Advances in Pharmacology and Therapeutics Journal
Shahid Sadoughi University of Medical Sciences, Yazd, Iran

SHAHID SADOUGHI UNIVERSITY OF MEDICAL SCIENCES

Original Article

A New Approach to the Investigation of Therapeutic Efficiency of Novel *Carum copticum* Nanoparticles against *Leishmania major* Promastigotes

Running Title: *Carum copticum* Nanoparticles against *Leishmania major*

Ali Fattahi Bafghi[1], Mohammad Reza Mozayan[2], Zahra Esmailei[3], Hamideh Emtiazi[4], Mojtaba Moghateli[5*]

[1]Medical Parasitology and Mycology Department, Infectious Diseases Research Center, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
[2]General Courses Department, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
[3]Biochemistry Department, Payame Noor University of Taft, Taft, Yazd, Iran.
[4]PhD in Organic Chemistry, Faculty of Pharmacology, Shahid Sadoughi University of Medical Sciences, Safaeeyeh, Yazd, Iran.
[5]Gratuatted MSc parasitology, Medical Parasitology and Mycology Department, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

ARTICLEINFO

Received: 04/08/2023
Accepted: 06/11/2023

***Corresponding author**
Mojtaba Moghateli; Medical Parasitology Department, School of Medicine, Shahid Sadoughi University of Medical Sciences, Safaeeyeh, Yazd, Iran.
Tel: +98-3538203410
E-mail
mojtaba.moghateli@gmail.com

Abstract

Current therapies for Leishmaniasis are associated with several side effects as well as drug resistance. Sensitivity and resistance of *Leishmania major* to Glutamine are referred to as those isolates which are responsive or non-responsive to one or two full courses of treatment by Glucantime systematically and/or intra-lesionally, respectively. In this study, We assessed a new approach to the investigation of the therapeutic efficiency of novel *Carum copticum* Nanoparticles against *Leishmania major* Promastigotes. First, the *Carum copticum* Nanoparticles were synthesized and liposomal *Carum copticum* was applied as a new therapeutic approach substituted for current therapy. In this experimental study, liposomal *Carum copticum* was prepared using the thin film hydration method and characterized based on encapsulation efficiency, size, and zeta potential. *Carum copticum* was successfully loaded into the liposome. The surface charge of the nanoparticle was neutral and the size of the nanoparticle was 176.5 nm. Liposomal *Carum copticum* beared spherical shape without any agglomeration. Results revealed that liposomal *Carum copticum* carried a significant effect, compared to the control sample, on parasite growth in both logarithmic and stationary phases. The result of this study signifies that the *Carum copticum* Nanoparticles induces a better and more tangible effect on the survival of *Leishmania major* promastigotes.

Keywords: *Carum copticum* Nanoparticles, *Leishmania major*, Nanoparticles, Promastigotes

Citation: Fattahi Bafghi A, Mozayan M, Esmailei Z, Emtiazi H, Moghateli M. A New Approach to the Investigation of therapeutic efficiency of Novel Carum copticum Nanoparticles against Leishmania major promastigotes. Adv Pharmacol Ther J. 2023;3(2): 75-83.

Introduction

Leishmaniasis is one of the world's 6 most important infectious diseases caused by different species of Leishmania (1). This disease can be transmitted to humans by the Sandfly bite of the genus of Phlebotomus (2). In 88 countries of the world including 22 countries in the modern world and 66 others in the traditional setting Leishmania species have appeared to be endemic infecting a population of nearly 12 million people (3). Several studies have shown that cutaneous Leishmaniasis in Iran and the world is increasing in recent years due to the emergence of resistance against the standard drugs that are predominantly pentavalent antimony compounds, for this reason. the treatment of Leishmaniasis has encountered many difficulties. One of the recurrent reports of the physicians is a lack of treatment or improper effect of drugs in patients (4). Most derivatives of the antimony Meglumine (Glucantime) and sodium Glucantime are at the frontline of treatment, i.e. The first choice for Leishmaniasis therapy, but in recent years, the effectiveness of these drugs has declined between 20-50%, and currently the emergence of resistance forms one of the main problems for therapeutic purposes (5,6). The resistant strains emergence has led to the introduction of new antileishmanial factors such as miltefosine, amphotericin B, ketoconazole, paromomycin, and other chemicals; however, none of these drugs are without side effects (7). Therefore, the use of plants lacking these disadvantages (resistance, side effects,...) seems to be of paramount importance. One of these plants deployed in traditional *Carum copticum* (*C copticum*) medicine is the one-year herbaceous plant with a height of 30 to 60 cm from Apiaceous (Umbeliferae) with brownish gray fruits. Its scientific name is *C copticum*. This plant is known by the Hindi names "Adjouan" and "Asprkay" in Baluchistan. Scientific reports on the pharmacological effects of this plant are limited and include histamine receptors inhibition, platelets aggregation inhibition as well as anti-fungal properties. In traditional medicine, this plant is known to bear multiple properties including antispasmodic, tonic, stimulant, and carminative. *C copticum* chemical composition comprises *thymol*, *p-cymene*, *gamma*, and *amitriptyline*. In *C copticum*, Beta-pinene sabinene is an essential oil in addition to protein and fat; it also contains kations including sodium, potassium, iron, calcium, magnesium, and zinc (8-10). In this study, We assessed a new approach to the investigation of the therapeutic efficiency of novel *Carum copticum* Nanoparticles against *Leishmania major* promastigotes.

Material and Methods

Leishmania parasite culture

Leishmania (L) major [MRHO/IR/75/ER] Promastogotes were cultured in RPMI-1640 medium supplemented with L-glutamine (Sigma Chemical Co., St. Louis, MO), 10% fetal bovine serum (FBS) (Sigma Chemical Co.), and Gentamicin (80 mg/ml) (Sigma Chemical Co.) at 27 ° C. The passages of promastigotes culture were maintained on the fourth day of incubation. An inverted microscope Olympus CKX 41) was used to monitor the daily growth of Leishmania (L) major [MRHO/IR/75/ER] Promastigotes.

DOI: https://doi.org/10.18502/aptj.v3i2.13459

Preparation of *Carum copticum* nanoparticles

Liposomal *C copticum* was prepared using the thin-film hydration method (11). In brief, 0.0934 g of soy phosphatidilcholoride, 0.0196 g of cholesterol, and 0.003 g of *C copticum* were dissolved in 3–4ml of chloroform which was then evaporated under vacuum condition at 45_ C using a rotary evaporator Release of *C copticum* from Nano- liposomal was investigated using dialysis method after immersing dialysis membrane containing liposomal *C copticum* in a phosphate buffer saline at 37 _C and pH 7.4 under gentle shacking condition. At predetermined time intervals until 72 hr., 1ml of surrounding phosphate-buffered saline (PBS) solution was withdrawn and replaced with fresh buffer. The released drug was determined using a spectrophotometer at 430nm wavelength and by comparing it with a standard curve of different concentrations of *C copticum* in isopropanol.

Antileishmanial activity assays (XTT assay)

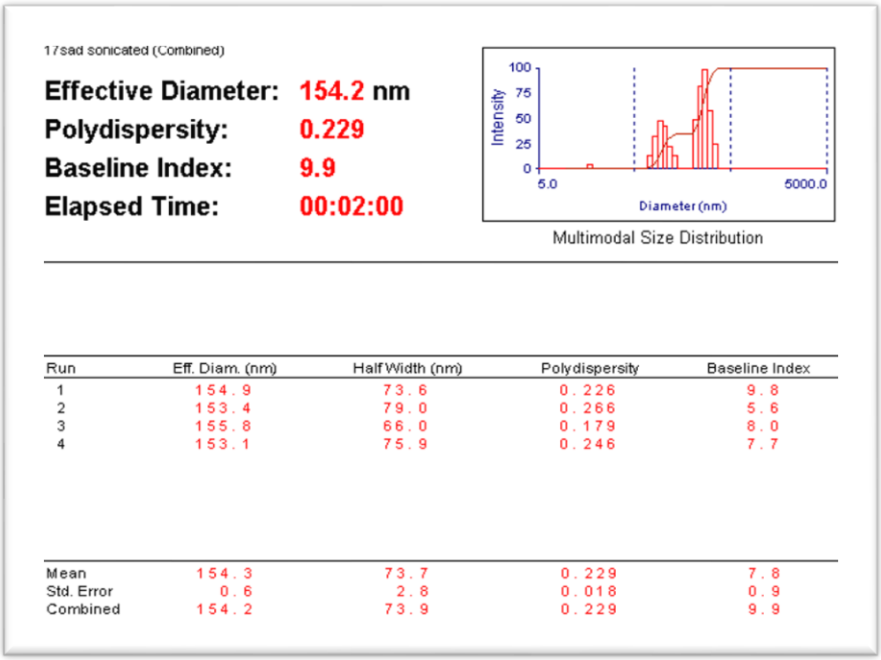
The antileishmanial activity in promastigotes was determined using XTT (sodium 3,39-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate). XTT is a colorimetric method containing salts that have modified Tetrazolium. This is much easier and more practical than the MTT test (12-13**)**. *Leishmania major* stationary-phase cultures in microplate 96 each being at a rate of 100 ml (Triplicates) were loaded and incubated for 4 hours. Parasite dilutions through water extract of *C copticum* and Glucantime were added to the already prepared microplate wells; this was done comparing with a negative control group without drugs. Other steps were performed based on the kit protocol.

Statistical analysis

All experiments were carried out in triplicate, and T-test analysis and ANOVA were used to compare the growth value in one group with the control group. A statistical difference of less than 5% (p-value <0.05) was considered as a statistical significance.

Results

Results of size specification: The two important parameters of particle size and Z potential analyses in water and organic solutions can be measured with Z-sizer. The size and distribution of the synthesized liposome particles before and after *C copticum* loading are displayed. The superficial charge of liposome was measured before and after *C copticum* loading using Z-sizer which was, on average, -4.62 before loading and -3.65 after loading. These results are presented in **Figures 1,2,3,4**.



Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	154.9	73.6	0.226	9.8
2	153.4	79.0	0.266	5.6
3	155.8	66.0	0.179	8.0
4	153.1	75.9	0.246	7.7
Mean	154.3	73.7	0.229	7.8
Std. Error	0.6	2.8	0.018	0.9
Combined	154.2	73.9	0.229	9.9

Figure 1. Results of liposome size before *C copticum* loading

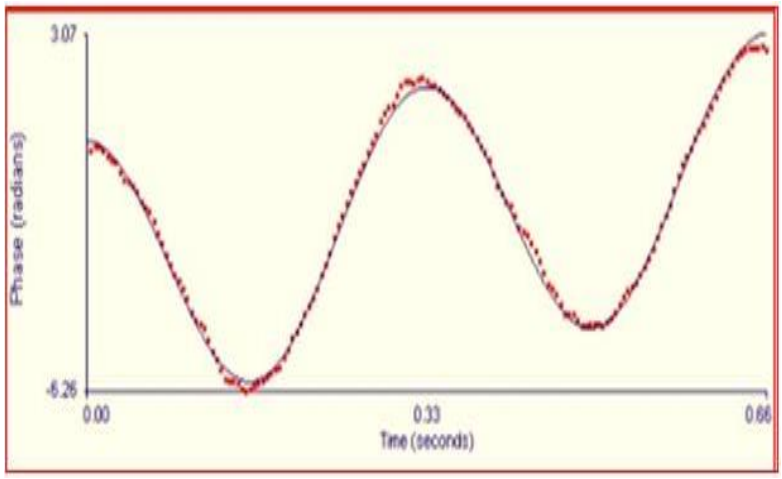


Figure 2. Results of liposome size before *C copticum* loading

DOI: https://doi.org/10.18502/aptj.v3i2.13459

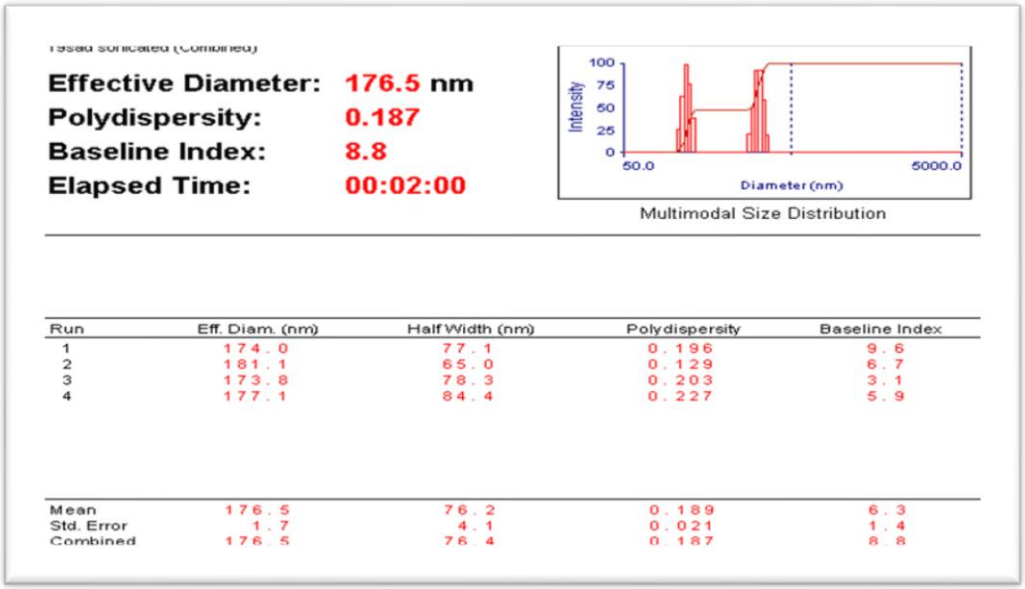


Figure 3. Results of liposome size containing *C copticum* (after loading)

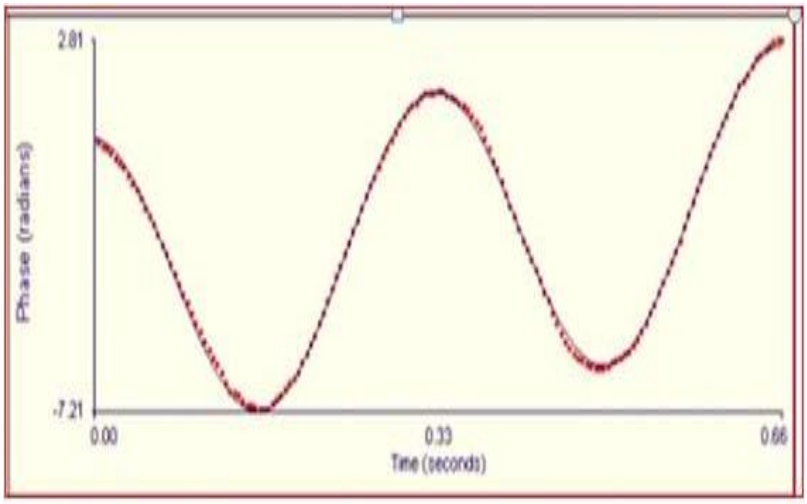


Figure 4. Results of liposome size containing *C copticum* (after loading

The percentage growth of logarithmic and stationary phases of *Leishmania major*[MRHO/IR/75/ER] on the *C copticum* is presented in **Figures 5** and **6**. The results demonstrated that the percentage growth is time-dependent in two phases. After 72 h, the percentage growth appeared to be higher than 90% in all doses in stationary and logarithmic phases. The result of the ELISA measurement is illustrated in **Figures 5** and **6**. As set out in these figures, after 48 h, the viability of parasites in stationary and logarithmic phases significantly decreased in *C copticum* compared to the control groups **(Figure 7)**.

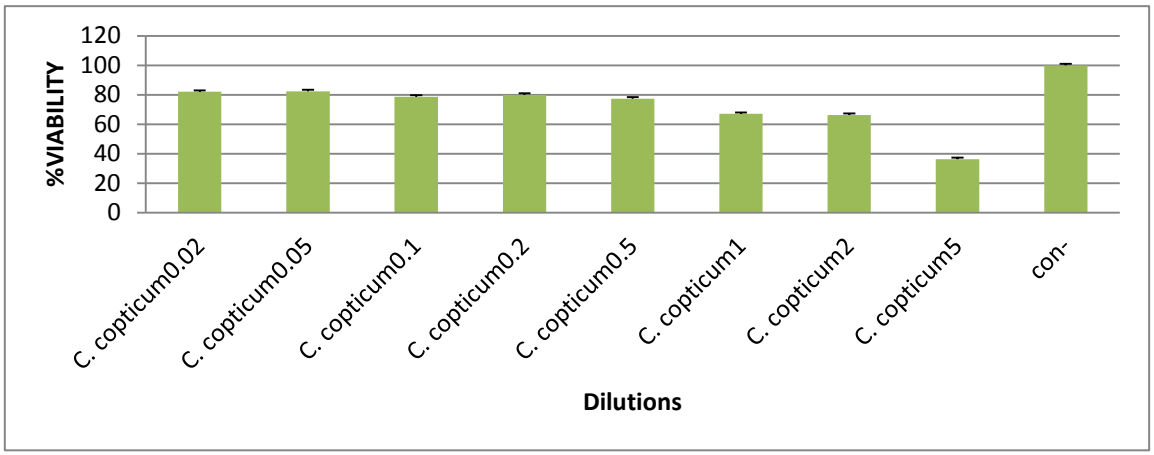


Figure 5. Average growth of *Leishmania major* by adding aqueous extracts of different dilutions by the average of the control group

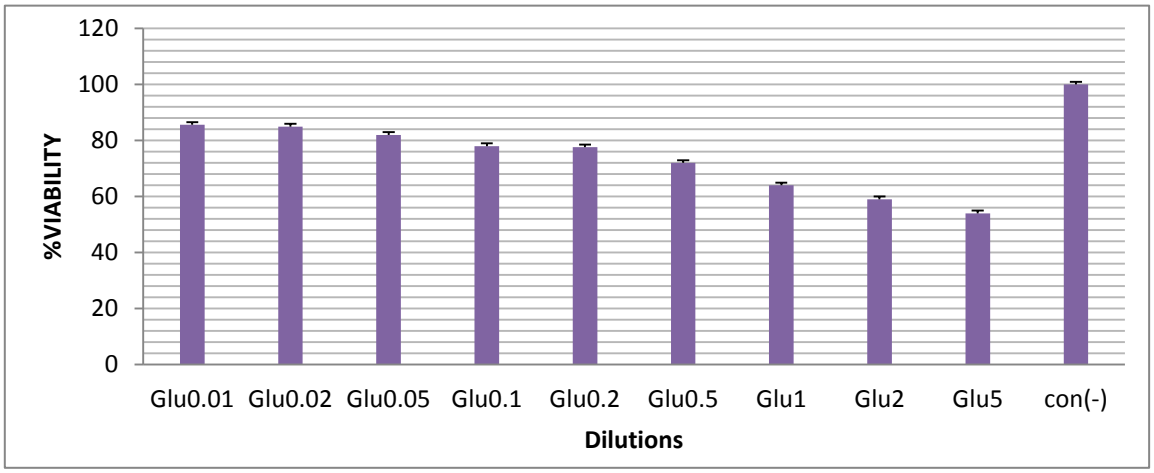


Figure 6. Average growth of *Leishmania major* by adding Glucantime different dilutions with an average growth control

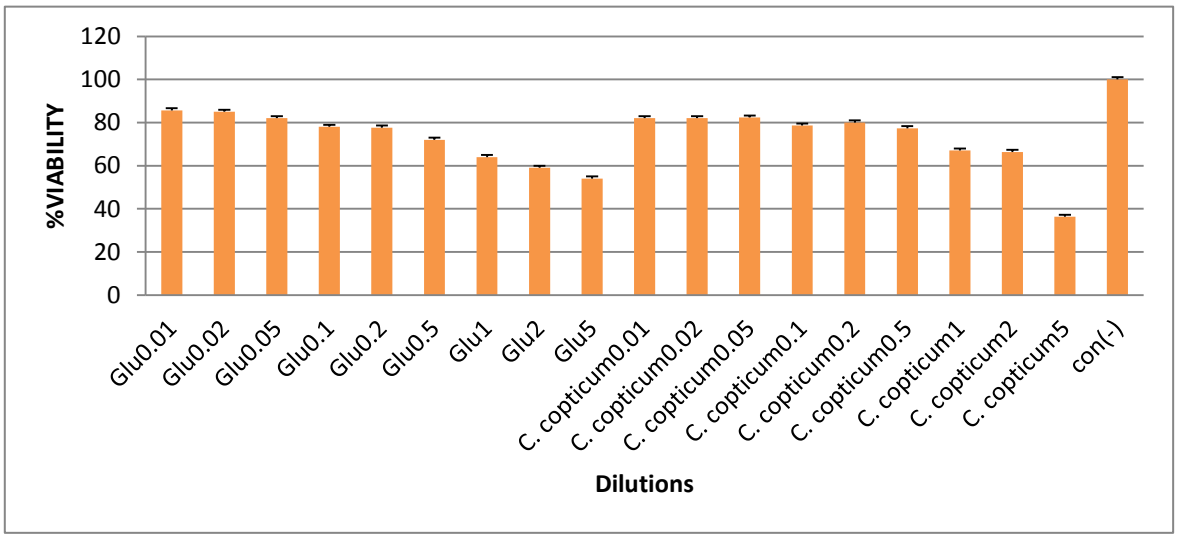


Figure 7. Comparing the average growth of *Leishmania major* with the average growth control by adding different dilutions of aqueous extract and Glucantime

DOI: https://doi.org/10.18502/aptj.v3i2.13459

Discussion

Leishmaniasis is considered **a** severe infection by the World Health Organization (14). Treatment of Leishmaniasis is difficult due to the intramacrophagic location of the infectious form. In the absence of a vaccine, there is an urgent need for effective drugs to replace or supplement those in current use. Currently-used drugs for Leishmaniasis include Amphotericin B and Pentamidine which suffer from some disadvantages such as high costs, toxicity, long-term treatment period, minor impact, and drug resistance (15). Despite giant strides made to prevent the disease, its prevalence is escalating in developing countries. So far, there is no effective drug available for the treatment of this infection. Hence, the greatest necessity at present is the preparation of a suitable, efficacious, definite, and cheap drug as a substitute. Herbal medicines and natural formulations are new candidates for treating Leishmaniasis. *C copticum* belongs to the ginger family and is one of the main ingredients of the rhizome of *C copticum* longa with antioxidant, antiparasitic, antifungal, antibacterial, antiviral, anti-inflammatory, and antiproliferative properties. To improve its efficacy, rapid absorption of the drug, maximization of its bioavailability in the target tissue, reduction of its toxicity, and decrease of the administered dose, drug delivery vehicles at the Nano-scale like **liposomes** are used as innovative drug delivery protocols (16). The present study has demonstrated the mean survival rate of the parasite with various concentrations of *C copticum* extract and Glucantime separately**.** So that if the intended extract shows a higher mean of survival in a specific concentration, this indicates the smaller effect of the extra on the parasite. In the case of a smaller mean survival rate of the parasite with the extract, it suggests the greater effect of the drug on the parasite. Previous studies have addressed the issue, as it is asserted in the study of Jose M. Perez-Victoria and Flora E. Arana in which the resistance rate of *Leishmania major* to Glucantime is up to 1.2 mg/ml. Also, Hadighi and Boucher reported that Glucantime-resistant individuals in Iran show a crossover reaction to other compounds of Glucantime and are resistant to them (17). Additionally, Sundar, S stated that in India, the number of Glucantime-resistant patients increased due to the number of patients with Kala Azar relative to treatment with Glucantime (18). Moreover, Sadeghian and Ziaei reported that the therapeutic effect of Glucantime shows a decrease in Cutaneous Leishmaniasis lesions with a secondary bacterial infection. They also indicated that, in the cases of unresponsiveness to treatment, the lesions should be evaluated for bacterial infection before repeating the treatment (19). Considering the studies by Kamiar Zomorodian. et al, it is asserted that the essential oils (EOs) obtained from Cutaneous Leishmaniasis a-Trepanned and p-cymene is identified as the main constituents of the EOs. Both Eos exhibited a broad spectrum of antibacterial and antifungal activity against the tested organisms. In addition, the EOs exhibited varying antifungal activities ranging from 0.25 to 16 µl/ml. Other studies by Bairwa and Rajawat reported that the extracted *C corpicum* bears some potentials like anti-Fungal, anti- Microbial, Nematicidal, ant-Helminthic, and anti-Filarial

properties (20). In the present study conducted in an ex vivo medium on the two phases of the life of the *Leishmania major*, it was manifested that Glucantime shows no effect on the parasite survival in 0.2, 1, 5, 25, and 125µg concentrations at 24 h and the growth of the parasite appears to be 100% at both logarithmic and fixed phases (P>0.05). However, the 250 and 125µg/ml concentrations had a significant effect on the parasite after 48 h in the fixed phase. After 72 h, only 125 µg/mL concentration was significantly effective for the parasite survival in the stationary phase. The percentage of survival rate for *Leishmania major* at various concentrations of *C corpicum* extract compared to the control group in the logarithmic and stationary phases at 24h were explored the results of which indicated that in the logarithmic phase, the percentage of parasitic survival at 600, 300, 150, 75, and 18.75 concentration of *C corpicum* extract turns out to be significantly different compared to the control group. Also, in the fixed phase at 24 h, the survival percentage of the parasite with 0.2, 1, 5, 25, and 125µg ml concentrations of *C corpicum* extract proved to be significantly different from the control group.

Conclusion

Our findings confirmed that liposomal *C copticum* demonstrates suitable Antileishmanial activity as well as lower side effects compared to the current drugs. The viability rate of promastigotes of rural Cutaneous Leishmaniasis, *Leishmania major*, treated by liposomal *C copticum* is time-dependent in both the logarithmic and stationary phases and leads to the killing of the parasite promastigotes. It was demonstrated that the *C copticum* aqueous extracts has a greater affinity for anti-Leishmania than the control group. The activity of *C copticum* aqueous extracts has good selectivity and high capacity in the treatment of Leishmania (L) major species. The other advantages of this treatment method are less toxicity and low cost.

Conflict of interest: The authors declare no conflict of interest.

Funding: This research received a specific grant with No: 4781 based on a thesis submitted in the School of Medicine, Yazd Shahid Sadoughi University of Medical Sciences. Yazd, Iran.

No competing financial interests exist.

Acknowledgments: The authors of this paper would like to express their gratitude to the authorities of Shahid Sadoughi University of Medical Sciences for their cooperation and financial support as well as the colleagues who assisted us in the experimental procedure, especially Mrs. Marziyeh Beigum Modares Sanavi, Dr. Mahdiyeh Emtiazi; and Mrs. Zahra Esmaili.

Ethical considerations: The experiments were confirmed by Ethical Committee in Vice Chancellor of Research in Shahid Sadoughi University, (Ethical No: IR.SSU.MEDICINE.REC.1395.333).

Authors' contribution: Study concept: AFB. Analysis and interpretation of data: MM and ZE. Drafting of the manuscript: AFB and MM. Critical revision of the manuscript for important intellectual content: MRM and HE. Statistical analysis: MM and AFB.

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DOI: https://doi.org/10.18502/aptj.v3i2.13459

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