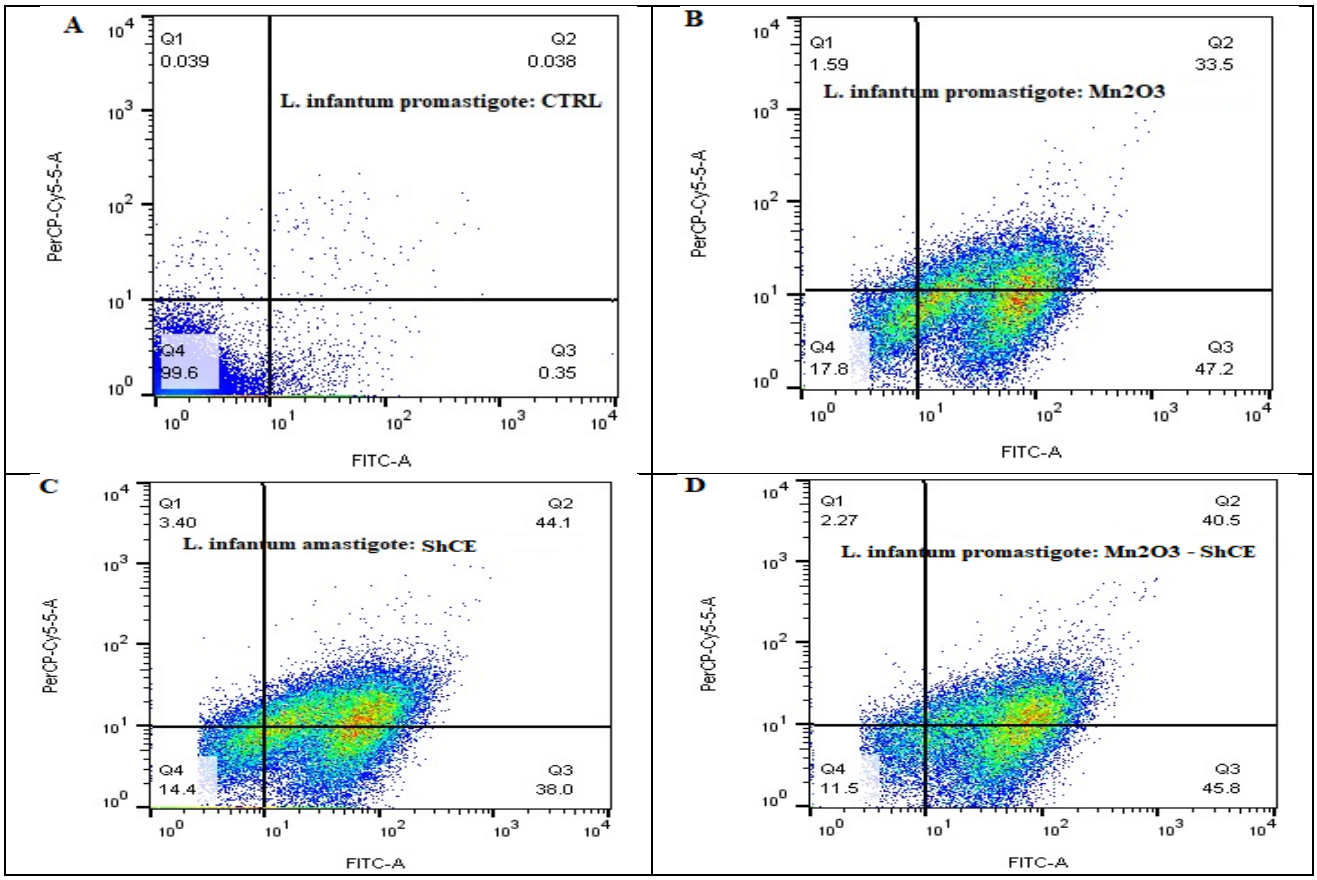

Fig. 4: Apoptotic profile of *L. infantum* promastigotes treated with IC_{50} concentrations of Mn_2O_3 NPs (B), and ShCE (C) and Mn_2O_3-ShCE (D) compared to the untreated control group (A)

In vivo assay

The results showed that Mn_2O_3 NPs, both with and without ShCE, effectively reduced the parasite burden in organs compared to negative control and positive control (GLU) groups (P<0.05). ShCE showed a significant difference compared to the negative control group, but not with the GLU group. Also, prophylactic administration of ShCE alone or in combination with Mn_2O_3 NPs significantly inhibited parasite growth and leishmaniasis development in a mouse model (Table 3). All mice in the control groups died within 12 weeks post-challenge. The survival time of mice treated with Mn_2O_3 NPs and ShCE was significantly longer than that of the control groups (P<0.05). A survival rate of 100% was maintained until the end of the 15-week challenge when Mn_2O_3 NPs were combined with ShCE (Fig. 5).

Table 3: Parasite burden in the tissues of BALB/c mice infected with *L. infantum* at 3-week intervals following treatment with studied combinations

Groups	Drugs	parasite count (mean±SD)/30 mg of tissue (-Log 10)	
		Spleen	Liver
Experimental	Mn_2O_3 NPs	2.8±1.2*	3.9±1.5*
	ShCE	4±1.1	4.2±1.5*
	Mn_2O_3 NPs-ShCE	1.7±1.5*	1.9±1.1*
Prophylactic	ShCE	1.7± 0.3*	1.9± 0.5*
	Mn_2O_3 NPs	3.1±0.8*	3.4±0.7*
Control	CTRL (-)-PBS	4.2±3.5	5.9±5.1
	CTRL (-)	4.2±3.6	5.9±5.2
	CTRL (+)	3.2±1.9	3.5±1.5

*Indicates a significant difference with the positive control group (P<0.05)

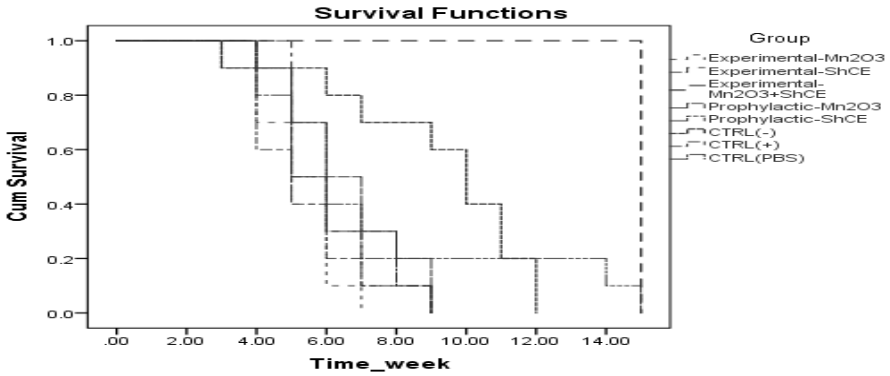

Fig. 5: Survival times using Kaplan-Meier analysis for infected and non-infected mice exposed to drugs

Discussion

Metal oxides NPs have attracted considerable attention due to their unique physicochemical properties and mechanisms of action, and have demonstrated efficacy against a broad spectrum of pathogens, especially Leishmania parasites (17, 18). The present study evaluated

the efficacy of Mn_2O_3 NPs, alone or in combination with ShCE, against *L. infantum* infection.

Our findings demonstrated that Mn_2O_3 NPs exhibited significant anti-leishmanial activity, markedly enhanced when combined with ShCE. The MTT assay revealed that Mn_2O_3 NPs, both alone and in combination with ShCE, significantly inhibited promastigote proliferation in a dose-dependent manner. The IC_{50} values for Mn_2O_3 NPs alone and in combination with ShCE were 19.8 ± 1.1 µg/mL and 5.9 ± 2.3 µg/mL, respectively. In our previous study on *L. major* promastigotes, the IC_{50} value of Mn_2O_3 NPs was 15 µg/mL (7). Similarly, manganese dioxide–conjugated curcumin–chitosan nanoparticles have been reported to significantly reduce parasite replication in murine models of leishmaniasis, particularly in the spleen, thereby demonstrating high therapeutic efficacy (18).

To assess the cytotoxicity in the macrophages, the SI was considered. The biological activity of a drug is not linked with any cytotoxicity when SI≥ 10 (19). In the present study, the combination of Mn_2O_3 NPs and ShCE demonstrated the highest SI (SI= 28.2). Furthermore, ShCE, both alone and in combination with Mn_2O_3 showed no cytotoxic effects within the range of our experimental concentrations (CC_{50} >100 µg/mL) and the SI was ≥10.

In the amastigote assay, a significant inhibitory effect was observed against intracellular amastigotes with increasing concentrations of Mn_2O_3 NPs, both alone and in combination with ShCE. The IC_{50} values were 29.9 ± 0.05 µg/mL for Mn_2O_3 NPs alone and 9.8 ± 0.08 µg/mL for the combination treatment. These findings are consistent with our previous observations in *L. major* (7). Furthermore, the results of the isobologram and CI analyses confirmed the synergistic effect of this combination. Other studies have also reported further improvement in the leishmanicidal activity of combination therapies in vitro and *in vivo* (20,21).

Flow cytometry analysis further supported these findings. At a concentration of 19.8 µg/mL, Mn_2O_3 NPs induced apoptosis in 80.7% of *L. infantum* promastigotes, while their combination with ShCE (100 µg/mL) increased apoptosis to 86.3%. Previous studies have demonstrated the dose-dependent induction of apoptosis following manganese treatment, including upregulation of FRXO3a-Bim/PUMA mRNA and activation of the caspase-3 pathway. Caspase-3 activation leads to proteolytic degradation and subsequent programmed cell death in *Leishmania* parasites. Our earlier work also reported significant apoptosis in *L. major* promastigotes after exposure to Mn_2O_3 NPs alone (7).

The *in vivo* results demonstrated a significant therapeutic effect of Mn_2O_3 NPs and their combination with ShCE in *L. infantum*-infected mice. Treatment with Mn_2O_3 NPs significantly reduced parasite burdens in the spleen and liver. In addition, prophylactic treatment with ShCE alone or in combination with Mn_2O_3 NPs significantly inhibited parasite growth and the progression of leishmaniasis in mice.

To date, no studies have evaluated the effects of Mn_2O_3 NPs in murine models of VL. Research on *L. major in vivo* remains limited. One study reported no significant reduction in wound diameter following intralesional injection of Mn_2O_3 NPs (22). However, other studies have shown that pre-treatment with Mn_2O_3 NPs (5 and 10 mg/kg for 14 d) before intraperitoneal parasite inoculation resulted in smaller lesions, and that post-infection treatment at the same doses led to complete wound healing. In the present study, administration of Mn_2O_3 NPs, both before and after infection, resulted in a significant reduction in parasite burden compared with the control group. Our findings are consistent with our previous research on murine cutaneous leishmaniasis, which also demonstrated significant wound healing following treatment with Mn_2O_3 NPs (7). Some studies have reported the antimicrobial effects

of manganese-based nanoparticles against various microorganisms (7,18).

The survival of *Leishmania* parasites within the host depends on maintaining redox homeostasis by balancing reactive oxygen and nitrogen species. Oxidative stress plays a key role in the host immune responses, whereas *Leishmania* employs antioxidant mechanisms to evade destruction. Exploiting antileishmanial drug-induced redox imbalance can increase parasite susceptibility and promote apoptosis (2,23).

Ultrafine nanoparticles like Mn2O3 can cause widespread cellular disorders through various mechanisms, including direct interaction with cell membranes, the release of toxic Mn^{2+} ions, and the generation of ROS, which can disrupt essential enzymatic functions or directly damage DNA. Despite these effects, the unique physicochemical properties of Mn2O3, such as its stability and catalytic action, make it a promising candidate for targeted antibacterial applications, drug delivery, and cancer therapy (24).

Other metal NPs, such as MgO, Fe_3O_4, NiO, Cr_2O_3, CuO, ZnO, Mn_2O_3, MnO_2, and TiO_2, exhibit remarkable properties, including high surface-to-mass ratios and the ability to adsorb drugs and proteins. They have been shown to possess antileishmanial activities against *L. major* and *L. infantum* through various pathways, although the mechanisms underlying some of these effects remain unclear. Overall, their antiparasitic action is associated with the generation of ROS, which can damage key protective structures of the parasites (25,26).

The occurrence of VL is associated with dysfunctional immune responses. Additionally, the immune system plays a synergistic role in VL treatment, enhancing the effectiveness of the drug. Moreover, the use of immunomodulators in combination with antileishmanial drugs has yielded promising therapeutic outcomes (9,27). In this study, mice treated with Mn_2O_3 NPs combined with ShCE showed significantly lower parasite burdens and better survival rates, supporting the immunomodulatory role of ShCE. The mechanisms of action of shark cartilage include anticancer effects such as directly targeting tumor cells and stimulating a Th1-mediated immune response. Furthermore, shark cartilage functions as a scavenger of reactive oxygen species, disrupts cell adhesion, and inhibits calcium channels (9,11).

This study has notable limitations, including the lack of investigation into *in vivo* toxic effects, such as DNA damage and oxidative stress. Furthermore, it did not assess different routes of administration or systemic toxicity using biochemical assays and histopathological analysis.

Conclusion

Our study demonstrated the synergistic effect of the Mn_2O_3-ShCE combination in inhibiting the growth of *L. infantum*. The findings also suggest that prophylactic use of Mn2O3-ShCE could prevent the development of *Leishmania* infection in a murine model.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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