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Original paper

In vitro evaluation of nanoliposomes berberine chloride against *Leishmania major* promastigotes

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ABSTRACT. Leishmaniosis caused by *Leishmania major* is one of the main infectious diseases that infected populations in developing countries around the world. We assessed the effectiveness of berberine chloride nanoliposomes (BcNLs) against *L. major* promastigotes *in vitro*. Nanoliposomal berberine chloride was prepared using thin film hydration method and characterized based on encapsulation efficiency, size and zeta potential. Anti-*Leishmania* effect of different concentrations (0.05–60 µg/ml) of BcNLs as studied in *L. major* (MRHO/IR/75/ER) at 24, 48 and 72 h using the hemocytometer technique. Berberine chloride was successfully loaded into nanoliposomes with encapsulation efficiency of 85.54%. The surface charge of nanoparticle is neutral and the morphology of nanoliposomal berbrine chloride is spherical without any agglomeration. Cell viability assay was performed on HFF cell line to show biocompatibility of liposome nanoparticles. IC50 of BcNPs at 24, 48 and 72 h against *L. major* were found to be 7.6, 5.96 and 3.19 µg/ml, respectively. BcNLs showed a significant anti-*Leishmania* effect and induced a better and more tangible effect on the survival of *L. major* promastigotes and could be suitable candidates for further investigation. The results showed that the BcNLs agent is effective against *L. major* promastigotes and may be a promising alternative to current treatments.

Keywords: *Leishmania major*, berberine chloride, nanoliposomes, cutaneous leishmaniosis

Introduction

Leishmania strains cause a range of human diseases in tropical and subtropical regions round the globe [1], manifesting as cutaneous leishmaniosis, cutaneous mucosal leishmaniosis, and visceral or kala-azar leishmaniosis [2]. It is induced by a type of obligate intracellular protozoan of the blood and tissue called *Leishmania* belongs to the order Kinetoplastida. Approximately 350 million people are at risk of suffering from this disorder worldwide, with 2 million new cases being reported annually [4,5]. It appears as two types of rural and urban leishmaniosis [6]. Antimonial compounds form the first line of defense since decades ago, a drug with toxic properties which needs repeated injections, is not very effective and promotes parasite resistance permanently. For example, in clinical variants of cutaneous leishmaniosis systemic pentavalent antimonial compounds are used [7]. For these reasons, other drugs, such as amphotericin and pentamidine, have come into use which have highly toxic effects on the liver, heart, and kidneys [8,9]. Hence, the provision of a more effective drug, with less complications and faster healing of the ulcer, seems mandatory

[10]. Consequently, man has turned to the use of herbal medicines to treat various infectious diseases for many years [11]. One of the herbal medicines is berberine chloride, which is the most important alkaloid in barberry with the scientific name *Berberis vulgaris* of the Berberidaceae family. The therapeutic properties reported for this herb are attributed to this alkaloid that exists in the roots, rhizomes, stem, and bark of the herb [12,13]. Its use dates back to 300 years ago in traditional Chinese medicine [14]. Several pharmacological properties of *Berberis vulgaris* were reported in general and its active component berberine [15]. Berberine comes as yellowish needle-like crystals with a melting point of 145°C, which is slightly soluble in cold water and highly soluble in hot water and alcohol [16]. Anti-*Leishmania* activity by berberine chloride through oxidative stress, probably due to inhibition of parasite mitochondria was very recently described [17]. Berberine hydrochloride has antimicrobial, antiparasitic, anti-cancer, anti-diarrhea, anti-diabetic, anti-inflammatory, anti-oxidant, anti-hypertensive, and anti-hyper-cholesterolemia effects (Fig. 1) [18].

Several methods have been applied to reduce its complications and improve the efficacy of its useful therapeutic constituent, one of which is nano-technology [19]. The advantage of pharmaceutical nanovesicles is the ability of nanoparticles to pass through capillary systems and penetrate the intended intracellular space due to their small size, leading to the accumulation of the drug at the desired site of action. Indeed, maximizing the efficacy of the drug and minimizing complications is the main goal of delivery of nanoparticles at the intended site [20]. A recent review by Mirhadi et al. [21] reported several types of nanocarriers for encapsulation of berberine. Among the various drug delivery vehicles, liposomes are used as the common drug delivery systems. The liposomal system protects the drug against destruction and controls drug delivery, easy release, and cellular absorption of the medicine [22]. This study aimed to investigate the anti-*Leishmania* effect of the new berberine chloride nanoliposomes against *L. major* promastigotes.

Materials and Methods

Parasite culture

L. major strain (MRHO/IR/75/ER) was obtained from the Iran Pasteur Institute and cultured in Novy-Nicolle-Mac Neal (NNN) medium. Subsequently, it was progressively adapted to RPMI-1640 medium (Thermo Fisher Scientific, MA, USA), supplemented with antibiotics, including penicillin (100 U/ml), streptomycin (100 µg/ml), and 20% heat-inactivated fetal calf serum (FCS), and glutamine at 25°C [23].

Preparation and characterization of nanoliposomes containing berberine chloride

The Bingham method, the water covering of a thin membrane, was used in this study for the production of nanoliposome. To synthesize the BcNLs, 0.0925 g of soy phosphatidilcoline, 3–4 cc of chloroform, and 0.0195 g of cholesterol were poured into a balloon. In addition, 0.003 g of berberine chloride was solved in methanol solution separately. Then, 2.5 ml of this solution was added to the balloon. The solvent phase was separated using a rotary evaporator device (Heidolph, Schwabach, Germany) (150 rpm, 45°C, and 120 min). During the homogenization, semi-vacuum, and total vacuum stages, a thin lipid film with yellowish-brown appearance was formed [1]. To produce spherical vesicles, 5.5 ml of sterile distilled water was added to the film construct (1, 100 µl of distilled water for each cc of suspension) and rehydrated using the rotary evaporator (150 rpm, 55°C, 30 min) [2]. The particle size is large in the hydration stage. To obtain particles of smaller size, the colloid liposome solution was sonicated for 15 min at a frequency of 60 Hz using probed sonication device. The resulting solution was passed through filters with 0.22 µm diameter to separate the larger particles from the smaller particles and also to produce a homogenous solution [2,3]. To separate the free drug from the liposome containing berberine chloride, the prepared suspension was poured into cellulose dialysis bags and stirred in a washer containing buffer (PBS 1×) pH=7.4 under gentle vibration for 1 h at 4°C in an iced container. Subsequently, to study the drug stored in the liposome, the prepared liposomes were mixed with isopropyl in various proportions and their absorption was read using spectrophotometer at 346 nm. Then, their load percentages were measured by plotting the standard curve using Microsoft Office Excel [2,3]. The diameter and *Z*-potential of berberine chloride containing nanoliposome was determined using Z-sizer (Brookhaven Instruments Corp, NY, USA). The determination of *Z*-potential is a parameter for the potential stability of the

Table 1. The standard equations of berberine chloride in 2-propanol

Correlation coefficient (R^2)	Standard equation (Y)
0.9961	0.0248X– 0.006

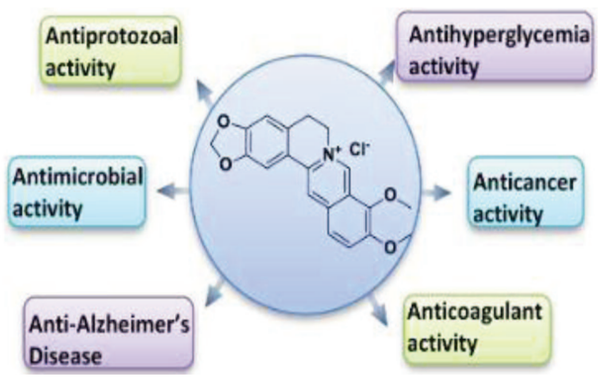

Figure 1. Chemical structure of berberine chloride and pharmacological applications

colloid system. The limit of the stability and instability of the suspension can be determined in terms of *Z*-potential [2,3]. To study the rate of drug release from liposome, the membranous diffusion method was used. In doing so, 1 ml of liposome suspension was poured into dialysis bag and then placed in a container with 10 ml of PBS buffer (pH=7.4). While the container was on the magnetic stirrer (18–26°C, gentle rotation), it was measured at specified time intervals including 72, 24, 8, 6, 5, 4, 3, 2, 1, and 0.5 h. In addition, 1 ml of the surrounding buffer was poured into a microtube and was again replaced with 1 ml of buffer. Finally, the absorption of the samples was read with a spectrophotometer at 346 nm and the release of the drug at various times was determined with the aid of the standard curve [1].

Challenging the BcNLs with rural wet Leishmania parasite

To evaluate the anti-*Leishmania* effect of nanoliposomes containg berberine chloride, the parasite was cultured in Novy-Nicolle-Mac Neal (NNN) medium. Subsequently, the promastigotes of the third passage from NNN medium were progressively adopted to RPMI-1640 medium (Thermo Fisher Scientific, MA, USA) supplemented with antibiotics, glutamine and FCS supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 20% heat-inactivated fetal calf serum (FCS) at 25°C [9]. Then, nanoliposomal berberine chloride was added in certain microbodies with a final concentration of 0.05, 0.1, 0.2, 0.4, 0.81, 1.62, 3.25, 7.5, 15, 30 and 60 µg/ml. In addition, PBS and the parasite were used as a negative control and amphotericin B (25 µg/ml) and the parasite used as a positive control. After 24, 48, and 72 h, the number of viable parasites was counted by 0.4% trypan blue using a hemocytometer and the 50% inhibitory concentration (IC_{50}) was calculated and graph was plotted using Sigma Plot™13 (Systat Software Inc, CA, USA). The percentage of growth inhibition (% GI) was calculated with respect to growth control as follows:

The experiments were performed in triplicate and repeated at least two times and single-blind conditions [2].

Ethical considerations

The experiments were confirmed by Ethical Committee in Vice Chancellor of research of Shahid Sadoughi University of Medical Sciences, (Ethical No: IR.SSU.MEDICINE. REC.1395.332).

Results

Loading rate of nanoliposomes containing berberine chloride

Various concentrations of BcNLs were prepared in 2-propanol. This solvent was used to break the lipid structure and drug release. Then, the photoabsorption of the concentrations was obtained with a spectrophotometer. The standard curve can be obtained by estimating the photoabsorption of the drug, indicating the relationship between concentration and photoabsorption. The percentage of drug loading can be obtained based on this relationship. According to this relationship, the mean percentage of drug load is 85.54% (Fig. 2).

The percentage of drug load compared to standard berberine chloride equations was determined and shown in table 1. The correlation coefficients (R^2) indicated that the Pearson´s correlation coefficients vary between –1 and +1. When the r-value approaches +1, it indicates a

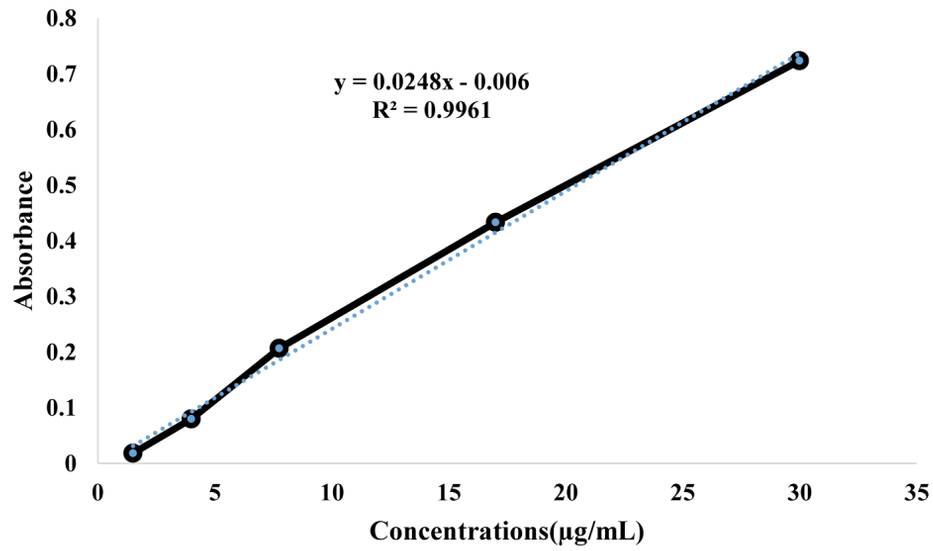

Figure 2. Standard curve of berberine chloride in 2- propanol solvent

positive linear correlation between the two variables and vice versa. The results demonstrated the good correspondence between the line and the experimental data.

Release of nanoliposomes containing berberine chloride

The amount of drug released from the liposome synthesized in PBS buffer with pH= 7.4 were estimated during time intervals of 72, 24, 8, 6, 5, 4, 3, 2, 1, and 0.5 h using the standard curve of PBS. The results are plotted in figure 2 which shows that the maximum amount of drug released from liposome during 72 h is 18.47%. It can be concluded, based on the reported release profile, that vesicles containing berberine chloride have controlled release under physiologic conditions (Fig. 3).

Size and zeta potential characterization of nanoliposomes containing berberine chloride

The two important parameters of particle size and Z-potential can be measured by Z-sizer in aqueous and organic solutions. The size and distribution of the synthesized liposome particle before and after berberine chloride loading is shown

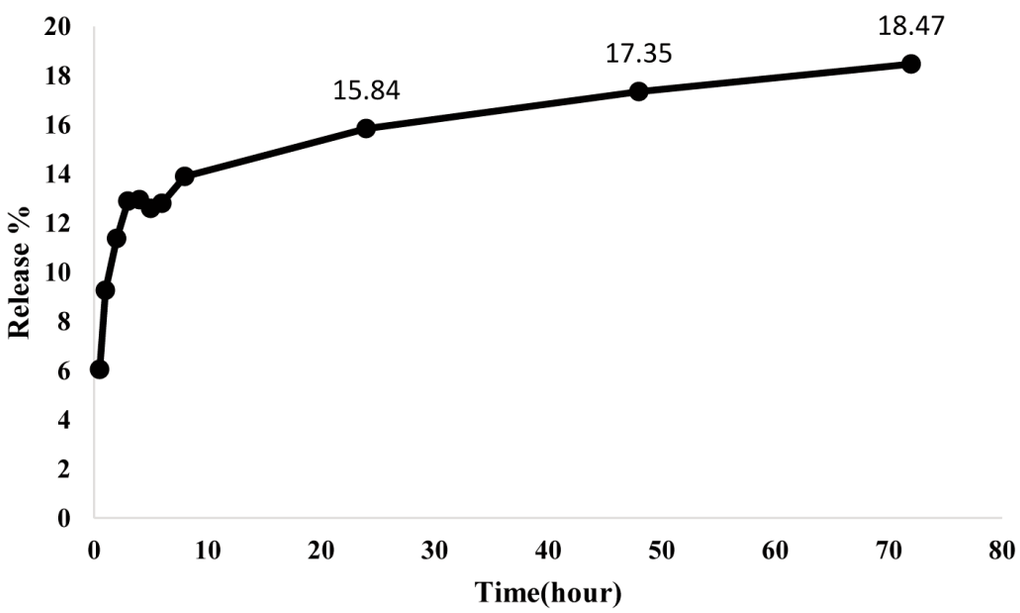

Figure 3. Curve of berberine chloride release from nano-liposome

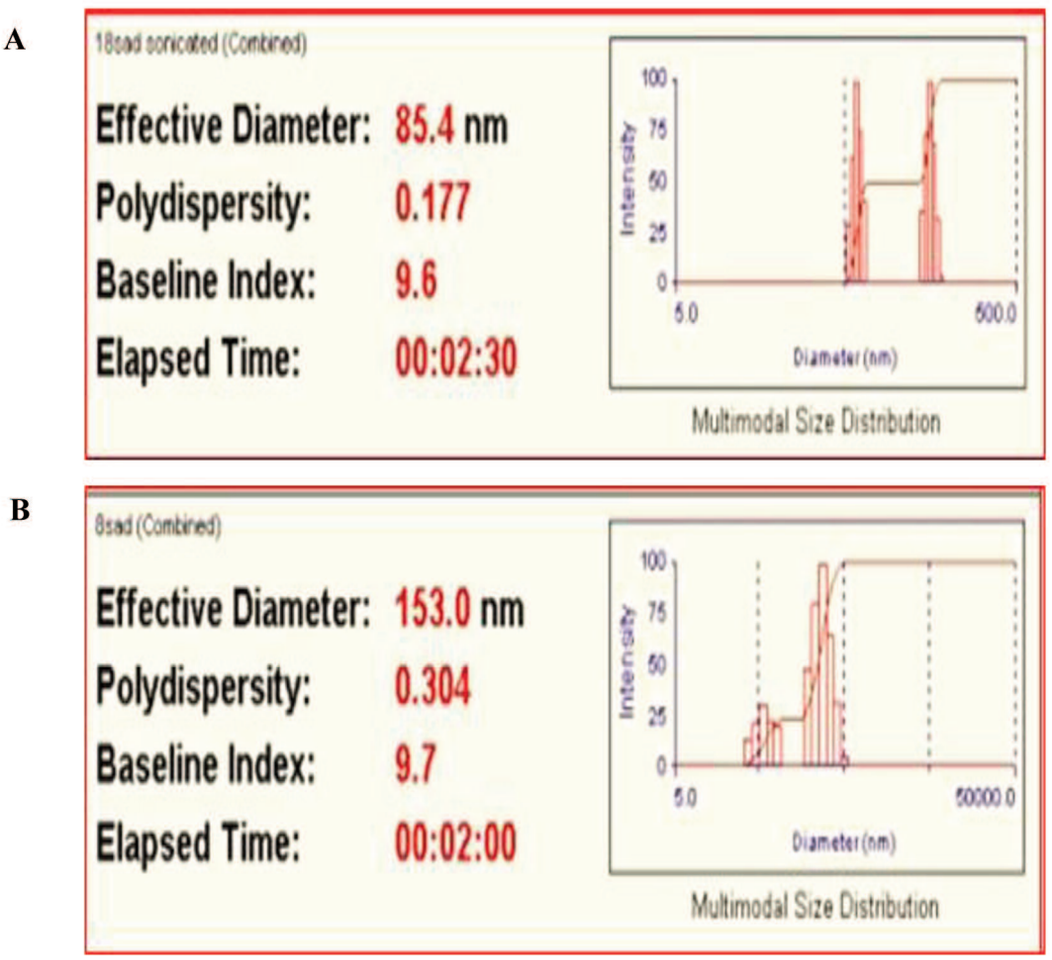

Figure 4. Results of liposome size before (A) and after (B) berberine chloride loading

in figure 4A and 4B. The superficial charge of liposomes was measured by Z-sizer before and after berberine chloride loading. The mean charge obtained was -3.24 before loading and -3.64 after BC loading, as displayed in figure 5A and 5B.

Effects of the nanoliposomes containing berberine chloride on the viability of L. major

The dose of BcNLs was (0.05–60 µg/ml). The IC_{50} of BcNLs for *L. major* promastigotes at 24, 48 and 72 h of incubation was equal to 7.6, 5.96 and 3.19 µg/ml, respectively (Fig. 6). The mean percentage of promastigotes viability after 24, 48 and 72 h at 0.05–60 µg/ml of BcNLs concentrations showed a statistically significant difference compared to the negative control (P<0.05).

Discussion

Although many studies have been conducted to prepare a drug for leishmaniosis, none of these treatments, including synthetic drugs, herbal medicines, and gene therapy, have been successful. For this reason, the preparation of a remedy for leishmaniosis is one of the most urgent needs. The antimony has been recommended as the main drug used to treat leishmaniosis, but there are some restrictions in this case, including high costs, side effects, frequent injections, and incomplete efficacy [10]. Despite recent developments, effective therapy for cutaneous leishmaniosis (CL) has still been based on long parenteral courses of these drugs for six decades, despite being reasonably expensive, toxic, and inconvenient to use, along with inadequate knowledge about their mechanism of action [7]. Our findings suggest that the anti-*Leishmania* activity of BcNLs are time-dependent, so that in the initial hours of exposure (24 hours) this is consistent with the results by Salehi et al. [33] on the effect of time on antiparasitic effects of barberry. Another similar study reported the anti-*Leishmania* activity of barberry, especially berberine. The study by Mahmoudvand et al. [34] indicated that the constituents of *Berberis vulgaris*,

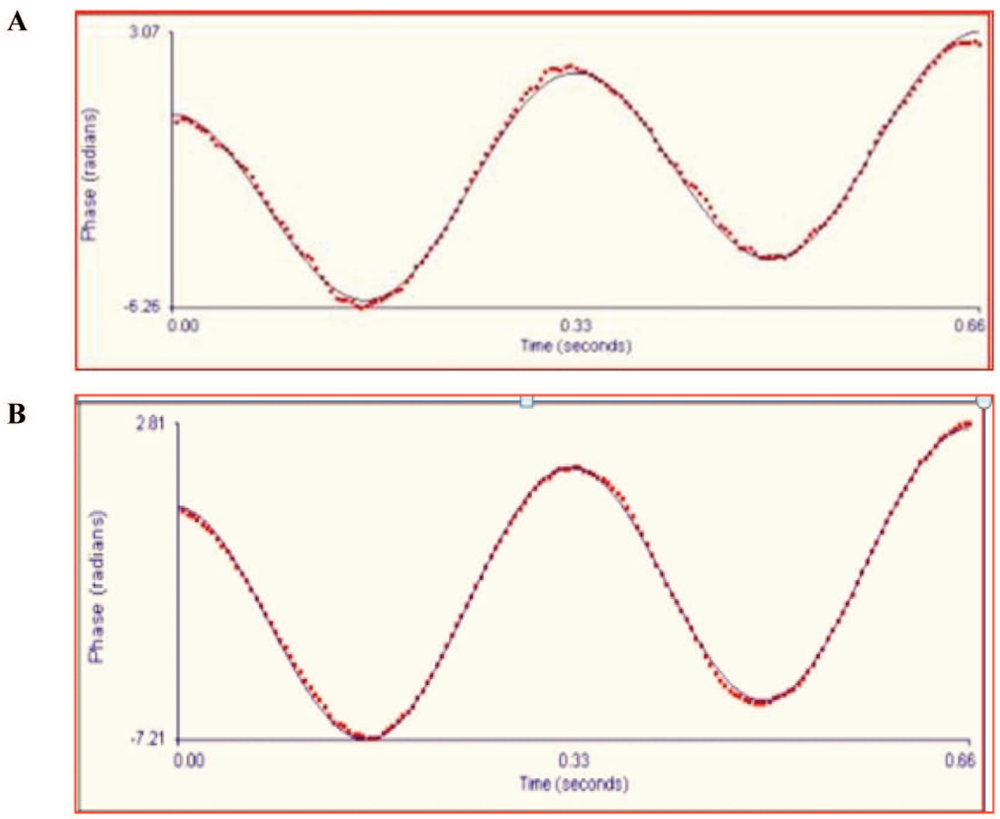

Figure 5. Results of liposome Z before (A) and after (B) berberine chloride loading

especially *Berberis* (P<0.05), reduced the speed of growth of *L. tropica* and *L. infantum* promastigotes compared to glucantime. Additionally, *Berberis vulgaris* and berberine significantly (P<0.05) decreased the mean number of amastigotes in any macrophage compared to the positive controls. The

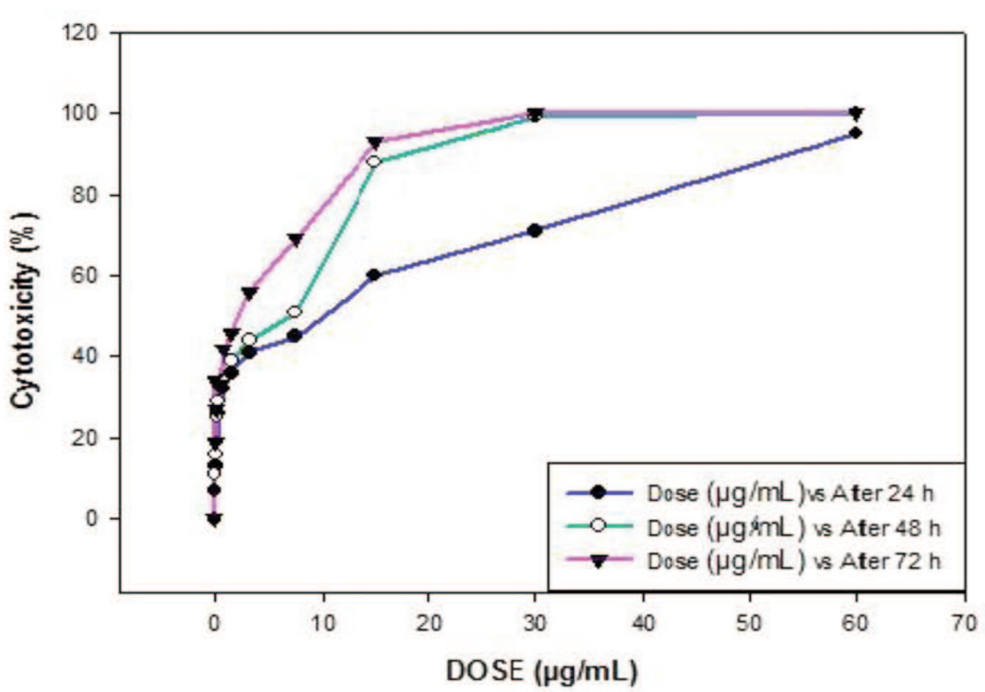

Figure 6. The IC50 of BcNLs for *L. major* promastigotes at various incubation times

mean viability of promastigotes decreased with increasing concentration of BcNLs, as Kazemi et al. [35] demonstrated that the alcoholic extract of barberry was more effective in diminishing both the size of the of ulcer *L. major* and promastigotes count in BALB/C mice in concentrations of 20%, 40%, and 80%, respectively [35]. Moreover, a study conducted in Health Research Institute in Washington on the effects of barberry extract on *L. donovani* on golden hamsters and pet dogs, showed that peritoneal injection of the extract on golden hamsters reduced the ulcer diameter by more than 50% [36] and decreased the parasite count in the spleen and liver by 90% compared to the control group. Also, the intra-ulcer injection of berberine extract four times a week proved to be significantly effective [37]. The effectiveness of BcNLs depends entirely on the time of exposure to promastigotes, so that the longer the time of exposure to the parasite, the lower the viability of *L. major* promastigotes. Many studies approve the gradual effect of the extract, among them Mahmoudvand et al. [38] investigated the lethal effect of barberry root and its main constituents, berberine, in various concentrations against *Echinococcus* protoscolex. Both berberine and methanol extracts with concentrations of 2 and 0.5 mg/ml, respectively, destroyed the entire protoscolex after 10 min of incubation [38]. Additionally, many studies have been reported on the effects of barberry ingredients, especially berberine chloride on prokaryotes. Among them, Zhao et al. [39] postulated that berberine in low concentration of 0.5 µg/ml triggers the inhibition of growth of *Candida albicans* while it completely inhibiting the growth of *Candida albicans* in concentrations above 0.75 µg/ml. In the study conducted by Birgani [40] the severity rate of lesions in the beberine group decreased significantly compared with the placebo group, so that the mean PASI (Psoriasis Area Scoring Index) decreased from 3.99 to 2.11 in the berberine group and from 3.98 to 3.71 in the placebo group. The difference in mean PASI before receiving beberine until the third month of intervention was not significant between the berberine and placebo groups, although it was significant during the fourth to sixth interventions (P=0.013). The results of this study showed that berberine was more effective in reducing cutaneous lesions in patients with psoriasis compared to placebo. In the studies by Ghaderi et al. [41], the anti-candidosis effects of aqueous and alcoholic extracts of barberry were explored and compared to clotrimazole *in vitro*. The findings of this study revealed that the barberry extract has anti-candidosis properties, with the most evident effects on the alcoholic extract compared to the aqueous extract.

Moreover, the study by Kachoei et al. [42] demonstrated that the aqueous and ethanol extract of the common barberry exerts an effect on bacteria resistant to multiple drugs and has antibacterial properties. Furthermore, the results of the study by Kiaei et al. [43] demonstrated that the barberry extract showed the optimal effect with the maximum mean diameter of the halo without growth of 4.29 against *Staphylococcus epidermidis*. Also, the findings of the study by Vaezi et al. [44] suggested that the administration of berberine hydrochloride for 6 weeks leads to improvement of cognitive disorders, especially memory and learning in the mice. Finally, the findings of the study by Kalalian et al. [45] showed that treatment with dose-dependent berberine improved cognitive and electrophysiological disorders in streptozotocin diabetic wild mice.

Considering the efficacy of the extract and the main alkaloid of barberry, i.e., berberine chloride, on microorganisms, especially eukaryotic ones, in most of the above-mentioned studies, it is clear that BcNLs possess much better anti-*Leishmania* activity, especially when compared to the current anti-*Leishmania* drugs as an alternative therapeutic agent in the future.

Acknowledgements

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