

Artemether-loaded nanostructured lipid carriers: preparation, characterization, and evaluation of *in vitro* effect on *Leishmania major*

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Abstract

Background and purpose: Cutaneous leishmaniasis is a global health problem. The discovery of new and highly efficient anti-leishmanial treatments with lower toxicity is globally needed. The current study was carried out to evaluate the anti-leishmanial effects of artemether (ART) and ART-loaded nanostructured lipid carriers (ART-NLCs) against promastigotes and amastigotes of *Leishmania major*.

Experimental approach: Solvent diffusion evaporation technique was applied to prepare ART-NLCs. These nanoparticles were characterized using a particle size analyzer (PSA), transmission electron microscopy (TEM), and dynamic light scattering (DLS). The antiparasitic activity on amastigote was assessed in J774 cell culture. The drug cytotoxicity on promastigote and macrophage was assessed using the MTT technique after 24 and 48 h and compared with NLCs, ART, and amphotericin B, as the control agents. The selectivity index was calculated for the agents.

Findings/Results: The DLS and PSA techniques confirmed that ART-NLCs were homogenous in size with an average diameter of 101 ± 2.0 nm and span index of 0.9. The ART-NLCs significantly heighten the anti-leishmanial activity of ART ($P < 0.001$). The IC_{50} values of ART and ART-NLCs on promastigotes after 24 and 48 h were 76.08, 36.71 and 35.14, 14.81 $\mu\text{g/mL}$, respectively while they were calculated 53.97, 25.43 and 20.13, 11.92 for amastigotes. Also, ART-NLCs had the lowest cytotoxicity against macrophages. Furthermore, among the agents tested, ART-NLCs had the highest selectivity index.

Conclusion and implications: ART-NLCs had lower cytotoxic effects than ART and amphotericin B, also its selectivity index was significantly higher. Based on the findings of the study, this formulation could be a promising candidate for further research into leishmaniasis treatment.

Keywords: Artemether; *L. major*; Leishmaniasis; Nanostructured lipid carriers.

INTRODUCTION

Leishmaniasis is a complex form of the infectious parasitic disease caused by intracellular protozoa of the *Leishmania* genus (1,2). *Leishmania* is a vector-borne parasite that is transmitted to the vertebrate host by the bite of an infected female phlebotomus flies (3). According to World Health Organization (WHO) assessment, leishmaniasis is reported

from 95 countries, nearly 12 million people are affected and also 350 million people are at high risk for this disease (4,5). Leishmaniasis is considered a neglected tropical disease with an annual report of 1.3 million new cases and 20,000-30,000 deaths (6).

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Leishmaniasis has a heteroxenic life cycle between humans and other definitive hosts and sand flies. Clinical manifestations are categorized into four major forms such as cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis, visceral leishmaniasis, and diffuse cutaneous leishmaniasis (7). Cutaneous leishmaniasis is the most common form of leishmaniasis in the world. In the old world, Iran is one of the most prevalent countries for cutaneous leishmaniasis caused by *L. major* and *L. tropica* (1).

The pentavalent antimonials such as sodium stibogluconate, pentostam or meglumine antimoniate, and glucantime have been considered as first-line of therapy, and amphotericin B (AMB), pentamidines, or paromomycin have been considered as the second-line of the treatment of leishmaniasis (8). Severe toxic adverse effects, high cost, long-term treatment with high doses, complex therapeutic scheme, and increased chemoresistance are some drawbacks that are associated with the conventional treatment recommended for leishmaniasis (9). Therefore, the discovery of new and highly efficient anti-leishmanial treatments with lower toxicity and costs is a global necessity (9,10). Nowadays, natural products have significant effects in the treatment of infectious diseases and are considered excellent foundations for novel drugs development (11). *Artemisia annua* (*A. annua*) is a plant that has medicinal properties (12). Artemisinin, a sesquiterpene lactone, is an active compound found in *A. annua* extracts (13). According to numerous published reports, artemether (ART), a methyl ether derivative of artemisinin, had an important antiparasitic effect *in vivo* and *in vitro* studies against *Plasmodium falciparum* and *Schistosoma japonicum*, and attracted particular attention for evaluating its antiparasitic effects on other parasites (14,15). The low water solubility of ART is a limiting factor in developing efficient formulation for clinical application. In this regard considering novel drug delivery systems such as nanostructured lipid carriers (NLCs) will resolve this limitation.

NLCs are the second generation of lipid nanoparticles that are formulated from a mixture of solid and liquid lipids. NLCs have so

many benefits such as zeta potential stability, long-term particle size stability, lower drug expulsion, high loading capacity, higher potential of skin penetration and drug deposition, construction of film on the skin surface, prevention of systemic adverse effects, and skin hydration (16). According to electron microscopic imaging, NLCs have interstitial holes and imperfections in their configuration that could induce a higher drug payload in comparison to solid lipid nanoparticles (SLNs) as the first generation of lipid nanoparticles (17).

Currently, the conveyance of drugs into the target cells with limited side effects on the other surrounding cells has been developed using nanoemulsions, niosomes, liposomes (18), and lipid nanoparticles (19-23), hydrogels (24) as drug delivery systems. Considering the importance of leishmaniasis in the world and the lack of cost-effective, affordable, and efficient therapies, this study aimed to use ART-loaded NLCs (ART-NLCs) to treat cutaneous leishmaniasis.

MATERIALS AND METHODS

Materials

Acetone, acetonitrile, and ethanol were purchased from Merck (domestic supplier in Iran); stearic acid and cholesterol were from Merck, Germany; Brij 35, Brij 72, and triolein were purchased from the domestic supplier of Sigma-Aldrich (St. Louis, Missouri, USA); dimethyl sulfoxide (DMSO) was from Sentmenat, Spain; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), RPMI-1640 medium (with L-glutamine and HEPES) and fetal bovine serum (FBS) were prepared from Sigma-Aldrich (St. Louis, Missouri, USA); AMB deoxycholate (conventional) and ART (Santa Cruz, USA), penicillin and streptomycin (Gibco, USA).

Quantitative analysis

All tests were undertaken in triplicate and the drug analysis was performed using high-performance liquid chromatography (HPLC, Agilent Technologies 1260 Infinity, USA) equipped with a UV detector. Acetonitrile:water mixture (75:25 ratio) was

used as mobile phase with a flow rate of 1 mL/min and method validation was performed for ART. R square in this work was calculated as 0.9999 that offers satisfactory linearity. Additionally, the limit of quantification (LOQ) was 2.5 µg/mL and the limit of detection (LOD) was 0.8 µg/mL. Accuracy was between 90-110% and specificity of this validated method for ART was assessed and drug retention time was 7 min.

ART-NLCs preparation

Solvent diffusion evaporation technique was considered to prepare lipid nanoparticles. In this technique, triolein (13.3 mg), cholesterol (6.6 mg), stearic acid (46.6 mg) (10:20:70 w/w ratios), and ART (3.3 mg) were dissolved in acetone as an organic phase. Brij 35 (62.3 mg) and Brij 72 (62.3 mg) (1:1 w/w ratio) as surfactants were mixed with distilled water (aqueous phase) and heated up to 70 °C, then acetone (10 mL) solution was added to the aqueous phase in water (20 mL) with the ratio of 1:2 at 1400 rpm mixing rate and during 15 s to achieve 100 nm of mean particle size. According to the Ghasemiyeh *et al.* protocol, heating was continued till the total volume of the mixture reached 33% of its primary volume, then, the solution was promptly cooled in an ice bath (< 5 °C) to solidify lipid nanoparticles (19,20). The particle size and size distribution of the obtained nanoparticles were freshly analyzed by particle size analyzer (PSA; SHIMADZU, SALD-2101, Japan) and rechecked in the predetermined interval and zeta potential was measured by Zeta-Chek (Microtract, ZC007, Germany).

ART-NLCs characterization

Particle size and size distribution analysis

The size of the nanoparticle was measured in five-time, exactly after preparation (freshly), after one week, 1, 3, and 6 months of preparation using a PSA. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) were also carried out to confirm the results of PSA. Span index was also measured according to the following equation (20):

$$\text{Span index} = \frac{D_{90}-D_{10}}{D_{50}} \times 100 \quad (1)$$

where, D90, D50, and D10 are 90%, 50%, and 10% undersized diameters, respectively.

ART-NLC stability

The Stability of particle size and drug expulsion were examined for 6 months at the refrigerator (2-8 °C) after preparation. In order to assess the drug expulsion from the lipid nanoparticles, ART was added to the lipid mixture at a level of 10% of total lipid.

Drug loading assessments

ART was added to the organic phase and ART-NLCs were prepared with an average diameter of 100 nm using the solvent diffusion evaporation method. Drug loading tests including entrapment efficiency (EE) and loading capacity (LC) were assessed in two steps. Once on the fresh sample and once a week after sample preparation in order to assess the loading stability during the first week. Five mL of ART-NLC with an average diameter of 100 nm were transferred in the superior cavity of centrifugal filter tubes (MWCO 10KDa, Amicon Ultra-4, Millipore Co., MA, USA) and centrifuged at 4000 rpm for 15 min. The filtrates were inspected using the HPLC technique to evaluate the quantity of the unloaded drug.

All experiments were performed in triplicate. EE% and drug LC% were calculated using equations below:

$$EE\% = \frac{\text{Loaded drug}}{\text{Total drug}} \times 100 \quad (2)$$

$$\text{Drug LC}\% = \frac{\text{Loaded drug}}{\text{Total lipid+loaded drug}} \times 100 \quad (3)$$

where, loaded drug (mg) is the total drug minus unloaded drug, total drug is the amount of drug (mg) that was added to the organic phase, and total lipid (mg) is the mixture of lipids used in the organic phase. The unloaded drug was calculated by filtrate analysis.

TEM

The morphology and size of ART-NLC were explored by TEM (Philips, Leo 906E, Germany, voltage of 80 KV). In this regard, ART-NLC particles were diluted with water (1:5 ratios) and 50 µL of the prepared sample was fixed in copper grids and stained with uranyl acetate (19).

Drug release assessment using in vitro model

Drug release from NLCs was evaluated using the dialysis membrane. ART-NLCs with average diameters of 100 nm were assessed in triplicate and outcomes were compared with the permeation of free drug through the dialysis membrane. The release medium having 200 mL volume consists of a combination of 80% phosphate-buffered saline (PBS, 10 mM, pH equal to 7.4) and 20% ethanol in order to maintain the sink situation. The temperature of the release medium was fixed at 37 °C and the medium was well mixed at 150 rpm during drug release assessment. Ten mL of freshly prepared ART-NLCs (containing 3.3 mg ART) was transferred to the dialysis membrane and was immersed in the release medium. Sampling (500 µL) was done at 0, 0.5, 1, 2, 4, 8, 24, 48, and 72 h and instantly was replaced with an aliquot amount of freshly prepared release medium at 37 °C. Samples were analyzed using the developed validated HPLC technique. ART release kinetics was also assessed (20).

Parasite culture

The promastigote form of *L. major* (MRHO/IR/75/ER) was provided from the Department of Parasitology and Mycology, Shiraz University of Medical Sciences, Shiraz, I.R. Iran. The promastigote was transferred from the liquid nitrogen tank to a cell culture flask containing 1 mL of RPMI-1640 medium supplemented with 10% heat-inactivated FBS, and penicillin-streptomycin (100 IU/mL) at 25 °C. The stationary phase promastigotes were subcultured to produce more parasites (8).

Macrophage culture

Mouse macrophage (J774 strains) cell lines were prepared by the Department of Immunology, Shiraz University of Medical Sciences, I.R. Iran. The cell lines were cultured in RPMI medium with CO₂ (5%) conditions at 37 °C.

In vitro anti-leishmanial effects of NLCs, ART, and ART-NLCs on promastigotes of L. major

Anti-leishmanial effects were evaluated using the MTT colorimetric test, as previously described by Valadares *et al.* (25). Briefly,

100 µL of suspension containing 1×10⁶ parasites/mL were added to the wells of a 96-well plate microplate and 100 µL ART, NLCs, ART-NLCs, and AMB at 2.5, 5, 10, 25, 50, and 100 µg/mL were poured into the well and the plate was incubated at 25 °C for 24 and 48 h. Cell viability was measured by adding 20 µL of MTT (5 mg/mL) and keeping it in a dark place for 4 h. Then, DMSO was added and after 20 min absorbance was measured using an ELISA plate reader (Biotek, Spain) at 570 nm. All tests were performed in triplicate. As the negative and positive controls, respectively, untreated wells and AMB were used.

The cytotoxicity of the NLCs, ART and ART-NLCs on J774 cell line

J774 cell lines (2 × 10⁴ cells) were seeded in 96-well plates and incubated at 37 °C for 24 h. In order to remove the unattached cells, the supernatants were removed and replaced with fresh RPMI 1640 medium containing 100 µL of 2.5, 5, 10, 25, 50 and 100 µg/mL of ART, NLCs, ART-NLCs, and AMB and then incubated at 37 °C with 5% CO₂ for 24 and 48 h. Untreated cells were chosen as the negative control. The cell viability and cell cytotoxicity were determined by MTT colorimetric test (26) and was calculated using the equations below:

$$\text{Cell viability (\%)} = \frac{OD_{\text{Test}} - OD_{\text{blank}}}{OD_{\text{Control}} - OD_{\text{blank}}} \times 100 \quad (4)$$

$$\text{Cytotoxicity (\%)} = 100 - \text{viability (\%)} \quad (5)$$

In order to calculate the concentration that caused 50% mortality in J774 macrophages (CC₅₀), 6 concentrations from 2.5 µg/mL to 100 µg/mL of ART, NLCs, ART-NLCs, and AMB were applied and their viability was assessed using MTT method and the CalcuSynv2demo analytical program. The selectivity index (SI) was considered by dividing CC₅₀ to IC₅₀. All experiments were carried out in triplicate.

In vitro anti-leishmanial effects of NLCs, ART, and ART-NLCs on intra-macrophagic amastigotes of L. major

The J774 cells (1 × 10⁶ cells/mL) in supplemented RPMI 1640 with 10% inactivated FBS, 200 U/mL penicillin/streptomycin were plated in 24-well culture plates (200 µL/well) and incubated at 37 °C and 5% of CO₂ for 24 h.

After incubation times, the cultures were washed to remove the unattached cells, promastigotes (2×10^6 cells/well) in stationary phase with a fresh RPMI 1640 medium was added and incubated at 37 °C for 24 h. The plate was washed to remove the non-internalized parasites and then 200 μ L different concentrations of NLCs, ART, ART-NLCs, and AMB were added to the infected culture and incubated at 37 °C for 24 and 48 h. The number of intracellular amastigotes and the number of infected macrophages were measured using a 1000 \times optical microscope after slide fixing by methanol and Giemsa staining (27). As a negative control, untreated infected macrophages were used.

Statistical analysis

The three independent experiments' numerical data were reported as mean \pm standard deviation (SD). Significant differences between the groups were statistically analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test using GraphPad Prism 8 software. *P*-values less than 0.05 were considered significant.

RESULTS

ART-loaded NLCs characterization

Particle size and size distribution analysis

The particle size of ART-NLCs was measured by DLS and PSA analysis. The results of DLS and PSA revealed that ART-NLCs were homogenous in size with an average diameter of approximately 101 ± 2.0 nm and span index of 0.9 (Fig. 1A and B). The final NLCs formulation was contained 5% ART.

ART-NLCs entrapment efficiency and loading capacity

ART-NLCs EE and LC were calculated by centrifugation ultrafiltration technique and measured as $89.57 \pm 0.66\%$ and $4.25 \pm 0.03\%$, respectively. After 7 days, the re-examination results revealed that the EE and LC of ART-NLCs were $88.73 \pm 0.6\%$ and $4.21 \pm 0.03\%$, respectively.

These results showed that ART-NLCs had acceptable stability during one week since the amount of loaded drug didn't change significantly during this period of storage as depicted in (Fig. 2).



Fig. 1. (A) Dynamic light scattering and (B) particle size analyzer graphs of artemether-loaded nanostructured lipid carriers with average diameter of 103 nm.

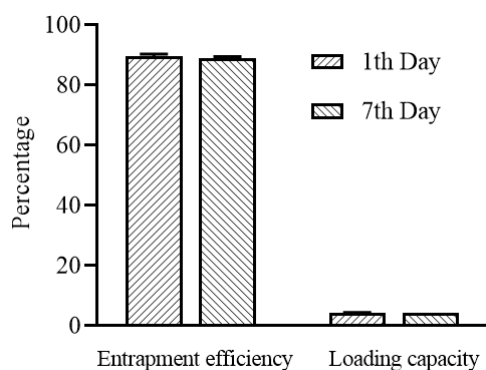


Fig. 2. The comparison of the artemether-loaded nanostructured lipid carriers entrapment efficiency and loading capacity between 1 and 7 days after production. Data are presented as mean \pm SD, n = 3.

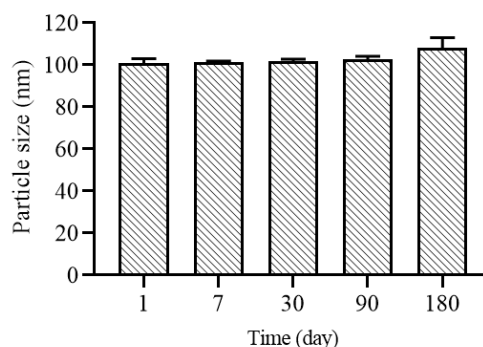


Fig. 3. Evaluation of the stability of artemether-loaded nanostructured lipid carriers during 6 months after productions. Data are presented as mean \pm SD, n = 3.

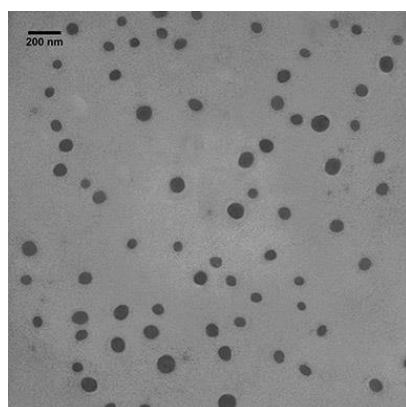


Fig. 4. Transmission electron microscopy of artemether-loaded nanostructured lipid carriers with mean diameter of 100 nm.

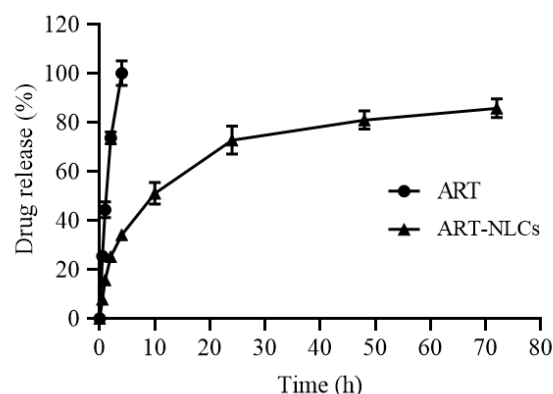


Fig. 5. The release pattern of the free ART and ART-NLCs through the dialysis membrane. Data are presented as mean \pm SD, n = 3. ART-NLCs, Artemether-loaded nanostructured lipid carriers.

Stability of the ART-NLCs

The stability of the ART-NLCs with an average diameter of 101 nm was examined after 1, 7, 30, 90, and 180 days of nanoparticles preparation. Our results indicated no significant changes in NLCs particle size after 3 months of storage, and after six months the enhancement in particle size was less than 8% (Fig. 3).

Zeta potential for ART-NLCs

The zeta potential of lipid nanoparticles was -33 ± 2 mV. The negative value of zeta potential is in agreement with other reports from lipid nanoparticles. Zeta potential with a negative charge could cause an electrical repulsive force between lipid nanoparticles, which in turn increases the physical stability of the nanoparticles during the storage periods and prevents the particles from merging with each other.

TEM

The morphological evaluation of NLCs through TEM image showed that these nanoparticles are completely spherical and uniform in shape. Also, as shown in (Fig. 4), the size of the obtained nanoparticles was completely in agreement with the PSA method.

In vitro drug release

Assessment of the drug release kinetic indicated that release from NLCs was significantly slower than drug permeation through the dialysis membrane. According to (Fig. 5), 100% of the free ART was passed through the dialysis membrane within 4 h, while 85.7% of the ART-NLCs was released within 72 h, indicating that these lipid-based nanoparticles are efficiently capable of promoting sustained drug release pattern in

comparison with free drug. According to (Table 1), drug release kinetics from NLCs was best fitted to the Higuchi model, which is a suitable model for matrix-based delivery systems, with the highest R square value.

The effect of ART and ART-NLC on the viability of the promastigote, amastigote, and J774 cells

The viability of promastigote, macrophage, and a load of the parasite (amastigote) in macrophage were assessed. The IC₅₀ values of ART, ART-NLCs, and AMB were calculated

on promastigotes after 24 and 48 h as 76.08, 35.14, 9.5 µg/mL and 36.71, 14.81, and 4.41 µg/mL, respectively. The results of 2-way ANOVA revealed that the differences between ART and ART-NLCs in all concentrations were statistically significant ($P < 0.001$). The results revealed that ART-NLCs had more anti-leishmanial effects in comparison to the ART alone and NLCs ($P < 0.05$). As showed in (Fig. 6), ART-NLCs significantly reduced the load of amastigotes in macrophage and was more effective than ART and also NLCs alone ($P < 0.05$, Fig. 6).

Table 1. Artemether release kinetics from nanostructured lipid carriers with an average diameter of 100 nm.

Kinetic Model	Zero-order	First-order	Korsmeyer-Peppas	Higuchi	Hixon-Crowell	Square root of mass	Three-second root of mass
R Square	0.7508	0.9031	0.6246	0.9234	0.8564	0.8311	0.8047

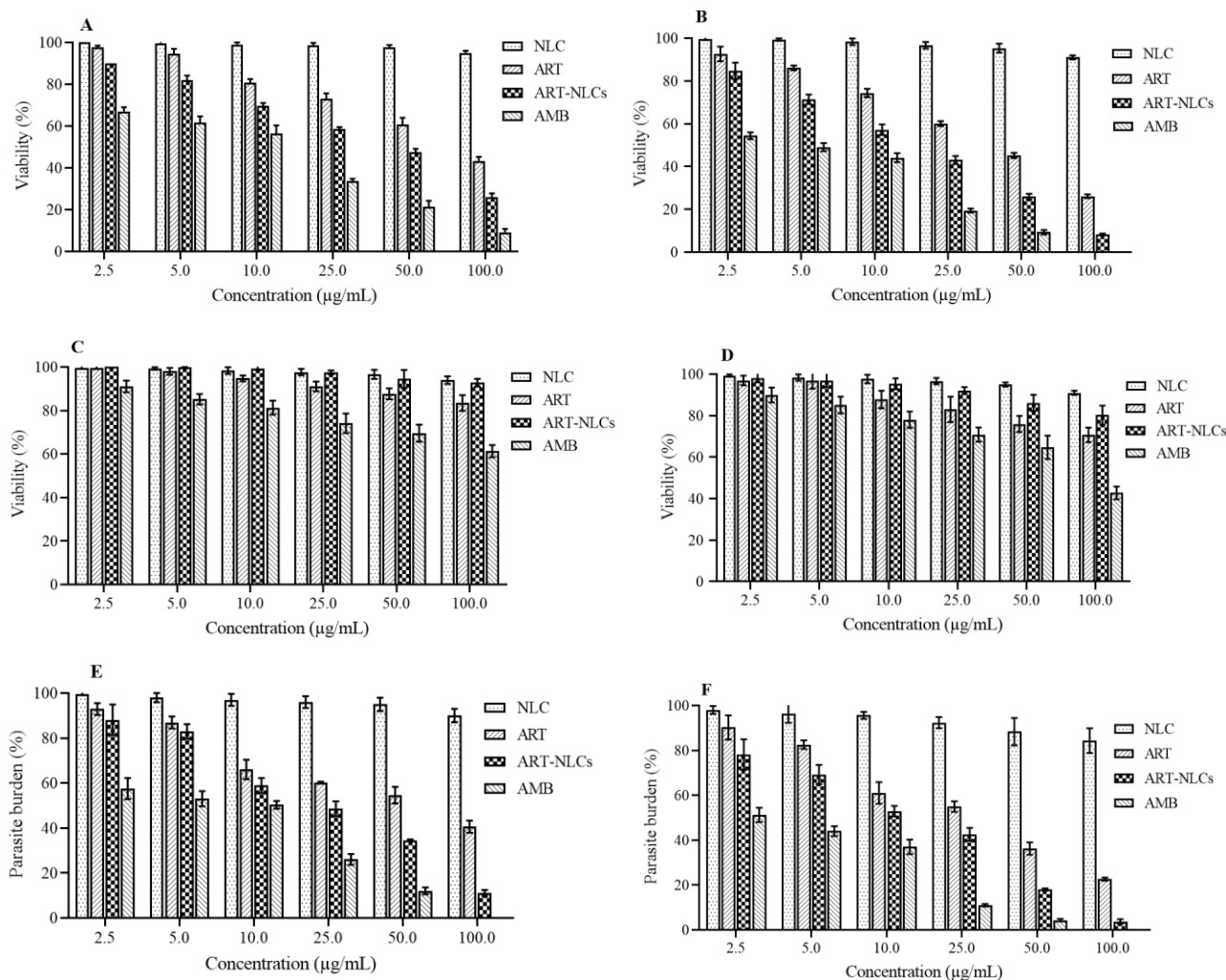


Fig. 6. The effects of NLCs, ART, ART-NLCs and AMB (A and B) on the viability of promastigote after 24 and 48 h, (C and D) the viability of macrophage after 24 and 48 h, and (E and F) a load of the parasite (amastigote) in macrophage after 24 and 48 h *in vitro* examinations. Data are presented as mean ± SD, n = 3. AMB, Amphotericin B; ART-NLCs, artemether-loaded nanostructured lipid carriers.

Table 2. IC₅₀ values for promastigote and amastigote, CC₅₀ and SI indexes of NLCs, ART, ART-NLCs, and AMB after 24 and 48 h. Data are presented as mean ± SD, n = 3.

IC ₅₀ /CC ₅₀ /SI	Time (h)	NLCs	ART	ART-NLCs	AMB
Promastigote IC ₅₀ (µg/mL)	24	2256 ± 146	76.08 ± 1.4	35.14 ± 0.3	9.5 ± 0.1
	48	1661 ± 112	36.71 ± 0.12	14.81 ± 0.1	4.41 ± 0.1
Amastigote IC ₅₀ (µg/mL)	24	2980 ± 132	53.97 ± 3.2	20.13 ± 0.3	5.7 ± 0.1
	48	1797 ± 95	25.43 ± 0.4	11.92 ± 0.18	3.41 ± 0.1
CC ₅₀ (µg/mL)	24	5303 ± 450	1178 ± 69	1969 ± 140	279.2 ± 11
	48	3121 ± 240	373.4 ± 14	708.3 ± 34	86.9 ± 5.6
Promastigote SI	24	2.35	15.48	56.03	29.39
	48	1.88	10.17	47.83	19.70
Amastigote SI	24	1.78	21.82	97.81	48.98
	48	1.73	14.68	59.42	25.48

AMB, Amphotericin B; ART-NLCs, artemether-loaded nanostructured lipid carriers, SI, selectivity index.

The IC₅₀, CC₅₀, and SI of promastigote and amastigote were presented in (Table 2). According to the results, the IC₅₀ values of ART-NLCs were significantly less than ART against both promastigote and amastigote after 24 and 48 h ($P < 0.05$).

DISCUSSION

Leishmaniasis is one of the most important parasitic diseases in tropical and subtropical areas. There are many approved treatments for leishmaniasis most of which show inevitable side effects that limited their use (8). Drug resistance is a new phenomenon that has been developed in the current two decades. So, the discovery of innovative treatments with a new mechanism of action is crucial. ART is a derivative of artemisinin extracted from *A. annua* that has a long history of clinical use (28). During recent years, there has been much investigational evidence, both *in vitro* and *in vivo*, indicating that *A. annua*, artemisinin, and its derivatives have noticeable antimicrobial activities. In a study conducted by Hensbroek *et al.* ART is used against *Plasmodium falciparum* in children with cerebral malaria and the results indicated that ART at the dose of 20 mg/kg treated 3.34% (7/209) of patients with cerebral malaria indicating that these results were equal to quinine (29). In recent years, ART showed acceptable antimicrobial effects against *Leishmania* and other parasites with low toxicity. Based on the results of these studies, ART could be an appropriate candidate for the treatment of leishmaniasis that can heighten the load of iron and production of free radicals and induce cell death (30). Ebrahimisadr *et al.*

reported the antileishmanial activity of ART in inhibiting the growth of *L. major* in an *in vitro* and *in vivo* trial with no cytotoxic effects (31). Although ART has good therapeutic potential, it has disadvantages such as low bioavailability, low water-solubility, slow and irregular absorption, short biological half-life (about 2 h), risk of acid degradation, and inherent toxicity. In this regard, the therapeutic potential ART following oral administration is significantly reduced (about 35%). One approach to reduce side effects, increase circulation half-life, and reduce drug toxicity is the recruitment of the nanotechnology-derived delivery system (32).

Nowadays, nanoparticles are considered efficient carriers for drug delivery purposes. Nanoparticles are capable to change the pharmacokinetics of drugs within the body, control drug release, protect the structure of drugs molecules, promote a long life circulation in the blood, and are able to overcome cell barriers to deliver the drug to the target site. Numerous materials are used in the preparation of nanoparticles such as metal particles, polymers, lipids, and proteins. The ability of lipid nanoparticles to target deeper skin layers is an intriguing aspect that can be used in the treatment of CL. Many researchers have demonstrated the ability of lipid nanoparticles to target the dermis, but the mechanism is still unknown. According to research, topical application of drug-loaded NLCs may result in targeted delivery of antileishmanial drugs to the site of action, which is infected macrophages in deeper skin layers (33). In this work, lipid nanoparticle was used for its ability to target deeper skin layers and delivery of the

antileishmanial drugs to the site of action, infected macrophages residing in deeper skin layers.

In the current study, ART-NLCs showed higher anti-leishmanial and lower cytotoxicity effects than ART. NLCs alone indicated no anti-leishmanial effect but using ART-NLCs profoundly enhanced anti-leishmanial activity of ART probably due to enhancing cell permeation and internalization of the ART. Furthermore, the effects of ART, ART-NLCs, and AMB were evaluated against amastigotes of *L. major* and the results indicated that amastigotes were significantly more sensitive than promastigotes.

Leishmania is an obligatory intracellular parasite and macrophages are its favorite host cell and the treatment of leishmaniasis must be designed for clearance of intracellular parasites. So, in addition to promastigotes, we evaluated the effects of ART and ART-NLCs on the parasitic load of macrophages. In the current study, ART-NLCs showed better anti-amastigote effects than ART. One of the most important factors in the evaluation of drug efficacy is the severity and prevalence of side effects, which unfortunately is one of the limiting factors during the recruitment of common treatments. In this study, the cytotoxic effects of ART and ART-NLCs on macrophage cells have been investigated by MTT and it has been shown that ART and ART-NLCs are relatively safe and their toxicity is much lower than that of AMB.

Another case that was examined in this study was the SI for the drug, which indicates which drug is more preferable to use. In the current study, ART-NLCs had higher SI in comparison to all tested treatments. In another investigation, Riaz *et al.* fabricated curcumin-loaded NLCs for topical drug delivery of CL in an *in vitro* study and reported that curcumin-loaded NLCs was highly effective against promastigotes and axenic amastigotes like cells with the IC₅₀ value of 105 and 190 µg/mL, respectively, whereas curcumin solution showed anti-leishmanial effects with IC₅₀ value of 165 and 243 µg/mL against promastigotes and axenic amastigote like cells, respectively (33). In a toxicity analysis on citral-treated mice, it was discovered that the nanostructured lipid carrier

encapsulated with citral (NLC-citral) has a slow-release mechanism, which increases citral solubility in water. There was also no toxicity detected by blood biochemistry study, flow cytometry immunophenotyping assay, or lipid peroxidation (34).

One of the important aspects in choosing an effective drug in addition to the antiparasitic effect is the toxicity of the drug. In the current study, ART-NLCs showed better anti-amastigote effects than ART and had lower antiparasitic effects than AMB, although it was less toxic than AMB, and the SI of ART-NLCs was significantly higher than that of AMB. As a result, ART-NLCs are better than AMB.

CONCLUSION

The ART and ART-NLCs at 25, 50, and 100 µg/mL revealed significant anti-leishmanial effects against *L. major* promastigote and amastigote in an *in vitro* study for the first time. Furthermore, our results indicated that ART-NLCs significantly heighten the anti-leishmanial activity of ART. Also, ART-NLCs had the lowest cytotoxicity against macrophages. Furthermore, these nanoparticles had the highest SI among other examined agents including ART, AMB, and NLCs. As a result, ART-NLCs are an effective formulation against *L. major* with low cytotoxicity. Although more animal and clinical assessments are needed to elucidate the usefulness of this delivery system in the treatment of leishmaniasis.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

M.H. Motazedian, S. Mohammadi-Samani, and Q. Asgari coordinated in designing the project and providing financial supports.

V. Rahnama, M. khazaei, P. Ghasemiyeh, and Q. Asgari gathered the information of mothers. V. Rahnama and P. Ghasemiyeh analyzed the data. V. Rahnama drafted the original manuscript and all authors read and approved the final version of the manuscript.

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