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Original Article

Fe₃O₄@Bio-MOF Nanoparticles Combined with Artemisinin, Glucantime®, or Shark Cartilage Extract on Iranian Strain of *Leishmania major* (MRHO/IR/75/ER): An In-Vitro and In-Vivo Study

Fatemeh GHAFARIFAR¹, *Soheila MOLAIE², Reza ABAZARI³, Zoheir-Mohammad HASSAN⁴, Masoud FOROUTAN⁵

1. Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
2. Arthropod Borne Diseases Research Center, Ardabil University of Medical Sciences, Ardabil, Iran
3. Department of Biochemistry, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
4. Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
5. Faculty of Medical Sciences, Abadan University of Medical Sciences, Abadan, Iran

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***Correspondence**

Email:

so.molaei@arums.ac.ir

Abstract

Background: In the present study, we examined the effects of Fe₃O₄@bio-MOF nanoparticle (Nano-FO) plus artemisinin (Art) and glucantime (Glu) or shark cartilage extract (ShCE) on *Leishmania major* in vitro and in vivo.

Methods: This experimental study was conducted at the laboratory of Department of Parasitology, Tarbiat Modares University, Tehran, Iran during 2016-2017. The promastigote and amastigote assays were performed were conducted at the presence of 3.12- 400 µg/mL of the drug combinations. According to in vitro IC₅₀ results, the combinations of 12.5µg/mL Nano-FO with 25 µg/mL Art as well as 200 µg/mL Glu and 0.5 mL of 20 mg/kg of ShCE were used to treat BALB/c mice. During and at the end of the treatment, the lesion sizes were measured. Parasite loads, cytokine levels were evaluated at the end of the treatment.

Results: The IC₅₀ of Fe₃O₄@bio-MOF-Artemisinin (Nano-FO/Art), Fe₃O₄@bio-MOF-Glucantime (Nano-FO/Glu), and Fe₃O₄@bio-MOF-Shark cartilage extract (Nano-FO/ShCE) on promastigotes were 12.58±0.12, 235±0.17, and 18.54±0.15, respectively. These results on amastigotes were 10.32±0.01, 187±0.03, and 338±0.07 µg/mL, respectively. The apoptosis percentage of these combinations were 32.54%, 20.59%, and 15.68% in promastigotes and 15.68%, 12.84%, and 3.51% in infected macrophages, respectively with no toxicity on uninfected macrophages. In vivo results showed that the size of lesions significantly decreased against all drugs combinations, but Nano-FO/Art combination with Selectivity Index of 23.62 value was safe, and more effective on healing of lesions than other drugs combinations (P=0.003).

Conclusion: This study suggested that Nano-FO/Art combination can be considered as an anti-leishmania combination therapy in CL induced by *L. major*.



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Introduction

Leishmaniasis is a neglected tropical disease caused by the *Leishmania* parasite including 20 genus. The most common form of leishmaniasis, which is a major public health problem in endemic areas, is cutaneous leishmaniasis (CL). CL appears on the exposed parts of body as papules or nodules, then develops to ulcerative lesions, leaving life-long scars and serious disabilities (1). The disease is endemic in 98 countries in the world (2) with new cases between 600,000-1000,000 worldwide annually (3). The situational study of CL in Iran showed that CL was distributed sporadically among all provinces, but it is endemic in some province in central, east and southwest of Iran (4).

The consumption of glu and pentostam as the first line of leishmaniasis treatment is accompanied with different systemic side effects, high toxicity, and treatment failure. So, it is necessary to find safer therapy with different delivery systems and action mechanisms (5-7). Nanoparticles (NPs) with their unique properties such as small size, large surface area, cost-effectiveness, and low toxicity can penetrate easily into macrophages, which the target cell in leishmaniasis (8-11). Additionally, high magnetic saturation, and ability in surface variation by various polymers have caused magnetite nano-ferro (Fe₃O₄) nanoparticles to be used widely (12). Our previous study showed the anti-leishmanial effects of Fe₃O₄@bio-MOF nano structure on *L. major* as well as other studies (10, 13-15).

Based on the hypothesis "Sharks Don't Get Cancer" in 1992 claimed by I. William Lane, Shark cartilage became a feeding supplement in cancer prophylaxis, an angiogenesis inhibitor for cancer treatment, a joint lubricant in arthritis, and also as an immunomodulation. These properties depend on its active chemical constituents, and its molecular weight (16). A fraction composed of two polypeptides with molecular weights of about 14 and 15

kDa from shark cartilage can induce Th1-type cytokines by modulating the ratio of CD4⁺ and CD8⁺ cells (17). We found positive effects of ShCE on murine visceral leishmaniasis (15, 18), Art alone (Data not published) and Nano-FO alone (10) on *L. major*.

We evaluated the anti-leishmanial activity of Nano-FO combined with Art, Glu, and ShCE on *L. major* in vitro and in vivo.

Materials and Methods

Synthesis of Nano-FO

The synthesis of Nano-FO was performed by sonochemically method, which described in our previous study (10). Briefly, sonication time, nano sizes, and morphology of the MBMOF nanocomposites were studied and optimised. Then, several methods including powder X-ray diffraction (PXRD), field emission scanning electron microscope (FE-SEM), fourier transform infrared spectroscopy (FTIR) were used to characterize the morphology, size, and crystalline structure of the magnetic nanoparticle. At the end, the crystal and the structure of the nanoparticles were evaluated by transmission electron microscopy (TEM), vibrating sample magnetometer (VSM), energy dispersive analysis of X-ray (EDAX), thermogravimetric analysis (TGA), and Brunauer-Emmet-Teller (BET).

Preparation of the drug

Fe₃O₄@bio-MOF-Artemisinin Combination (Nano-FO/Art)

Various concentrations of Nano-FO or Art were used to prepare the drugs combinations (3.12- 400 µg/mL) in in vitro (12, 19). For in vivo experiments, mice were administered with 25 µg/mL oral Art daily (15, 19). At the same time, 12.5 µg/mL of Nano-FO were used on lesions in ointment form according to in vitro IC₅₀ results (10).

Fe3O4@bio-MOF –Glucantime Combination (Nano-FO/Glu)

The same method was used for the preparation of Nano-FO/Glu combination within the range of 3.12- 400 µg/mL as described in the previous section. For in vivo experiments, mice were injected intralesionally (*I.L.*) of Glu with 20 mg of active substance/kg/d (18).

Fe3O4@bio-MOF- Shark cartilage extract combination (Nano-FO/ShCE)

The ShCE was prepared, that was described in our previous studies (10, 15, 20, 21). In in vitro, 400 µg/mL ShCE and 3.12-400 µg/mL of Nano-FO were used. 20 mg/kg body weight of ShCE was administered orally in mice daily with 12.5 µg/mL ointment on the lesions (22).

Parasite Culture

Iranian strain of *L. major* (MRHO/IR/76/ER) was cultured in RPMI1640 medium which enriched with 12% (v/v) fetal bovin serum (FBS) and 100 units/mL penicillin or 100 µg/mL streptomycin, and incubated at 24 °C.

In vitro Experiments

Evaluation of the drug combinations on promastigotes and MTT assay

The promastigotes of *L. major* at logarithmic phase were used for evaluation of drugs inhibition on promastigotes after 24, 48, and 72 h and MTT after 72 h incubation (10, 15). After the time points, IC₅₀ of the drugs were determined by promastigote counting. The optical density of formazan crystal was read at 570 nm.

The effects of the drugs combinations on macrophages

J774 A1 macrophages (CGBR80052901) were cultured in RPMI1640 medium supplemented with 10% FBS, 100 IU/mL of penicillin G, 100 µg/mL of streptomycin, 25 mM HEPES buffer, 2 mM L-glutamine. The effect

of drug combinations on uninfected and infected macrophages was performed as mentioned already (12, 19).

Flow cytometry analysis

Necrotic and apoptotic cells were identified using Annexin-V FLUOS Staining Kit (Bioscience, USA) according to the manufacturer protocol at stationary-phase of promastigotes after 72 h against designated concentrations.

In the in vitro experiments, we used Amphotericin B at 10µg/mL as positive control.

In vivo experiments

Anti-leishmanial effects of the drugs combinations in BALB/c mice

Overall 40 Female BALB/c mice (mean weight:18–22 g, age: 6–9 wk) were purchased from Pasteur Institute of Iran and maintained in a standard. The mice were randomly divided into five groups (8 mice/group) including Nano-FO/Art, Nano-FO/Glu, Nano-FO/ShCE, Ethanol (drug solvent), and control (infected-untreated). 2×10^5 parasites were injected subcutaneously into mice tails in the test groups. After the lesions emerged (visible after 14 d), the treatment was performed for three weeks and followed up for six weeks. The mean diameters of lesions were measured by Vernier Caliper. Seven weeks after inoculation, five mice in each group were sacrificed to determine the parasite burden (23, 24).

Evaluation of cytokines

Seven weeks after inoculation, IFN-γ and IL-4 levels were measured in spleen-extracted lymphocytes, which stimulated by Soluble *Leishmania* Antigens (SLA) of *L. major* promastigotes at stationary-phase, according to the manufacturer's protocol (10, 19, 24).

Ethical approval

All applicable international, national and institutional guidelines for the care and use of animals were followed and approved by Medical Ethics Committee of Tarbiat Modares University (No: 52D/3593/TUMS).

Results

The effect of the drugs combinations on promastigotes

The proliferation of promastigotes was reduced at all time points in presence of all concentrations of Nano-FO/Art in a dose- and time-dependent manner ($P < 0.05$). The only exception was at 3.12 $\mu\text{g}/\text{mL}$ after 24 h.

Nano-FO/Glu at 50-400 $\mu\text{g}/\text{mL}$ reduced the number of promastigotes after 24 h. A significant reduction was observed for all combinations of Nano-FO/Glu after 48 and 72 h ($P < 0.05$) (Fig.1 a,b,c). The IC_{50} of Nano-FO/Art, Nano-FO/Glu, and Nano-FO/ShCE on promastigote were 12.58 ± 0.12 , 235 ± 0.17 , and 18.54 ± 0.25 $\mu\text{g}/\text{mL}$, respectively, after 72 h.

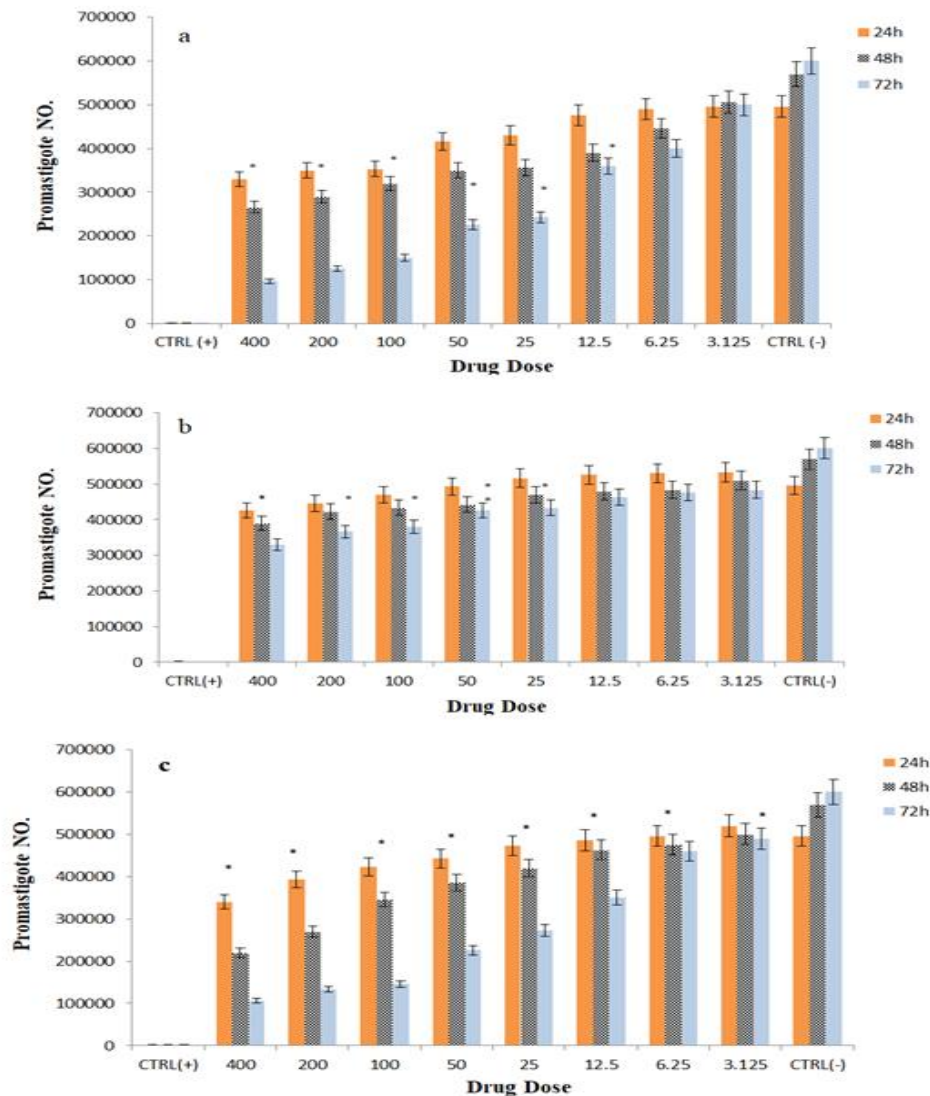


Fig. 1: Mean \pm SD ($n=3$) of the number ($\times 10^4$ / mL) of *L. major* promastigotes after 24, 48 and 72h incubation. CTRL (-): Negative control group (200 μL of 1×10^6 / mL parasite, CTRL (+): Positive control group, a) Nano-FO/Art b) Nano-FO/Glu c) Nano-FO/ShCE. *significant at $P < 0.05$ compared with CTRL (-)

MTT results showed noticeable effects after treatment with all concentrations of drugs.

There was also significant difference between the groups ($P=0.01$) (Fig. 2).

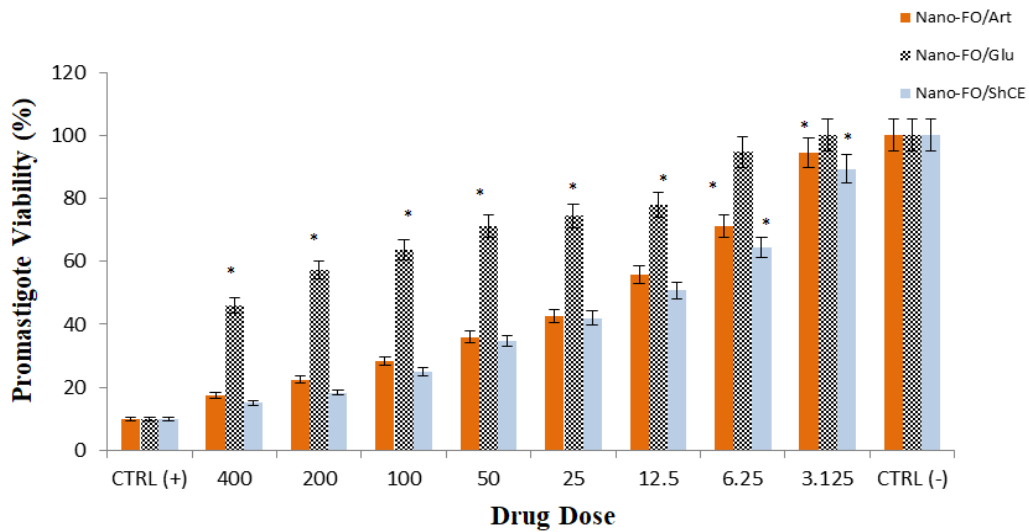


Fig. 2: Cell viability of *L. major* promastigotes after 72 h incubation. CTRL(-): Negative control group (200 μ L of 1×10^6 /mL parasite), CTRL(+): Positive control group, *significant at $P < 0.05$ compared with CTRL (-)

The effect of the drugs combinations on uninfected macrophages

Viability percentage of J774 macrophages showed an inverse correlation of drugs concentrations with macrophage viability in a

dose-dependent manner. These effects were statistically significant compared to the control group ($P < 0.05$) (Fig. 3). The IC50 and CC50 of each drug combination were assessed (Table 1).

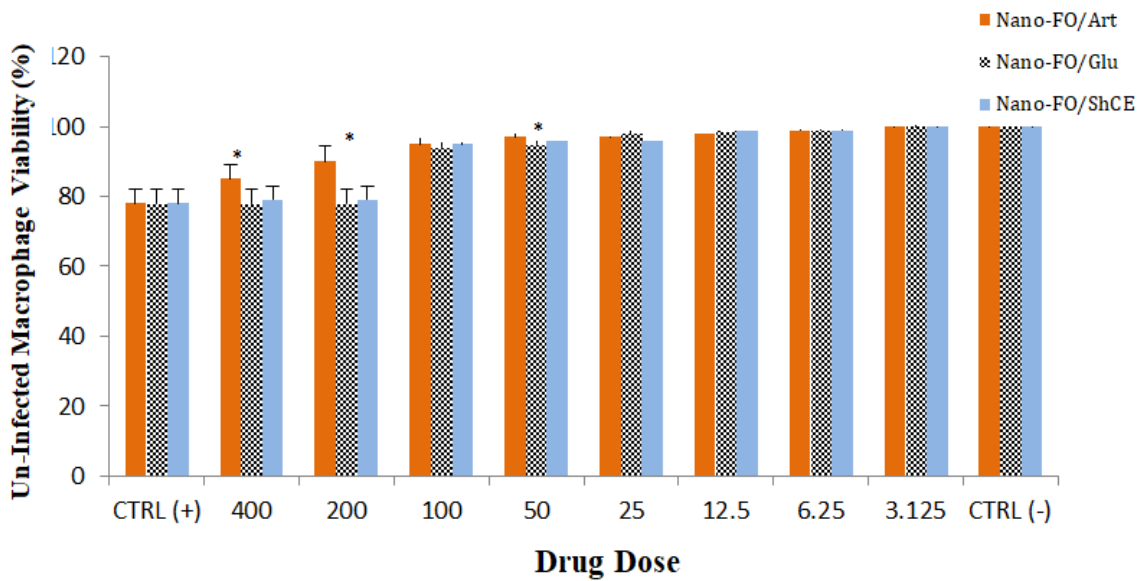


Fig. 3: Viability percentage of J774 macrophages at different concentrations after 72 h incubation. CTRL (-): Negative control group (200 μ L of 1×10^6 /mL parasite), CTRL (+): Positive control group, *significant at $P < 0.05$ compared with CTRL (-)

Table 1: Comparison of the IC50, and CC50 values of drugs combinations on macrophage and SI index

<i>Drug Groups</i>	<i>Amastigote</i>	<i>Promastigote</i>	<i>J774 Macro-phage</i>	<i>SI</i>
	IC50± SD (µg/mL)	IC50± SD (µg/mL)	CC50± SD (µg/mL)	Selectivity Index
Nano-FO/Art	10.32± 0.01	12.58± 0.12	243.84± 0.15	23.62
Nano-FO/Glu	187±0.03	235± 0.17	317.28± 0.21	1.69
Nano-FO/ShCE	338± 0.07	18.54± 0.25	256.54± 0.25	0.75

IC50: 50% Inhibition Concentration of promastigotes and amastigotes; CC50:50% Cytotoxicity Concentration of drugs on macrophages; SI (Selectivity index), the ratio between CC50 on J774 cells and IC50 against *L. major* amastigotes (SI=CC50/IC50≥10 non-toxic).

The effect of the drugs combinations on infected macrophages

Drugs combinations showed reduction of the infected macrophages and also, amastigotes in a dose dependent manner. The results showed significant differences between

the test and the control groups ($P<0.05$). IC50 of Nano-FO/Art, Nano-FO/Glu, and Nano-FO/ShCE for amastigotes were 10.32±0.01, 187±0.03, and 338±0.07µg/mL, respectively (Table 2).

Table 2: The anti-leishmanial effects of various concentrations of drugs combinations on infected macrophages and amastigotes after 72 h

<i>Variable</i>	<i>Nano-FO/Art</i>		<i>Nano-FO/Glu</i>		<i>Nano-FO/ShCE</i>	
	Infected Mφ (%)	No of amastigotes	Infected Mφ (%)	No of amastigotes	Infected Mφ (%)	No of amastigotes
400	36± 2.7	4± 0.8*	34± 2.7	3± 0.8*	40± 2.7	6± 0.8*
200	45± 0.8	6± 0.3	41± 1.2	3± 0.5*	47± 0.8	6± 0.5
100	54± 1.1	7± 0.6*	54± 1.2	4± 0.5*	55± 0.8	6± 0.6*
50	63± 2.6	8± 0.5*	60± 2.6	5± 0.5*	60± 2.1	8± 0.8
25	72± 1.1	9± 0.5	67± 1.7	6± 0.3	69± 1.2	9± 0.9
12.5	74± 1.5	9± 1.1	71± 1.2	7± 1.4	71± 2.1	10± 0.6
6.25	75± 2.6	8± 0.6	71± 1.8	8± 1.3	72± 1.4	11± 0.6
3.125	78± 2.7	9± 0.2	71± 0.8	8± 1.4	74± 1.1	11± 0.4
CTRL (-)	77± 0.5	12± 0.4	77± 1.1	9± 1.3	77± 1	12± 0.6
CTRL (+)	26± 2	3± 0.1	26± 0.9	3± 0.8	26± 2	3± 0.5

CTRL (-): Negative control group (200 µL of infected macrophage cells, CTRL (+): Positive control group, *non-significant at $P > 0.05$ compared with negative control

Flow cytometry assay

The highest rates of promastigote apoptosis was observed in Nano-FO/Art (32.54%) and then in Nano-FO/Glu (20.59%), and Nano-FO/ShCE (15.68%) compared to the control group ($P<0.05$). The apoptosis rate in infected macrophages were 15.68%, 12.84%, and

3.51% following treatments with Nano-FO/Art, Nano-FO/Glu, and Nano-FO/ShCE combination, respectively. There were significant differences in apoptosis and necrosis rates in all drugs groups compared with the control ($P<0.05$) (Fig. 4).

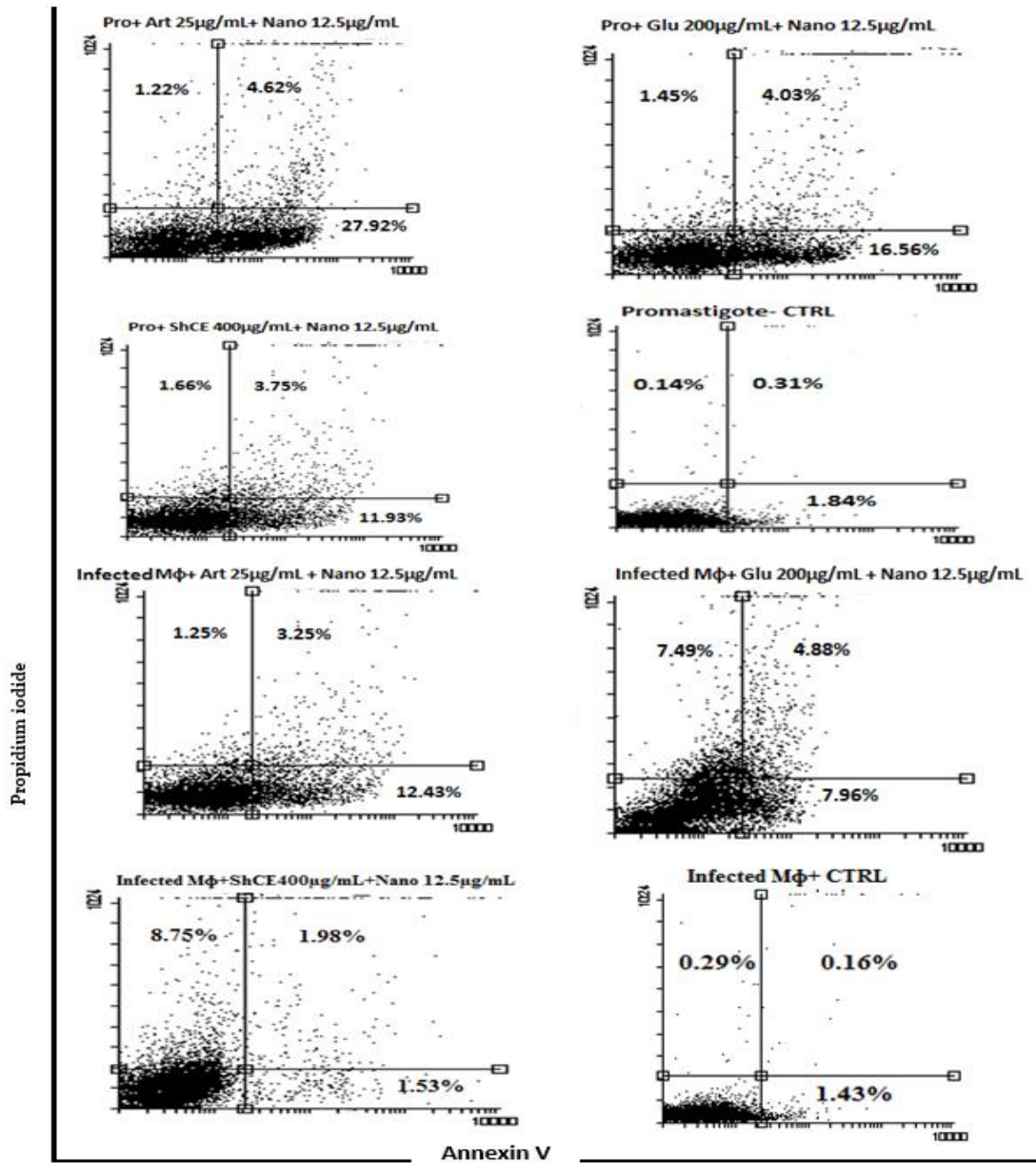


Fig. 4: Flow cytometry data analysis on promastigotes and infected macrophages. Early apoptosis, late apoptosis, and necrosis were showed after 72 h

Anti-leishmanial effects of the drugs combinations in BALB/c

All of the drugs combinations reduced the parasite loads in organs. The reduction rate in spleen were 81%, 76%, 48%, 11% and in liver

77%, 65%, 45%, and 14% for Nano-FO/Art, Nano-FO/Glu, Nano-FO/ShCE, and the control group, respectively. The efficacy of the drugs were higher in the spleen than in the liver ($P<0.05$).

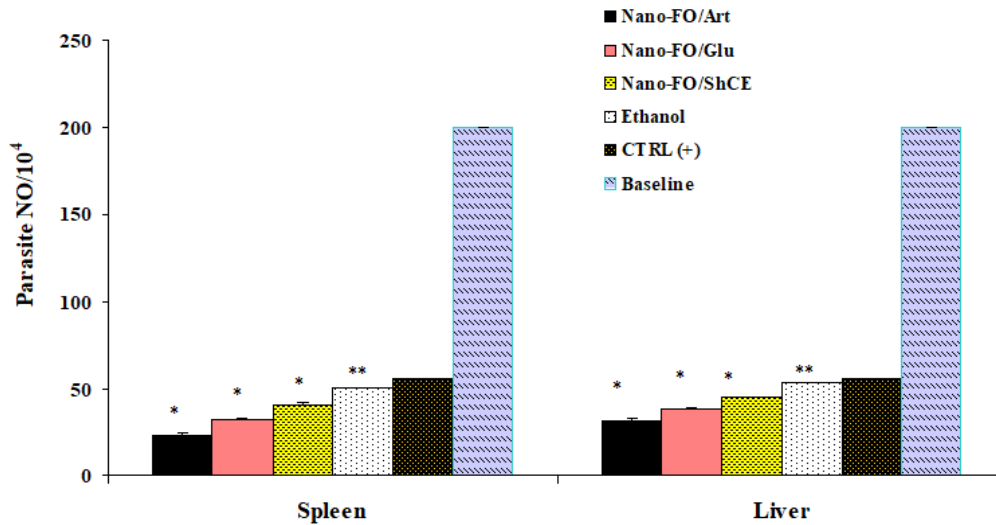


Fig. 5: The parasite number in the spleen and liver of infected mice (n=5 in each group). Baseline: (2×10⁵) parasites were injected subcutaneously into mice tails. *significant at *P*<0.05 compared with control group

The anti-leishmanial effect of the drugs combinants on lesions showed that topical application of vaseline-based nanoparticle ointment simultaneously with the aforementioned drugs caused a significant reduction in

lesion sizes after 4 wk of treatments compared to the control groups receiving ethanol or untreated (Table 3). Effects of the drugs were more significant at the first and last weeks (*P*<0.001).

Table 3: The effects of the drugs concentrations on the lesion sizes (mm) of murine cutaneous leishmaniasis (n=5 in each group)

Group	Before treatment	1-week	2-week	3-week	4-week	5-week	<i>P</i> value
Nano-FO/Art	4.5± 0.2	5.7± 0.5	6.3± 1.1	6.8± 1.2	5.5± 0.19	3.2± 0.15	0.38
Nano-FO/Glu	5.3± 0.57	4.8± 0.51	7.4±0.45	9.3± 0.31	10.3± 0.92	8.3± 1.1	
Nano-FO/ShCE	5± 0.43	4.6± 0.33	6.6± 0.55	9.2± 0.85	7.9± 0.79	7±0.54	0.21
Ethanol	5.1± 0.58	6.1± 0.36	7.6± 0.30	12.3± 0.57	13.3± 0.47	11.6± 1.1	0.05*
CTRL	6.4± 0.69	4.4± 0.47	5.9± 0.51	8.9± 0.55	13.3± 0.35	13.1± 0.28	0.06

Values are represented as mean ± SD; *significant at *P*< 0.05 compared with control group

Evaluation of cytokines

Cytokine assay indicated that the production of IL-4 was higher than INF-γ in positive, negative, and ethanol controls. On the contrary, in the test groups, INF-γ level was higher than IL-4 level (*P*<0.05). The highest

level of INF-γ was observed in Nano-FO/Art and then in Nano-FO/Glu (*P*<0.001). Also, the highest level of IL-4 was observed in Nano-FO/Glu, and the highest level of INF-γ was observed in Nano-FO/Art group (*P*<0.001) (Fig. 6).

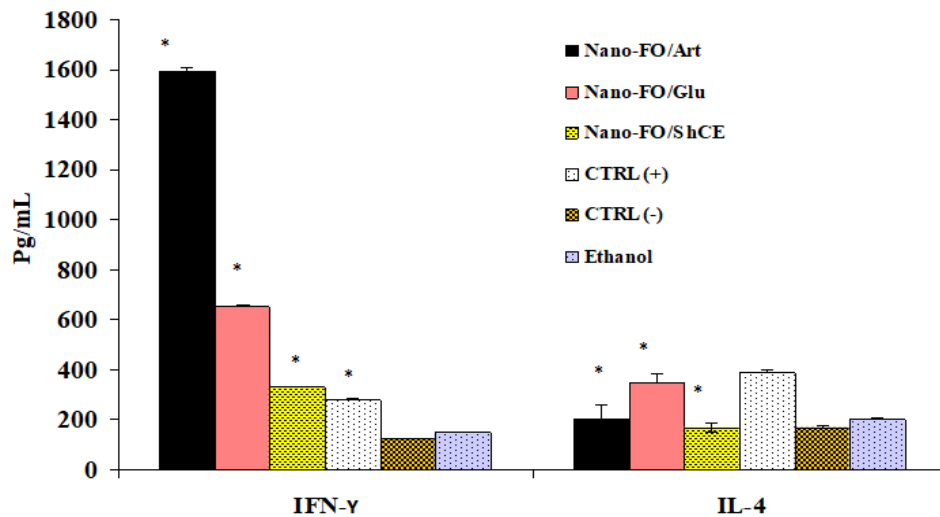


Fig. 6: IFN- γ and IL-4 levels (n=5 in each group) at the end of treatments. Values are represented as mean \pm SD; *significant at $P < 0.05$ compared to control group

Discussion

In the in vitro experiments, promastigote inhibition and MTT tests showed that each combination had toxic effects on *Leishmania* parasites. Promastigote inhibition test demonstrated that after point times, the number of parasites were reduced in a dose- and time-dependent manner in the presence of all drugs combinants as well as MTT results. Nano-FO/ShCE were more effective than Nano-FO/Glu or Nano-FO/Art on promastigotes as well as Art only (14, 25-26).

In amastigote assay, not only the number of infected macrophages, but also the number of intracellular amastigotes decreased in a dose-dependent manner after 72 h. In addition, Nano-FO/Glu was more effective than other combinations in reducing the intramacrophage amastigotes. In contrast, promastigotes showed less sensitivity to Nano-FO/Glu. These differences are related to both structural, and biochemical properties of parasite and Glu.

Several studies showed promising anti-leishmanial effects of NPs on *L. major*. Recently, Tavakoli et al. reported the cytotoxic effects of Mn₂O₃ NPs on *L. major* promastigotes and amastigotes (27). In another

study the anti-promastigote effects of biogenic antimony sulfide nanoparticles at 62.5 μ g/mL has been shown (5). Nano-FO has been found to have high anti-leishmanial effects in vitro and in leishmaniasis animal models (13, 28).

Similar to our previous studies, ShCE showed positive effects on promastigotes not amastigotes against drugs combinations. The possible mechanism of action of shark cartilage is obvious. The therapeutic effect of ShCE is related to low-molecular-weight proteins which we used in this study (10-20 kDa) (29).

The results of flowcytometry analysis showed the occurrence of early and late apoptosis in promastigotes and infected macrophages following treatment with the drugs combinations. The high rate of early or late apoptosis in promastigotes was observed in Nano-FO/ShCE combination, but in infected macrophages, Nano-FO/Glu showed a higher apoptosis rate than other combinations. Phosphatidylserine (PS) play a main role in apoptosis. In live cells, PS is located in the inner side of cell membrane. During apoptosis, PS is translocated to the external surface of cell membrane and thus is exposed to annexin which has a high affinity to PS (30). Apoptosis is the main form of cell death in *Leishmania*

parasites (31) in response to diverse agents including chemotherapeutic effectors, and oxidants like H₂O₂(9). In infected macrophages, intracellular *Leishmania* parasites can inhibit apoptosis. Nano-FO/Art/Glu/ShCE may prevent these effects by inducing apoptosis in the infected macrophages and promastigotes, and thus, eliminate the intracellular parasites. Art alone (32) and Nano-FO alone had significant leishmaniacidal effects as well as other nanoparticles (14, 33).

The high concentration of the drugs combinations showed significant toxicity on uninfected macrophages, especially in Nano-FO/Glu combination. High viability of uninfected macrophages was observed in the presence of Nano-FO/art compared to other combinations. The cytotoxicity of Mn₂O₃ NPs on *L. major* has been reported at IC₅₀ value of 40 µg/ mL (28).

Nano-FO at ointment administration combined with artemisinin or ShCE orally in BALB/c mice showed the reduction in lesion sizes following treatment with studied drugs in the early stages, and third weeks post-treatment as well as drugs only (34). Ag-NPs could reduce the size of the lesions at a single dose of 40 ppm (5). Jebali and Kazemi also reported similar effects of several nanoparticles on *L. major* with cytotoxic effects on macrophage cells (35).

The results of cytokine assay showed increased of IFN-γ and decreased of IL-4 level especially in Nano-FO/Art. Other studies have shown the same results following treatment of mice with artemisinin (19, 36). The main cells involved in *Leishmania* infection are macrophages, which are activated by IFN-γ as Th1-producing cytokines in animal models, and play the key role in the control (IFN-γ) or expansion (IL-4) of *L. major* infection in BALB/c mice (37, 38). However, the cytokines production depends on drug dose, the route of administration, treatment duration, the number of parasites, and site of inoculation (39).

The very small size of nanoparticles facilitates their penetration into macrophages and activates them towards phagocytosis. Therefore, the results showed that nanoparticles acts against the parasite by mechanisms other than cytokines, among which nanoparticles absorption by macrophages may be responsible (40).

Conclusion

Fe₃O₄@bio-MOF nanoparticles combined with artemisinin can be one of the promising anti-leishmanial alternative therapeutic. We believe this drug combination can be introduced as new therapeutic agent for the treatment of cutaneous leishmaniasis, although more studies are required to prove its leishmaniacidal effects.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) were observed completely by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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