The study of the inhibitory effects of aqueous and ethanolic extracts of *Ziziphora tenuior L.* on the proliferation of *Leishmania major* amastigotes

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Background: Leishmaniasis is a parasitic disease originated from species of the genus *Leishmania* from the Trypanosomatidae family, with three main clinical forms of cutaneous, mucocutaneous and visceral. Every year, many new cases of the disease are reported in endemic areas. Nowadays, in non-endemic regions, the incidence of the disease has also created tension. Medicine side effects, reports of resistance against currently used drugs, and the absence of a putative vaccine have made researchers look for new effective drugs. The objective of the present study was to evaluate the effects of the ethanolic and aquatic extracts of the *Ziziphora tenuior L.* on the prolifration of *Leishmania major* amastigotes.

Methods: The J774 cell lines were infected by promastigotes, at stationary phase, and treated with different concentrations of both extracts. After 12, 24 and 48 hours (h) at 37°C, the macrophages were stained with Giemsa, and the mean number of amastigotes in the macrophages was determined and compared with the control group. Finally, the ED50 of the extracts was calculated through statistical analysis.

Results: Aquatic and ethanolic extracts of *Ziziphora tenuior L.* reduced the number of amastigotes within the macrophages. Following 48h of treatment, the ED50 of the aquatic and ethanolic extracts of the plant were 15.75 mg/ml and 15 mg/ml, respectively. Hence, there was no significant difference between the ED50 of the aquatic and ethanolic extracts of the plant $P<0.05$).

Conclusion: Aquatic and ethanolic extracts of *Ziziphora tenuior L.* have a considerable anti-*Leishmania* effect, hence the significnace of further studies using the animal model of CL.

Keywords: *Leishmania major*, *Ziziphora tenuior L*, amastigote

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INTRODUCTION

Leishmaniasis is a parasitic disease caused by various species of the genus *Leishmania* from trypanosomidae family, and can be observed in various clinical forms of cutaneous (CL), visceral and mucosal lesions [1,2]. The World Health Organization has recommended and supported research on various aspects of this major tropical disease. According to the world health organization (WHO), more than 12 million people are affected by the disease every year, 350 million people are at risk, and 2 million new cases are annually discovered [3,4]. The first line drugs in the treatment of leishmaniasis are pentavalent antimonials (pentavalent Sb or SbV), including meglumine antimoniate (glucantime) and

sodium stibogluconate (pentostam) [5]. Affecting parasitic enzymes, specifically interrupting the phosphokinase enzyme, these compounds prevent the production of adenosine triphosphate and the beta-oxidation of fatty acids [6].

Over the recent years, the lack of adequate response to treatment, renal and cardiac toxicity, the high cost of the foregoing drugs, and the increased resistance to sbv, all have led to the introduction of new anti-leishmaniasis drugs such as miltefosine, amphotericin B, paromomycin and ketoconazole. These drugs are, more often than not, costly and inaccessible [7,8]. Such restrictions in the treatment of leishmaniasis have led researchers to focus on new antiparasitic drugs, particularly medicinal herbs [9]. Some herbs have extensively been used in traditional medicine due to their antibacterial, antiparasitic and anti-inflammatory effects: Artemisia siberi Besser and Scrophuiaria striata Boiss [10], herbal extracts, pepper seeds, almond powder and Castor oil [11], nettle, Artemisia, Tarijah, Case, Tarragon, Garlic and Eucalyptus [12] and garlic extract [13] have all proved to be effective in eliminating parasites.

Ziziphora tenuior, a family of mints, is a herb with small, reciprocal, less or leaner petiolate leaves, and full of small white, pink and purple flowers . All parts of the plant are used as pharmaceutical agents, especially its flowers and leaves. An intestinal disinfectant, *Ziziphora* plant has many therapeutic benefits, including antioxidant, antibacterial, anti-inflammatory and anti-parasitic effects [14]. The most effective ingredient in the aerial part of the plant is polgen and among other components are flavonoids, saponins, terpenoids, alkaloids, menton, isomenton, neuizomenton, pyropytonone, thymol, xenylene, antioxidants, sulfide compounds [15], Methyl cyclopentane, carboxylic acid and Camphor [16]. In 2012, Shaafei et al. identified the chemical compounds of *Ziziphora* essence to investigate its antimicrobial effect on *Chloromycosis marxanus* yeast by the blot method presented in plate. It was shown that this plant has an inhibitory effect on the yeast, and its antimicrobial compounds are phenolic oils and terpenes. As a result, their mechanism of antimicrobial activity is similar to other phenolic and terpenic compounds [17].

The aim of this study was to investigate the anti Leishmania effect of the aquatic and ethanolic extracts of *Ziziphora tenuior* on the proliferation of *Leishmania major* amastigotes in the amastigote/macrophage model.

MATERIALS AND METHODS

Parasite culture

This experimental study was initially designed for the mass production of parasites *Leishmania major* (MRHO/IR/75/ER) inoculated into the base of inbred Balb/c mice tail. After approximately 4 weeks at the inoculation site, a lesion of leishmaniasis became the source of the parasite for culture. This work was performed to avoid using parasites that have repeatedly been under the passage in the medium. The culture was done by the autopsy of the infected mice, and their hemogenised popliteal lymph nodes were employed as affected tissues for inoculation into NNN modified culture medium with penicillin and streptomycin (1%). The culture flasks were incubated at 25 ± 1°C. Following a complete propagation of the parasites, the muliplied promastigotes were transferred to enriched RPMI 1640 medium supplmented with Fetal calf serum (FCS), penicilin 100 unit/ml and streptomycin 100 µg/ml and stored at 25 ± 1°C for 6-9 days to produce metacyclics.

Preparation of the extracts

Ziziphora plant was collected from Oshtorankoh of Lorestan province, Iran. The confirmation code was developed by the Research Center for Agriculture and Natural Resources and the Department of Botany of the University of Isfahan, Iran.

For extraction, the aerial parts of the plant were washed three times in water, and dried in shade; ethanol and water solvent fractions were extracted via Succillus method [18].

Macrophage culture

The J774 cell line was obtained from Pasteur Institute of Tehran, Iran, cultured in cell culture flasks containing RPMI 1640 medium supplemented with %20 inactivated fetal calf serum (FCS), and incubated at 37 ˚C and 5% carbon dioxide. The cells were then harvested from the flasks using cell scrapers, and a number of 1×10^6 cells were transferred to each 3.5 cm well of the six-well

plates. Prior to transferring the macrophages, the bottoms of the wells were covered with sterilized 22x22 coverslips.

Metacyclic promastigotes of *L. major* (MRHO/ IR /75/ER) were added to the J774 macrophages cell line culture at a ratio of 7: 1. After 24 hours of plate incubation at 37 ˚C and % 5 CO2 condition, metacyclic promastigotes that did not enter the cells were removed by PBS and fresh complement RPMI 1640 medium was added to the wells.

Treatment of infected cell lines with aquatic and ethanolic extracts of *Ziziphora* plant

Wells containing macrophages and amastigotes were treated with aquatic and ethanolic extracts of *Ziziphora* in concentrations of 5, 10, 15, 20, and 30 mg/ml. For this purpose, the outer medium was drained and 2 ml of the fresh culture medium containing the mentioned concentrations of extracts was added; three wells were further considered as controls. After 12, 24 and 48 hours, the coverslips were removed from wells' bottoms,fixed using methanol, and stained with Giemsa.

A direct microscopic method was used to count the intracellular amastigotes in the control and test groups. In this way, 100 macrophages per each well were investigated and the existing amastigotes were further counted. Finally, the average number of amastigotes in a macrophage was calculated. The final result was obtained by the mean number of the three wells considered for each concentration (triplicate wells). Only healthy and distinct macrophages were examined with grayish cytoplasm and purple-red nucleus. The amastigotes inside were oval-shaped along with kinetoplast. It is to be noted that 0.2% DMSO, identified as safe for amastigotes in previous studies, was used for the preparation of different concentrations of ethanolic extract; the same amount of DMSO was added to the control group.

The results of the experiment were analysed by T-test ANOVA and Tukey HSD test at a confidence level of $p \leq 0.05$. Data were ultimately analyzed through the use of SPSS software, and further specified was the ED50 (Effective Dose 50%), the drug concentration that removed 50% of the parasites.

RESULTS

Contamination of J774 cells with promastigotes

The results of this step are shown in Figure 1, where the promastigotes penetrating the macrophages and transformed into the amastigotes are also depicted.

The results concerning the anti-amastigote effect of the aquatic extract are shown in Figure 2, with respect to the mean number of amastigote per infected macrophage.

Figure 3 demosntrates the results related to the anti-amastigote effect of the ethanolic extract in terms of the mean number of amastigotes present in each infected macrophage.

The results of ED50 obtained from the aquatic and

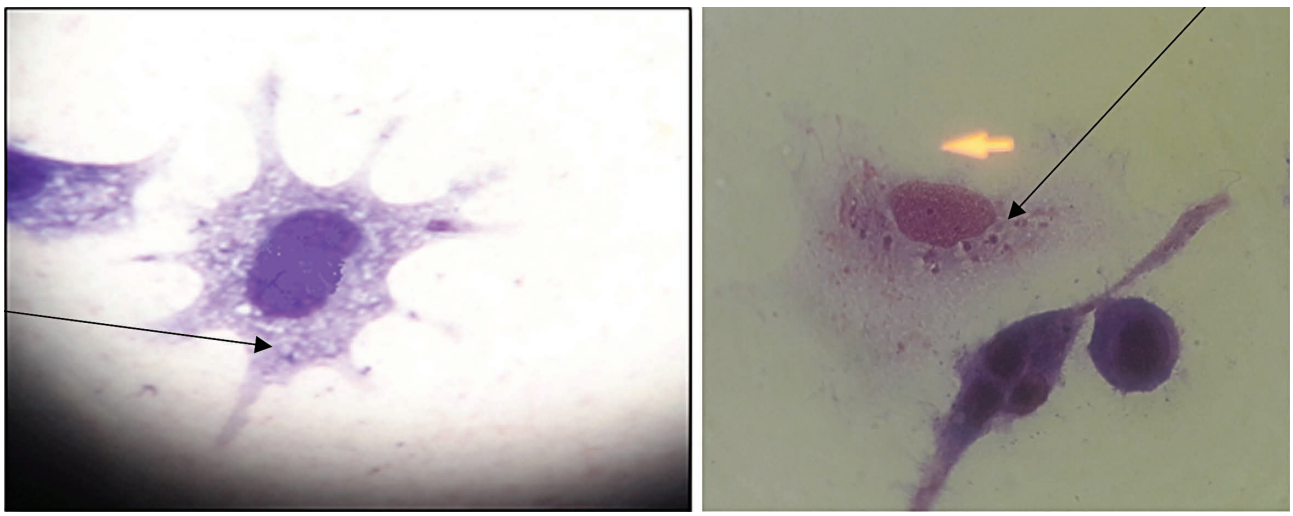

Figure 1. Macrophages containing phagocytysed parasites which are transformed from promastigotes to amastigotes, magnification ×100

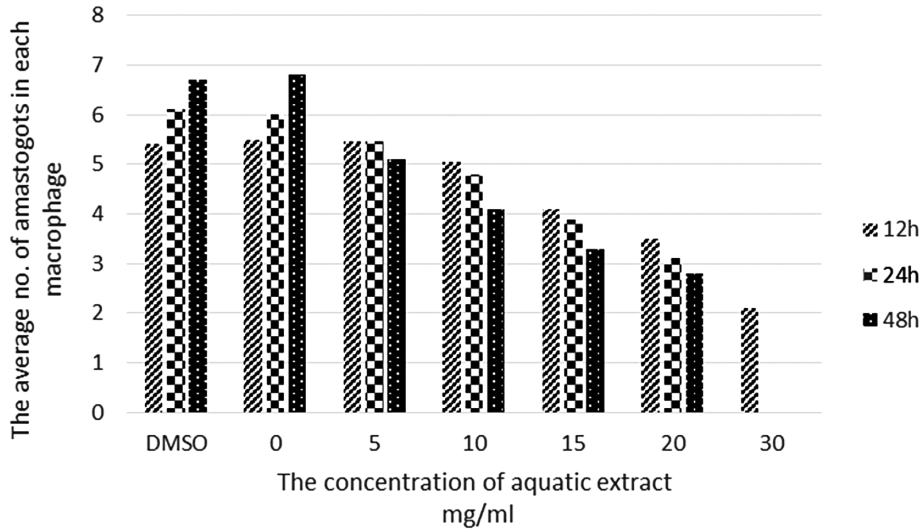

Figure 2. The mean of amastigotes per macrophage in various concentrations of aquatic extract of the plant *Ziziphora*

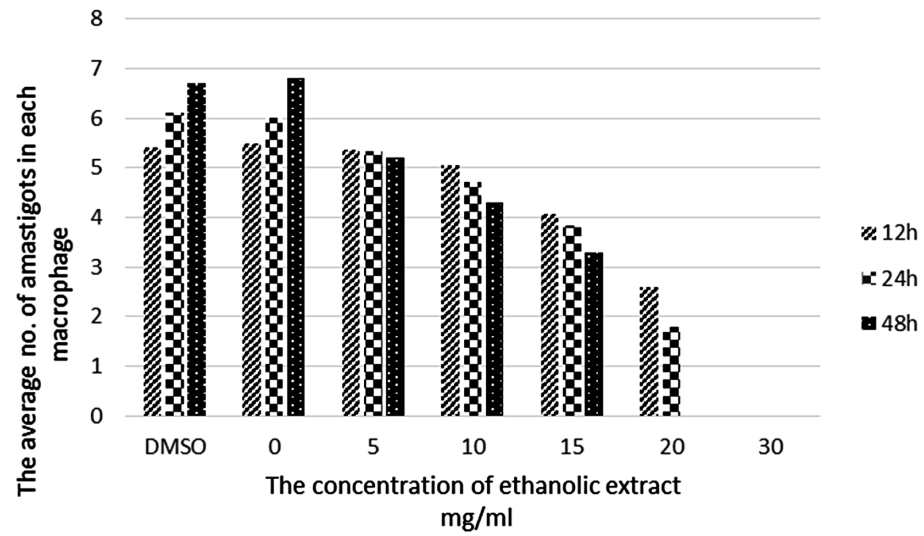

Figure 3. The mean number of amastigotes per macrophage in various concentrations of ethanolic extract of the plant *Ziziphora*

ethanol extracts of *Ziziphora* are listed in Table 1.

Investigating the effect of the aquatic and ethanolic extracts of *Ziziphora* plant on macrophages, it was observed that, at concentrations above 30 mg/ml, each extracthad a cytotoxic effect on macrophages.

Table 1. ED50 of aquatic and ethanolic *Ziziphora* extracts on amastigote of *Leishmania major* after 24 and 48 h

ED50	Incubation time		
	12h	24h	48h
ED50 of aquatic extract (mg/ml)	20	18	15.75
ED50 of ethanolic extract (mg/ml)	19	17	15

DISCUSSION

Despite the myriad studies conducted on leishmaniasis, it is still a major health problem in the involved countries. The main therapeutic treatments against leishmaniasis are pentavalent antimony (sbv) compounds, containing meglumine antimoniate or glucantime [19]. However, the toxicity and side effects of glucantime, as well as a high incidence of non responses and resistance to the drug have been reported [20].

Nowadays, according to the World Health Organization, over 80% of the world's population use herbal local remedies to treat CL. It has also been shown that 50% of medications are herbal

drugs either derived directly from plant extracts or synthesized on the basis of plant compounds [21].

Based on various studies, plants are known as effective and available sources of anti-parasitic compounds, containing anti-leishmaniasis effects. Research has shown that increased cellular and humoral immunity from plant extracts, due to compounds such as flavonoids, saponin, terpenoids, and alkaloids in plant extracts, augments the level of interleukin 2 and interferon gamma in macrophages [22,23].

Among the herbs, *Ziziphora tenuior L.* is a family of mints, all parts of which (especially its flowers and leaves) are utilized as pharmaceutical substances. The most important active ingredient in the *Ziziphora* organs is pulgen; among other compounds are flavonoids, saponins, terpenoids, alkaloids, menthon, isomenton, neuizomenton, pypentinone, thymol, xenylene, antioxidants, sulfide compounds [24], and alpha and beta-toedone Methyl cyclopentane, carboxylic acid and camphor [16,25].

So far, no study has been conducted to confirm the anti-leishmaniasis effect of the *Ziziphora tenuior L.*. In the present research, using the macrophage-amastigote infectious model, the effect of aquatic and ethanolic extracts of *Ziziphora tenuior L.* on reference strain of *L. major* (MRHO/IR/75/ER) was investigated. The results showed that the number of eliminated parasites has a direct relationship with the the extract concentration.

Amanzadeh and colleagues examined the effect of the aquatic and alcoholic extracts of shallot on the growth of *L. infantum* under *in vitro*, stating that its inhibitory effect on parasite growth is due to the sulfide content of the extract [26]. As was mentioned, the *ziziphora* plant contains sulfide compounds as well, to which the inhibitory activity of the *ziziphora* extract can be attributed [15].

Shamsuddini et al. investigated the effect of *Mimozatnoee flora* extract on the proliferation of *Leishmania in vivo;* it was shown that the plant had anti-inflammatory effects, which coupled with the formation of collagen fibers, resulted in normal wound healing [19]. Owing to the anti-inflammatory and antioxidant compounds of the *Ziziphora tenuior L*, it is safe to say that the extract of this plant has a role in collagenization, conducing to the repair of the lesions caused by leishmaniasis [14]. Studies on 27 Lebanese plant strains in 2008 showed that these species have anti-leishmanial effects due to the presence of antioxidant genes [27]. Plants belonging to the mint family, including cacti, also contain antioxidant compounds, which appear to be effective in its anti-leishmanial activity [14].

Dalimi et al. studied the effect of Artemisia extract on *Leishmania major* growth in laboratory conditions and showed that certain chemical compounds, such as alpha and beta-tudon, methyl cyclopentane carboxylic acid and camphor, have antimicrobial, antiviral and antiparasitic activities [10]. Some of the foregoing compounds are present in the *Ziziphora* plant, to which the anti-parasitic activity of the extract of this plant can be attributed [16].

The present results showed that the aquatic and ethanolic extracts of the *Ziziphora tenuior L.* had does- and time-dependent inhibitory effects on the proliferation of the amastigotes within the macrophages. Comparing the ED50 of ethanolic and aquatic extracts, we conclude that the former extract, having less ED50, is more effective than the aquatic extract, a difference not statistically significant.

CONCLUSION

The aquatic and ethanolic extracts of *Ziziphora tenuior L.* are able to reduce the number of amastigotes present in macrophages, an effect which is dose and time dependent. It is worth noting that in concentrations higher than 30 mg/ml, the disintegration of macrophages may occur, hence the fact that the use of high doses should be considered with greater precision and caution. Finally, *Ziziphora* plant probably has the ability to control and treat leishmaniasis, a fact which requires more tests and research on animal models and volunteers alike; its effect on the stimulation of the host immunity system should further be specified.

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Conflict of Interest: None declared.

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