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Research Article

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Nanoliposomal miltefosine for the treatment of cutaneous leishmaniasis caused by *Leishmania major* (MRHO/IR/75/ER): The drug preparation and *in vitro* study

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ABSTRACT

Cutaneous leishmaniasis is caused by intracellular parasites of Leishmania. Leishmania major is one of species that causes zoonotic cutaneous leishmaniasis and antileishmanial treatments have not provided acceptable results yet. This study was aimed to design, synthesis and evaluate nanoliposomal miltefosine in vitro conditions against Iranian strain of Leishmania major (MRHO/IR/75/ER) for the first time. To design nanoliposome particles that can penetrate into the dermal infected macrophages and intracellular Leishmania major, nanoliposomes were prepared with a combination of 1,2- dioleoyl-sn-glycero-3-phosphocholine, pure powder of miltefosine, cholesterol, stearylamine and vitamin E with the ratios of 32.6:65:13:6.5:1.9 mol % by the lipid film hydration and extrusion method. The size and ζ-potential of nanovesicles were measured by photon correlation spectroscopy. The encapsulation efficiency and drug loading of miltefosine in liposomes and nanoliposomes was determined by LC/MS. Inhibitory concentration and cytotoxicity of them were evaluated against intracellular Leishmania major amastigotes. The nanoliposomes had 56.7 nm size,+15.5 mV ζ-potential, a high encapsulation efficiency of 95.3 % and an IC50 of 1.6µM.Our findings show that antileishmanial effect of nanoliposomal miltefosine is stronger than miltefosine thus, nanoliposomes as a desirable topical drug delivery system can be used for the loading, transportation and diffusion of miltefosine towards the reproduction sites of parasite in the dermal macrophages of susceptible laboratory animals.

Keywords: Drug loading , *Leishmania major*, Miltefosine, Nanoliposome**,** Particle size, ζ-potential

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INTRODUCTION

Leishmaniasis is one of the most important zoonotic diseases awoken in many of the world zones.(1) Cutaneous leishmaniasis (CL) is the most common form of this disease. Leishmania species are endemic disease in 98 countries

around the world and two million new cases of leishmaniasis occur annually within which more than 1.5 million are CL.(2) *Leishmania major* is one of the important parasites causing acute CL in the Old World. Although CL is a self-healing disease, healing takes a long time and healing times have been reported even up to 2 years.(3) Also, because of transformation disease to severe forms such as Lupoid and disfiguring occurred
on the face in half of the cases, treatment is indispensable.(4)

Meglumine antimoniate (Glucantime®) is the first choice treatment for CL.(5) But due to inconsistent results and significant side effects, multiple injections that are painful and intolerable by most of the patients and resistance to pentavalent antimonials,(6) miltefosine (HePC) has become the center of attention due to its significant therapeutic effects on leishmaniasis.(7) Miltefosine (Figure 1) developed as an anticancer drug. But, now it is the only oral drug registered for the treatment CL.(8) So that, in studies conducted by Mr. Mohebali for the treatment of cutaneous leishmaniasis caused by *Leishmania major*, miltefosine *in vitro* and in animal models and eventually in humans has been successfully used.(9,10) However, these treatments have not provided a strong consistent result due to the rapid clearance of drugs from the site of action and side effects in high doses.(11) On the other hand, miltefosine should be used for 28 days at a rate of 2.5 mg/kg of body weight and it had harmful effects such as gastrointestinal adverse effects. Therefore, to reduce complications and increase the therapeutic effect of miltefosine, a nanoliposomal formulation of HePC was developed. Liposomes are an important delivery system to overcome these problems. By modulating the pharmacokinetic characteristics of drugs, Liposomes can reduce side effects of drugs and improve their activity.(12) Liposomes passively target drugs to macrophages. Therefore, they have been used for delivery of antileishmanial drugs to macrophages.(13,14) The objective of the study was design, synthesis and evaluate the effectiveness of different concentrations of nanoliposomal miltefosine against Iranian strain of *L. major* (MRHO/IR/75/ER) *in vitro* conditions. Nanoliposomes HePC were prepared by lipid film hydration and extrusion method. Then, size, ζ- potential, encapsulation efficiency (EE), drug loading (DL), loading capacity (LC) and as well as their impact on the cell viability of macrophages and amastigotes of *L.major* were characterized.

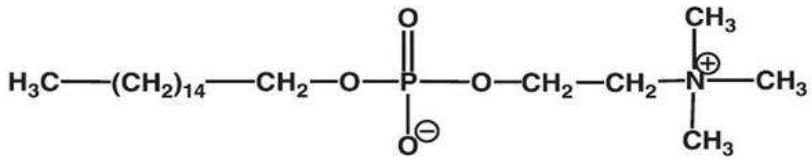

Figure 1: Chemical structure of miltefosine

MATERIALS AND METHODS

2.1 Materials

Cholesterol (Cho), methanol, chloroform, stearylamine (SA) dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma Aldrich (St. Louis, Missouri, USA). Polycarbonate nanopore filters in sizes 50, 100, 200 and 1000 nm were purchased from Avanti-polar (alabaster, alabama, USA). HEPES buffer, vitamin E, RPMI 1640 medium and penicillin–streptomycin were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Foetal bovine serum (FBS) was purchased from Gibco company (Gibco, USA). Amicon Ultra-15, PLQK Ultracel-PL Membran , 50 kD, centrifugal filter devices were purchased from Millipore (Cork, Ireland). Miltefosine was from Zentaris GmbH (Frankfurt, Germany). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Lipoid GmbH (Germany). 8-well culture chamberslides (Lab-tek Nunc Inc, USA) was purchased from Nunc company. All lipids were of the highest purity available and all other chemicals used were of analytical grade and used without further purification.

2.2 Parasite culture

Leishmania major promastigotes (MRHO/IR/75/ER) were from the Department of Parasitology, Tehran University of Medical Sciences, Iran. For mass production, the promastigotes were cultured in RPMI 1640 medium (Sigma, USA) supplemented with 10 % heat-inactivated FBS (Gibco, USA), 1 % penicillin-streptomycin (Sigma, USA) in a CO2 incubator at 28°C, 5 % Co2 and 80 % relative humidity.

2.3 Production of miltefosine liposomes and nanoliposomes

Liposomes and nanoliposomes were prepared by the lipid film hydration and extrusion method.(15) Briefly, components of liposomes containing DOPC (65 mol%), Cho (13 mol%), HePC (32.6 mol%), SA (6.5 mol%) and vitamin E (1.9 mol%) were dissolved in 5 ml of chloroform using a round-bottomed flask, respectively. Then, the solvent was evaporated by rotary evaporation until a thin and homogeneous lipid film was formed. For the production of liposomes, thin-film was hydrated with HEPES buffer (pH=7.4) at 70°C for 30 min to get

multilamellar vesicle dispersion. HEPES buffer up to 100 % (5 ml) was slowly added to the lipid film and mixture was vigorously vortexed so that all its components were very well mixed. 500 µl of this liposomal dispersion was diluted 20 fold with HEPES buffer. Finally, the last concentrations of liposomal components were 5 mg/ml DOPC, 1 mg/ml Cho, 0.5 mg/ml SA, 2.5 mg/ml HePC and 0.15 µl VE. Liposomal dispersion was cooled for 30 min when it was being vigorously vortexed and liposomes were maintained to mature for 24 h at room temperature. Then, they were successively extruded with eleven times extrusion through polycarbonate membrane with 1000 nm pore size to produce liposomes and seven to eleven times extrusion through polycarbonate membranes with 200,100 and 50 nm pore sizes to reduce the size of liposomes by using a thermo barrel extruder at 70°C (Avanti Polar Lipids ,Inc , USA), respectively. The same procedure was used to prepare drug-free liposomes and drug-free nanoliposomes with using the drug-free liposomes, except that HePC was omitted.

2.4 Determination of particle size, zeta potential, encapsulation efficiency and drug loading in liposomes and nanoliposomes containing miltefosine

The particle size and ζ-potential of HePC liposomes, HePC nanoliposomes and drug-free nanoliposomes were measured at 25°C and at the duration used 60 **s** by photo correlation Spectroscopy by using a photon correlation spectrometer (Zetasizer Nano-ZS). Data were analyzed by the MALVERN software. 50 µl of each sample was diluted immediately with 2 ml of HEPES buffer (40 fold) to get a proper concentration of particles and preventing the multi scattering events. The obtained homogeneous suspension was examined to find the volume mean diameter, size distribution and polydispersity. For the measurement of ζ-potential, the Samples were Correspondingly diluted with distilled water. Measuring the size and ζ-potential of each sample was performed 3 times and results were expressed as a mean values ± standard deviation.

The concentration of HePC encapsulated in liposomes and nanoliposomes was measured by the centrifree filtration method and liquid chromatography coupled to tandem mass spectrometry system (LC/MS),(16,17) a HP1100 liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA) consisting of a binary pump, degasser and autosampler. The HPLC system was connected to an Agilent 6410 triple quadrupole mass spectrometer equipped with a combo source electrospray (ESI) and atmospheric pressure chemical ionization (APCI) which are in the same rate. The quadrupoles were operated with unit resolution in the positive ion mode. Briefly, the amount of non-encapsulated HePC separated by filtration and centrifugation was determined by LC/MS. Then, the % EE, % DL and LC of HePC were calculated by the formulas. The filtration procedure eliminates large liposomes and drugs entrapped in them. So, to find the encapsulation efficiency of HePC, measuring the concentration of HePC in filters is essential. The filters used for the production of liposomes and nanoliposomes (50, 100, 200 and 1000 nm filters) were separately massed. One ml of methanol was added to each filter and filters were vortecxed for 1 min to dissolve liposomes and drugs trapped in them. Then, 10 µl of each sample was directly injected into the LC/MS system. Also, to find non-encapsulated HePC, 1.5 ml of liposomal and nanoliposomal formulations was separately added to any Amicon Ultra-15 filter device (50 kD, 15 ml, Millipor, Germany). Amicons were centrifuged at 7000 g for 30 min. Then, 10 µl of solution accumulated in the bottom filter cup was injected in LC/MS system. For all samples, the specific mass of miltefosine was monitored. All samples were analyzed in duplicate and encapsulation efficiency and drug loading were calculated based on the following formula:

$$\%\,\boldsymbol{DL} = \frac{\boldsymbol{mt} - \boldsymbol{mf}}{(\boldsymbol{mt} - \boldsymbol{mf}) + \boldsymbol{mi}} \times \mathbf{100}$$

$$\%\,\boldsymbol{EE} = \frac{\boldsymbol{mt} - \boldsymbol{mf}}{\boldsymbol{mt}} \times \mathbf{100}$$

In here *mt* is the total amount of HePC, *mf* the amount of non-encapsulated HePC (drug-free) in the filtration and centrifugation and *mi* the original amount of lipids. The LC of liposomes and nanoliposomes was calculated as the ratio of the amount of entrapped drug in them to the total weight of the formulations:(18)

$$\boldsymbol{LC} = \frac{\boldsymbol{Total\ amount\ of\ HePC\ (\mu g)}}{\boldsymbol{Total\ dry\ weight\ of\ liposomes (mg)}}$$

2.5 Drug susceptibility testing and Cytotoxicity

Drug susceptibility of intracellular amastigotes to HePC nanoliposomes, nanoliposomes without drug and HePC liposomes was examined by adding promastigotes of *L. major* (MRHO/IR/75/ER) in the stationary phase to macrophage cultures (8×10^4 cells per well) in 8-well culture chamberslides (Lab-tek Nunc Inc.) at a ratio of 10 parasites per macrophage. The mixtures were incubated in a CO_2 incubator at 37°C, 5 % CO_2 and 80 % relative humidity for 4 h. Excess promastigotes were then removed by two washings with medium and macrophages were incubated for 24 h in fresh RPMI 1640 medium. Then, medium was discarded and cells were incubated at 37°C for 48 h in fresh medium that contained different concentrations of samples (20, 10, 5, 2.5 and 1.25 µM). Finally, the

medium and chambers were removed and the slides were fixed with methanol and stained with Giemsa. The percentage of infected cells and the number of amastigotes per 100 macrophages were determined in duplicate cultures with a microscope. The results were presented as a ratio of infection between the treated and non-treated macrophages or multiplication index (MI). The inhibitory concentration 50 % of cell growth (IC50) was determined by linear regression.

$$\boldsymbol{MI} = \frac{(\boldsymbol{Number\ of\ amastigotes\ in\ experimental\ culture/100\ macrophages})}{(\boldsymbol{Number\ of\ amastigotes\ in\ control\ culture/100\ macrophages})} \times \mathbf{100}$$

The cytotoxic effect of liposomes and nanoliposomes containing HePC on macrophages was examined by methyl The 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.(19) MTT test is a method that measures the ability of cells in transforming the yellow tetrazolium crystals to insoluble blue formazan dye by the mitochondrial electron transport chain and dead cells are incapable of this action. Peritoneal macrophages of male Balb/c mice were cultured in RPMI 1640 medium containing 10 % FBS and 100 µg/ml penicillin-streptomycin at 37°C in a CO_2 incubator in 5 % CO_2 and 80 % relative humidity. Briefly, macrophages were seeded in 96-well plates and maintained for 24 h at 37°C. The cells were then infected with *L.major* (MRHO/IR/75/ER) promastigotes at a ratio of three promastigotes per macrophage and plates were incubated at 37°C in 5 % CO_2 for 4 h to allow internalization of the parasites in the cells. Next, 200 µl of dilutions of 1.25, 2.5, 5, 10 and 20 µM of HePC nanoliposomes, nanoliposomes without drug and HePC liposomes was added to 96-well plates and plates were incubated for 48 h in 37°C. Then, 20 µl of MTT solution (0.5 mg/ml) was added to plates and after a 4 h incubation at 37°C, supernatants were aspirated and discarded. Finally, 100 µl of DMSO was added to the wells to dissolve the formazan crystals and obtaining a homogeneous solution suitable for measuring the absorbance with an ELISA plate reader (lQuant, BioTek, Winooski, USA) at wavelength 540 nm. Every test was done in triplicate and the results were expressed as the means and standard deviations. The optical density was set in the absence of the drugs as the 100 % control value. Relative numbers of live cells were determined based on the absorbance of the treated and untreated samples and blank wells using the following formula:

$$\textbf{Viable cells}\ (\%) = (\boldsymbol{AT} - \boldsymbol{AB})/(\boldsymbol{AC} - \boldsymbol{AB}) \times \mathbf{100}$$

Where *AT*, *AC* and *AB* are the absorbance of the treated samples, the control samples and the blank wells, respectively. The selectivity index (SI) was determined based on the equation cytotoxicity concentration (CC50) / inhibitory concentration (IC50).

2.6 Analysis

All data represent the means ± standard deviations (SD) of two or three independent experiments. The means and SD obtained, using Microsoft Excel 2007 software (Microsoft, Redmond, WA). Data were analyzed using Student's t-test, one-way and two-way ANOVA followed by the scheffe *post hoc* tests, using IBM SPSS Statistics 20 software (IBM Statistics SPSS collection) for Windows. P-Value less than or equal to 0.05 was considered statistically significant.

RESULTS

3.1 Measuring the size and surface potential

Liposomes and nanoliposomes were fabricated as described in article with DOPC as main lipid constituent and SA to prepare cationic liposomes. After the last extrusion of samples through polycarbonate filters, the mean diameter of HePC nanoliposomes and drug-free nanoliposomes were less than 100 nm (Figure 2a and 2e). Also, The ζ-potential of these compounds was positive and drug-free nanoliposomes showed the highest ζ-potential among these compounds (Figure 2 and Table 1).

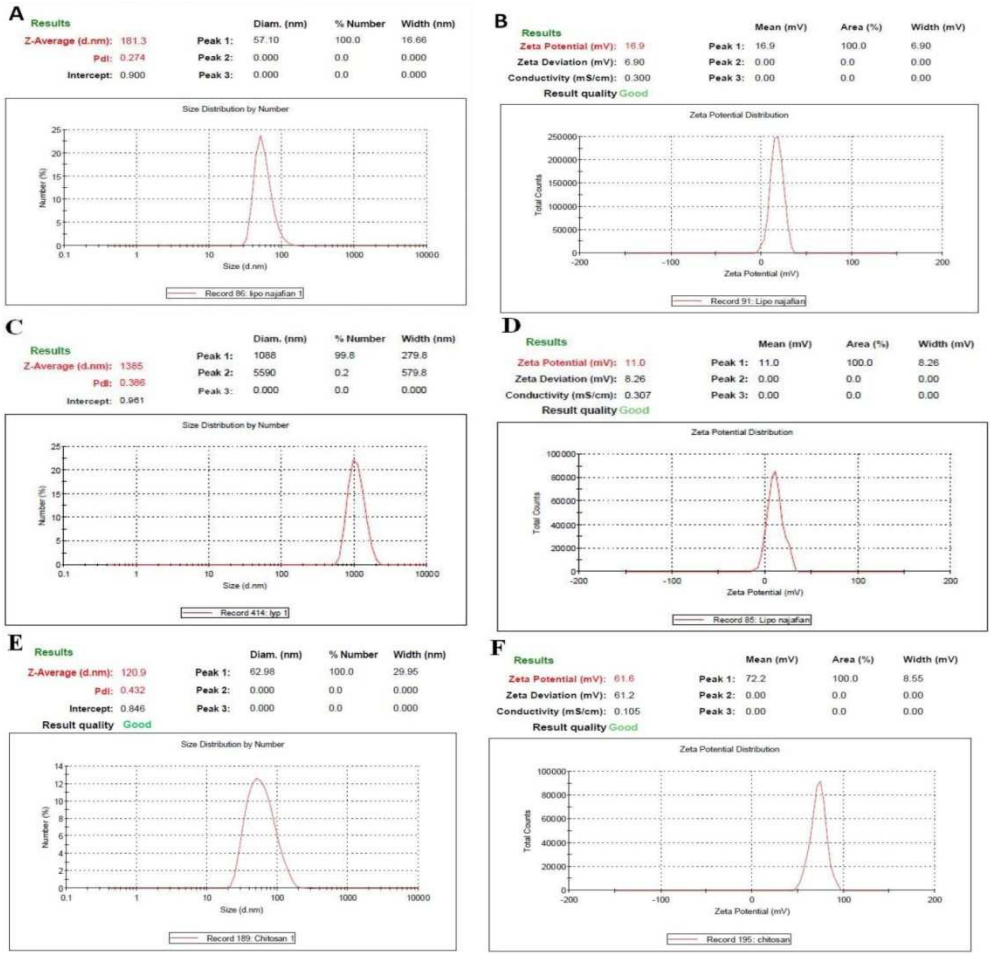

Figure 2: The particle size and distribution of liposomes and nanoliposomes measured by photo correlation Spectroscopy and the ζ-potential measured with a combination of laser Doppler velocimetry using ZetaSizer Nano-ZS (Malvern Instruments Ltd., Red Lable,Worcestershire, UK). Panel a, c and e are representative of the Size analysis of HePC nanoliposomes, HePC liposomes and drug-free nanoliposomes, respectively. Panel b, d and f are representative of the ζ-potential of HePC nanoliposomes, HePC liposomes and drug-free nanoliposomes, respectively

Table 1 Composition of liposomal formulations (molar ratio), average size, polydispersity index (PDI) and ζ-potential of lipid nanoparticles (n=3)

Formulation	composition (mol%)	Extrusion filter size (nm)	Average size (nm)	z-average (d.nm)	ζ-potential (mV)	PDI[a]
Nanolip-HePC	HePC/DOPC/Cho/STA/VA (32.6:65:13:6.5:1.9)	50	56.72±20.35	108.7	+15.5±3.65	0
Nanolip-drug free	DOPC/Cho/STA/VA (75.2:15:7.5:2.2)	50	62.98±31.92	120.9	+61.6±3.65	0.432
Lip-HePC	HePC/DOPC/Cho/STA/VA (32.6:65:13:6.5:1.9)	1000	1088±279.8	1385	+11.0±0.07	0.386

[a]*PDI, polydispersity index. polydispersity index is a measure of the heterogeneity of the sample. Its values range from 0 (homogeneous population) to 1 (completely heterogeneous population).*

3.2 The concentration of miltefosine in the samples and determination of % EE, % DL and LC

The concentration of HePC in liposomes and nanoliposomes was measured by LC/MS. The results of this analysis were shown in figure 3: representative chromatograms of extract of HePC in samples of A: standard sample of HePC ; B and C: filters used in the production of HePC liposomes and nanoliposomes, respectively; and D and E: samples centrifuged by Amicon Ultra-15 filter device.

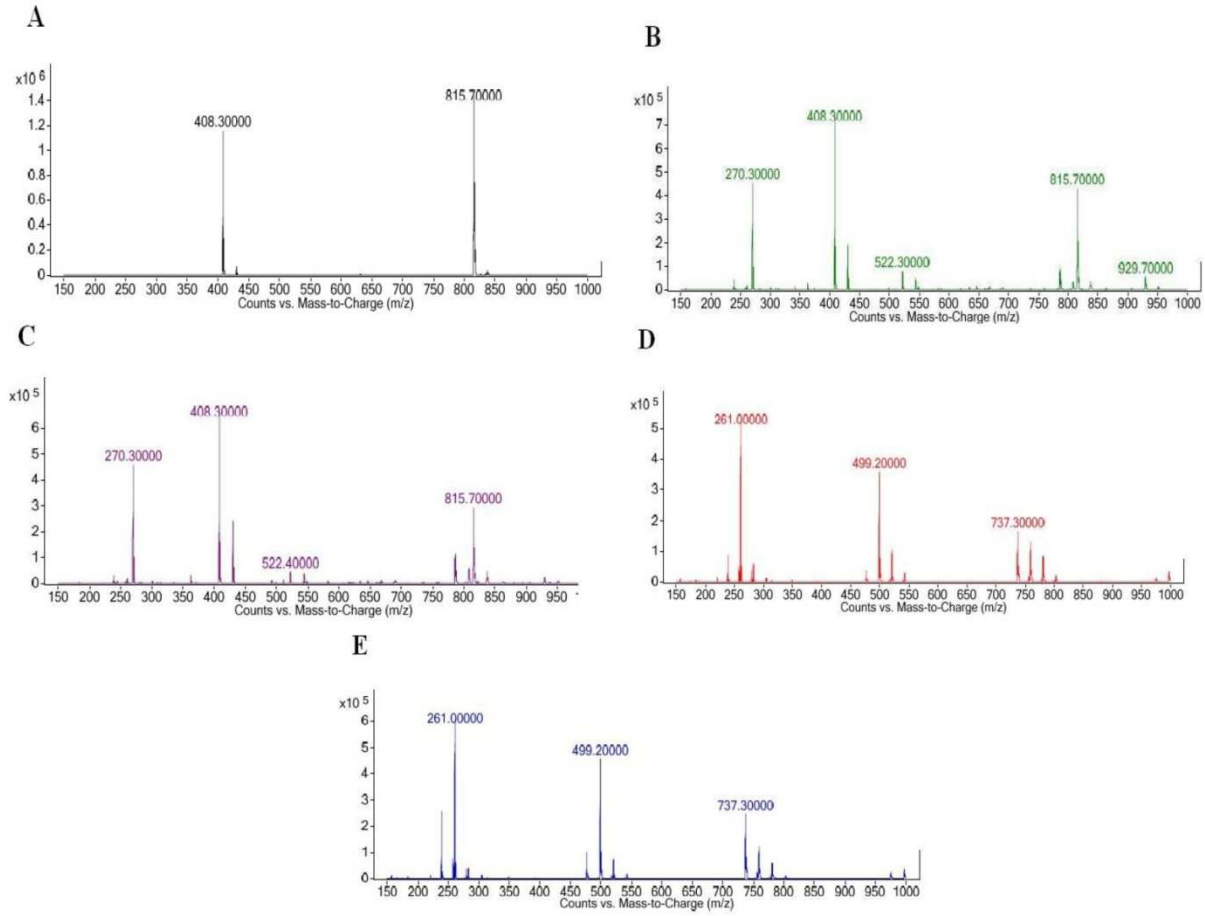

Figure 3: LC/MS mass scans after direct injection of samples; mass range: *m/z* 150-1000. The *m/z* of 408 corresponds with HePC $[M+H]^+$. Panel a displays the chromatogram of monomer and dimer Ions of HePC extracted from the standard sample of 200 µg/ml HePC in methanol. Panel b and c show HePC-ions in the mass spectra *m/z* 408 and *m/z* 815 in filters used in the production of HePC liposomes and nanoliposomes, respectively. panel d and e show no HePC-ions (either monomer or dimer) in the mass spectra obtained from centrifugal samples of liposomal and nanoliposomal HePC by Amicon Ultra-15 filter device, respectively. The recurrent masses that are recognizable in the spectra of the extracts of the centrifuged samples are *m/z* 261, *m/z* 499 and *m/z* 737 (all unidentifiable)

In the standard sample of 200 µg/ml of HePC in methanol and in filters used in the production of HePC liposomes and nanoliposomes, HePC $[M+H]^+$ at *m/z* 408 is the most abundant ion with a very high intensity, but also a HePC dimer $[2M+H]^+$ at *m/z* 815 is visible (in Figure 3a, 3b and 3c**)**. On the other hand, the samples extracted from the liposomes and nanoliposomes centrifuged by using Amicon Ultra-15 filter device (Figure 3d and 3e) do not show any peak at the HePC mass indicating that there is no HePC in these samples. 117.1 ± 5.05 µg/ml and 111.6 ± 8.08 µg/ml of 2.5 mg/ml of HePC used in formulations were only found in filters used in the production process of liposomal and nanoliposomal HePC, respectively (n = 2). By using results from the measurement of the concentration of HePC in samples and using formulas described in the earlier section, the encapsulation efficiency, drug loading and loading capacity of HePC in liposomes and nanoliposomes were determined (Table 2).

Table 2 :Chemical characteristics of HePC-loaded liposomes and nanoliposomes

formulation	HePC loading (%)	Encapsulation efficiency (%)	Loading capacity ($\mu g/mg$)[a]
Nanolip-HePC	26.88±0.094	95.53±0.32	264.83±0.9
Lip-HePC	26.82±0.043	95.31±0.20	264.23±0.55

Data are reported as mean ±SD (n = 3). [a]Loading capacity of HePC is based on total weight of liposomes and nanoliposomes.

3.3 in vitro cytotoxicity

The cell viability of macrophages was estimated by MTT test in the different concentrations of drug-free nanoliposome, HePC nanoliposome, HePC liposome and HePC (1, 2.5, 5, 10 and 20 µM). The cytotoxicity of these compounds at 48 h incubation with infected macrophages in a dilution 1.25 µM was 1.01 %, 4.98 %, 12.18 % and 26.76 %, respectively. But their cytotoxic effect in a dilution 20 µM was 17.98 %, 26.36 % , 33.68 % and 46.66 %, respectively. In the present study, the CC50 of these compounds against macrophages after 48 h of incubation was >20 µM.

3.4 leishmanicidal activity and selectivity index

The IC50 of HePC nanoliposome for *L. major* amastigotes at 48 h incubation was 1.6 µM according to the liner regression was shown in figure 4. The IC50 of HePC liposome and HePC were 2.1 µM and 2.35 µM, respectively. Also, the IC90 of HePC nanoliposome, HePC liposome and HePC for the *L.major* amastigotes after 48 h incubation were 7.4 µM, 8.43 µM and 9.58 µM according to the liner regression, respectively. A ratio of cytotoxicity to biological activity (CC50/IC50) was used to find the selectivity index of HePC nanoliposome, HePC liposome and HePC, that HePC nanoliposome with the selectivity index 12.5 showed the highest SI among these compounds (Table 3).

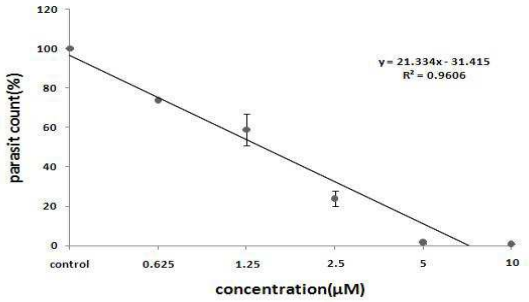

Figure 4: The inhibitory effect of different concentrations of HePC nanoliposome on the proliferation of amastigotes of *L.major* at 48 h incubation

Table 3: Inhibitory concentrations (IC50 and IC90) and selectivity index (SI)

Formulation	IC50[a] µM	CC50[b] µM	IC90[a] µM	SI [c]
Nanolip-HePC	1.6±0.0	>20	7.4 ±0.14	>12.5
Lip-HePC	2.1±0.62	>20	8.43±0.44	>9.52
HePC	2.35±0.21	>20	9.58±0.59	>8.51

IC50, IC90 and SI of HePC, liposomal HePC and nanoliposomal HePC against intracellular amastigotes of *Leishmania major*. [a]Data are IC50 and IC90 values in µM ± standard deviation. These data represent the average of 2 independent experiments. [b]CC50 values (50 % cytotoxicity concentration) on macrophages ± standard errors of the mean. [c]selectivity index (SI): CC50 of macrophages / IC50 of amastigotes of *Leishmania*.

DISCUSSION

4.1 Physicochemical characterizations of liposomes and nanoliposomes containing miltefosine

The performance of liposomes in biological environments is generally determined by their physicochemical properties, especially reaction and their membrane permeability in the skin depends on these physicochemical properties.(20) Also, detailed physicochemical characterizations are important to make sure the stabilization and efficacy of the liposomes. Therefore, particle size and ζ-potential are parameters that indicate the physical stability of liposomes.

To generate liposomes, a lipid formulation was first selected in an effort to increase the efficiency of the loading of HePC. DOPC, Cho and SA were selected for producing liposomes. DOPC, a zwitterionic phospholipid, was chosen as main lipid for two major reasons: first; because it is an amphiphilic molecule which can form stable monolayers; second; its oleic chains and phosphocholine polar head group are major components in biological membranes in eukaryotic cells. HePC, Cho and DOPC were substantially miscible. Because, sedimentary layer, turbidity or insoluble mass were not observed after the production of liposomes and nanoliposomes and during their storage for 2 months at 2 to 8°C.

Cho is known to form complexes with both drugs and phospholipid membranes and stabilizing them, as is well known condensation between Cho and zwitterionic phospholipids, including DOPC (Cho-DOPC).(21) Beside, Rakotomanga,(22) showed that there is a high affinity between HePC and Cho. Therefore, Cho was encapsulated into the nanovesicles for its high affinity with HePC and DOPC, and Cho mixed (13 mol %) with DOPC and HePC increased significantly their affinity for combining with each other (Table 2). Furthermore, due to the complex interplay of different molecules in the development process of a leishmania parasite, usually the administration of synergistic combinations is indispensable to improve antileishmanial activity and prevention of disease recurrence. In this regard, we incorporated SA as one of the components in lipid nanovesicles for its synergistic action with HePC, since SA-bearing cationic liposomes (PC/SA) have been reported to have antileishmanial potential.(23, 24)

HePC is a lipid analogue belonging to the Alkylphosphocholines group and similar to phosphatidylcholine.(25) Due to the fact that HePC is an amphiphilic molecule and structural part of liposome bilayer,(26) its ability to play both structural and therapeutic roles was also considered. Therefore, it was predicted that the encapsulation efficiency of HePC would be completed. Results obtained from this study show that this forecast was exact, because HePC was effectively encapsulated in the liposomes and nanoliposomes (Table 2). Also, the results display that the high encapsulation and loading of HePC are dependent on both the main lipid used (DOPC) and the structural role of HePC. On the other hand, the presence of SA in the liposomal formulations made of Cho and phosphatidylcholine reduces the drug leakage by reducing their membrane fluidity.(27) On the other hand, HePC was encapsulated with a loading capacity more than 260 µg/mg, and there was not observed significant difference between the loading capacity of liposomes and nanoliposomes (Table 2) ($p > 0.05$). This high loading capacity of HePC is related to the main lipid used in liposomes and nanoliposomes. This ability of DOPC can be due to its unsaturated nature which is primarily composed of unsaturated phospholipids. DOPC is made of the one of the longest fatty acid tails and the chain length may be a determining factor in this performance of DOPC.(20) Anyway, changing the size of the vesicles does not effect on loading capacity, and this is our liposomal formulation components that play a decisive role in the drug-loaded HePC, not the size of liposomes. These findings clearly show that HePC has been mixed with the highest possible value in lipid formulations.

The performance and physical stability of liposomes *in vivo* strongly depend on their size. Beside, liposomes with diameter less than 0.6 µm can penetrate in the skin.(27) Therefore, the size of produced liposomes and nanoliposomes was determined (Table 1). It can be concluded from data in Table 1 and Table 2 that the size of the liposomes and nanoliposomes does not have any effect on the drug loading. Because, despite the significant difference in the size of the liposomes and nanoliposomes ($p<0.05$), their DL and EE did not show significant differences ($p>0.05$). The size of drug-loaded nanoliposomes was compared with the drug-free nanoliposomes, and there was no significant difference between them ($p>0.05$). Therefore, it can be concluded that the presence of HePC alone does not role in reducing the size of nanoliposomes. In fact, the liposome size is reduced by the high affinity among Cho, DOPC and HePC, as well as the penetration of SA (charge inducer) between the hydrocarbon chains of phospholipids. (28)

The surface charge of liposomes is one of the most important factors in their skin penetration. (22) The positive charge of liposomes is helpful for their binding with the negatively charged skin cells and hair follicles.(29) Shanmugam,(30) showed that the steady flow of drugs through the skin is further in cationic liposomes Compared to anionic and neutral liposomes. The enhanced skin penetration of cationic liposomes has been attributed to the selective permeability of skin. (30) The lack of surface charge can reduce the physical stability of the liposomes by increasing their aggregation. The positively charged surfaces of liposomes increase not only the vesicular stability but also the intracellular uptake of liposomes by cells. (28) The net charge of DOPC, Cho and HePC is zero. But, SA used in formulation is a positive charge inducer. (28,31) Thereby, our liposomal and nanoliposomal formulations showed a posetive ζ-potential (Figure 2) measured by zeta sizer. There is a significant difference between the ζ-potential of HePC nanoliposomes and drug-free nanoliposomes ($p<0.05$), while they are roughly equal in size (Table 1). So, the size of nanoliposomes has no role in their ζ-potential difference. The amount of materials used in the production of nanoliposomes was constant, but to produce nanoliposomes without drug, HePC removed. It seems that the elemination of HePC and thereby increasing the molar ratio of SA (6.5 mol% → 7.5 mol%) can be responsible for the higher ζ-potential of nanoliposomes without drug than HePC nanoliposomes (Figure 2b and 2f).

4.2 In vitro cytotoxicity assessment

The analysis of information obtained from the cytotoxic effect of HePC, HePC nanoliposome, HePC liposome and drug-free nanoliposome in dilutions of 1.25, 2.5, 5, 10 and 20 µM on the macrophages, using two-way ANOVA followed by the scheffe *post hoc* test, showed statistically significant differences between control group and 2.5, 5, 10 and 20 µM dilutions ($p<0.05$). Also, there were found significant differences between 5, 10 and 20 µM dilutions and 1.25 µM dilution ($p<0.05$). But, no statistically significant differences were found among 5, 10 and 20 µM dilutions ($p>0.05$). Also, HePC and drug-free nanoliposome showed the highest and lowest toxicity in all dilutions on the infected macrophages, respectively. The cytotoxicity of HePC at 48 h incubation with the peritoneal macrophages Balb/c mice showed a significant difference with the cytotoxicity of HePC nanoliposome ($p = 0.0001$), HePC liposome ($p = 0.002$) and drug-free nanoliposome ($p = 0.0001$). Also, the cytotoxicity of HePC nanoliposome showed a significant difference with the HePC liposome ($p = 0.016$), but this difference was not significant in comparison with drug-free nanoliposome ($p = 0.598$). In a study that HePC was used as the reference drug by using the MTT test, the 50 % cytotoxicity concentration (CC50) of HePC was 54.7 µM**,** and in another study that the Alamar Blue micromethod was used to estimate the CC50 of HePC in macrophages, the CC50 was 92.7 µM after 68 h of incubation.(32,33) In accordance with these studies, the cytotoxicity of HePC, HePC liposome and HePC nanoliposome at the highest concentration studied (20 µM) was between 27.98 % and 46.66 % , and despite the fact that HePC showed the highest cytotoxicity on the macrophages, but it did not cause the 50 % cytotoxicity. So, the

CC50 of HePC, HePC liposome and HePC nanoliposome were more than 20 µM. These results show that the use of HePC into a nanoliposomal new form can reduce drug cytotoxicity. Because, in comparison with other HePC compounds, HePC nanoliposome had the least toxic effect on the macrophages. Therefore, the use of nanoliposomal HePC can not only increase the effect of HePC drug but also reduce its cytotoxicity.

4.3 Toxicity and anti-parasitic effect

In most laboratories, the screening for leishmanicidal compounds is carried out with leishmania promastigotes or axenic amastigotes. However, the best approach to find leishmanicidal compounds is the use of amastigotes residing in macrophages. So, the effect of nanoliposomal HePC, liposomal HePC and HePC on the L.major amastigotes after 48 h incubation was studied and IC50 and IC90 them were determined. Analysis of results of this study by using of one-way ANOVA followed by the scheffe *post hoc* test showed that different concentrations of nanoliposomal HePC had been reduced the proliferation of *L. major* amastigotes compared with the control wells and these differences were statistically significant ($p = 0.0001$ and $p = 0.004$). Also, significant differences were observed between 1.25 µM and 2.5 µM concentrations and 2.5 µM and 5 µM concentrations of nanoliposomal HePC (Figure 5). By comparing the inhibitory effect of different concentrations of nanoliposomal HePC and HePC on the amastigotes of *L.major*, all the concentrations of these two compounds (0.625, 1.25, 2.5, 5, 10 and 20 µM) reduced the number of amastigotes in comparison with control wells, but this anti-parasitic effect was stronger in nanoliposomal HePC. So that, significant differences were found between nanoliposomal HePC and HePC in 2.5 µM ($p = 0.03$) and 5 µM ($p = 0.04$) concentrations (Figure 6). This finding proves that anti-parasitic effect of nanoliposomal HePC is stronger than HePC. Drug-free nanoliposome also had inhibitory effect on intracellular amastigotes, but no IC50 value obtained (Figure 5). In agreement with these findings, Esmaeili et al.(9) reported that after 48 h incubation, the IC50 of HePC was 2.20 µM that is close to the IC50 of HePC obtained in our study (2.35 µM). On the other hand, the IC50 of nanoliposomal HePC was 1.5 times less than the IC50 of HePC ($p = 0.02$). Also, significant difference was observed between The IC90 of nanoliposomal HePC and HePC ($p = 0.03$).While, no significant differences were observed between the IC50 and IC90 of liposomal HePC and HePC ($p > 0.05$). The resultes show that anti-parasitic power of nanoliposomal HePC is more than HePC and among the compounds investigated, nanoliposomal HePC has the highest inhibitory effect on the intracellular amastigotes.

According to Weninger et al.(34) the biological efficacy of the tested drug is not attributable to cytotoxicity when SI ≥ 10. In general, all compounds showed properly selective toxicity and they were more destructive for intracellular parasite than the murine cells (Table 3).But, nanoliposomal HePC was 12.5 times more toxic for the amastigote forms of L. major than murine macrophages and its SI showed a significant difference with SI of HePC ($p = 0.007$) and liposomal HePC ($p = 0.01$). No statistically significant difference was found between the SI of HePC and liposomal HePC ($p = 0.339$). In total, in comparison with the HePC and liposomal HePC, nanoliposomal HePC showed the highest anti-amastigote activity with the least anti-macrophage activity.

CONCUSION

In the present study, the incorporation of HePC, an amphiphilic molecule, into the unilayer of liposomes and nanoliposomes was evaluated with the aim of developing a topical delivery vehicle for HePC. Liposomes were selected as the ideal vehicle for drug delivery due to their ability to efficiently encapsulate amphiphilic and lipophilic molecules. Our findings indicate that nanoliposomes are the suitable carriers for the loading and transportation of HePC drug to eliminate *L. major* intracellular parasites.These features, along with the use of SA in lipid formulations, strengthen the antiparasitic effect of HePC against intracellular parasites, and reducing the size of liposomes and the production of nanoliposomes loaded with HePC has a key role in better penetration of HePC into the site of parasite's reproduction. The topical application of nanoliposomal HePC can make faster treatment of cutaneous leishmaniasis and reduce the duration of therapy for the cutaneous lesions, thereby reducing the risk of disease relapse. Further, studies are required to clarify the role of nanoliposomal concentrations of HePC in treatment of CL in susceptible laboratory animals and humans.

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