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Original Research

Treatment Role of Terpenoid Fraction of *Perovskia abrotanoides* Kar. on Zoonotic Cutaneous Leishmaniasis in Animal Model

Masoud Sadeghi Dinani[1], Javad Rezapour[2], Simindokht Soleimanifard[3]*

[1]Department of Pharmacognosy, School of Pharmacy, Isfahan University of Medical Sciences & Health Services, Isfahan, Iran
[2]Research Committee, School of Pharmacy, Isfahan University of Medical Science & Health Services, Isfahan, Iran.
[3]Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences & Health Services, Isfahan, Iran

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Abstract

Leishmaniasis is one of the infectious diseases in tropical countries that is seen as cutaneous, mucocutaneous and visceral forms. Due to the side effects, costs and effectiveness of anti-Leishmaniasis drugs, many clinical trials have been conducted on the importance of plant-based substances against Leishmaniasis. Therefore, the following study was performed to evaluate the therapeutic effects of *Perovskia abrotanoides* Kar. terpenoid fraction on cutaneous *Leishmania*sis in Balb/c mice. The ethanol: water (80:20) extract of the plant flowers was fractionated by Medium Pressure Liquid Chromatography using RP-18 column. The terpenoid-rich fraction was detected by TLC and HNMR analyses and evaluated for healing effects on cutaneous Leishmaniasis in mice with concentrations of 0.8%, 1.6% and 3.2 µg/mL. The effect of that was evaluated using ANOVA statistical tests. The results indicate that terpenoid of *P. abrotanoides* in 3.2% concentration is effective in treating cutaneous Leishmaniasis in mice and also increases the lifespan and decreases the parasite burden of infected mice. According to this result, it is suggested that the effectiveness of this extract on the treatment of cutaneous Leishmaniasis to be evaluated as a clinical trial in humans.

Keywords: Cutaneous leishmaniasis; *Perovskia abrotanoides* Kar.; Treatment; Parasite load

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*Corresponding Author: Simindokht Soleimanifard
Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences & Health Services, Isfahan, Iran
Email: Soleimanifard@med.mui.ac.ir
Tel: +989132943369

Introduction

Leishmaniasis is a parasitic disease caused by a *Leishmania* protozoan of the Trypanosomatidae family and is a disease transmitted by arthropods. Types of this disease occur in the form of cutaneous, mucocutaneous and visceral Leishmaniasis. World Health Organization (WHO) has identified Leishmaniasis as one of the ten most important parasitic diseases in the world. The disease is present in more than 88 countries in tropical and subtropical regions and the number of cases of the disease annually is estimated at 1.5 million cases of cutaneous Leishmaniasis and 500,000 cases of visceral Leishmaniasis. More than 350 million people are at risk of developing with the disease. Different species of *Leishmania* cause cutaneous Leishmaniasis, including *Leishmania major, L. tropica* and *L. aethiopica* [1,2]. Despite the fact that this disease is self-limiting, its treatment has been considered by WHO for various reasons, including the unsightly scars, as well as the lack of improvement in some cases of the disease and its chronicity. The first line of treatment for this disease is pentavalent antimonial salts [3]; however, significant side effects of these drugs, as well as the lack of improvement in more than 43% of patients, have raised the need to use other drugs [4,5]. Other drugs used in the treatment of this disease include Amphotericin B, Pentamidine and Miltfusine [5]. These drug treatments have limitations such as high cost, painful injection, patient rejection, as well as significant drug side effects, and these limitations have increased the need for research to achieve new drugs. Today, herbal remedies have received less attention despite the significant effects they have shown in the treatment of many diseases, as well as the lower cost of treatment and fewer side effects. In Iran, many plants have been used to treat Leishmaniasis, including *Artemisia annua* L., *Thalictrum alpinum* L., *Curcuma longa* L., *Camellia sinensis* (L.) Kuntze, *Pentalinon andrieuxii* (Müll.Arg.) B.F.Hansen & Wunderlin [6]. The medicinal plant *Perovskia abrotanoides* Kar. (Barazambal in Persian) belongs to the Lamiaceae family, which grows in the northern, northeastern, eastern and central regions of Iran and in countries such as Afghanistan, Pakistan and Turkmenistan. In Iran, three species of the genus Perovskia are found, of which *P. abrotanoides* is the most abundant. Previous investigations on this plant resulted in isolation of bioactive tanshinones with leishmanicidal, antiplasmodial and cytotoxic activities. There are also other reports of isolation and identification of two triterpenes with a novel carbon skeleton from *P. abrotanoides* [7]. The compounds found in this plant include alkaloids, tannins, flavonoids and saponins. Cryptotanshinone, 1-oxomiltirone, 1-oxocryptotanshinone and 1-β-hydroxycryptotanshinone are also active compounds extracted from the roots of this plant and have anti-*Leishmania* effects [8,9]. In Persian medicine, Barazambal has been used alone or in combination with other medicinal plants to treat many diseases, including cutaneous Leishmaniasis and malaria. Methanolic and ethanolic extracts of this plant have been shown to be effective on *L. major* [10]. In addition, this plant is used as a tonic, disinfectant, cooling, rheumatic pain

reliever, anti-inflammatory, worm repellent, and antibacterial [8,11]. However, many studies are needed to identify the therapeutic effects and compounds in this plant [6]. As mentioned earlier, plants of the Lamiaceae family have anti-Leishmaniasis effects. Also, the terpenoid part of the aerial part of Barazambal plant extract showed leishmanicidal results in vitro. [12]. No study has been done so far to investigate the effects of Barazambal in the treatment of Leishmaniasis in animal models; thus, in this study, we aimed to investigate the effects of terpenoid fraction of aerial parts of Barazambal on the improvement of cutaneous Leishmaniasis in an animal model.

Materials and Method

This study is an experimental study to evaluate the effect of the terpenoid fraction of *P. abrotanoides* on lesion healing caused by *L. major* (MRHO / IR / 75 / ER) and its comparison with standard glucantime in Balb/c mice in 1398. The study has been done in the Faculty of Pharmacy of Isfahan University of Medical Sciences with the approval of the ethics committee of that university (code: IR.MUI.RESEARCH.REC.1398.327).

Extraction and preparation of terpenoid fraction

Barazambal plant (*P. abrotanoides*) with the cooperation of Kashan city natural resources department expert from Shahsavaran region of Kashan city, was collected and after identification and approval, an herbarium sample was prepared and registered under voucher number 2277 in the herbarium of Pharmacognosy Department of Isfahan School of Pharmacy. The aerial parts of the plant including flowering branches were dried in the shade and pulverized by an electric mill. Then, 1300 g of plant powder was extracted by maceration method. For this purpose, the plant powder was placed in an extraction tank in the presence of ethanol: water (80:20) at a rate of 4 liters for 3 days. Each step was repeated 4 times. The filtered extracts were added together and concentrated as much as possible by a rotary apparatus. Finally, the solvent residue was separated from the Barazambal plant as a dry powder using a freeze dryer [13]. The resulting dried extract was then fractionated by Medium Pressure Liquid Chromatography (MPLC). For this purpose, the glass column of the device containing RP-18 silica gel (Lichroprerp®, 25-40 μm) was used as a fixed phase and the linear gradient of water and methanol was used as a moving phase. 25 primary fractions were obtained and after examining the fractions by TLC on GF254 silica gel plates and using BAW solvent system (butanol: acetic acid: water in 4: 1: 5 ratio) and Cerium (IV) sulfate reagent, based on the similarity of their compounds, the resulting fractions were prepared in the form of 6 final fractions and finally concentrated by a rotary machine and completely dried by a freeze dryer (lyophilizer). According to the results of TLC and also the study of the initial HNMR spectrum of the fractions, finally the fractions P.A.4, PA5 and PA6 containing terpenoid compounds were detected which due to the remarkable similarity of the compounds were mixed and for Anti-*Leishma-*

nia effects were used.

Preparation of topical form of terpenoid fraction

In order to prepare a topical drug form, the terpenoid fraction obtained from the total aerial extract of Barazambal was added to methanol in a ratio of 1 to 10 and added to eucerine base so that the final three topical forms with concentrations of 0.8%, 1.6% and 3.2% of the fraction were prepared. Considering that on average 12.5 mg of ointment is enough to cover the wound, this amount of ointment contains 100 µg, 200 µg, and 400 µg of the fractions obtained from the total extract of the aerial parts of Barazambal.

The effect of fractions on healing of Leishmaniasis lesion

Cultivation of L. major promastigotes

Frozen promastigotes of *L. major* (MRHO/ IR/75/ ER) were obtained from the Department of Parasitology & Mycology, School of Medicine, Isfahan University of Medical Sciences. Promastigotes were first cultured in NNN (Novy-MacNeal-Nicolle) medium at 24 ± 1 °C and then for mass production were introduced into RPMI1640 (Roswell Park Memorial Institute Medium 1640) containing Fetal Bovine Serum (FBS) (10%) Streptomycin (100 µg/mL) and penicillin (100 units/mL).

Leishmaniasis ulcer in laboratory model

Twenty-five female Balb/c mice 4 to 6 week old were purchased from the Royan Institute of Isfahan and placed in the animal nest of the Faculty of Medicine, Isfahan University of Medical Sciences. The standard *Leishmania major* strain (MRHO / IR / 75 / ER) was injected subcutaneously into the tail base of each mouse at 2×10^6 after culture and reaching the static phase. After 2 to 3 weeks, a small, hard nodule formed at the site that turned into a lesion after about 2 weeks. Direct sampling on the slide and microscopic observation were used to ensure the presence of parasites in the wound.

Mice were divided into 5 groups, so that the first, second and third groups received ointments containing 100, 200 and 400 µg terpenoid fraction respectively, the fourth group received glucantime for the positive control at a dose of 60 mg/kg [14]. The fifth group as a negative control did not receive any medication.

The treatments were applied topically twice a day. The mean wound diameter of mice was measured with a caliper before the start of treatment and then during the treatment period weekly up to 4 weeks after its end. On day 21, one mouse from each group was randomly selected and its spleen was examined for parasite burden or parasite load. If in the average of 10 microscopic fields of view with oily lens, 1 Leishman body was seen +1, if 1 to 10 Leishman bodies were seen +2, if 11 to 100 were seen +3 and if 101 to 1000 were seen it was considered +4 and if no parasites were seen in the whole slide, it was considered negative [14]. The results of lesion size, spleen parasite burden and also the number of days of survival of mice after treatment were compared in the treated groups and the group receiving glucantime and the control

group receiving no treatment by one-way analysis of variance (ANOVA). And if there was a significant difference, the groups were compared by Post hoc test Tukey. P value < 0.05 was considered as a significant level.

Results

Results of measuring wound diameter and number of days of survival of mice

Post hoc test showed that the mean wound diameter (mm) of mice in the second to fourth weeks in the treated groups was significantly lower than the control group. From the second week of treatment, the wound area decreased and statistical analysis showed that there was a significant difference between the 5 groups and with increasing the concentration of fraction in the ointment, the average wound diameter decreased ($p < 0.05$) (Figure 1). Also, the mean of wound size (mm) in the group treated with glucantime was statistically similar to the group treated with ointment with a concentration of 3.2% (Table 1 and Figure 2).

Table 1. Mean diameter of wound size in different groups

P value	Positive control (glucantime)	Negative control (without treat)	3.2 Concetration of fraction	1.6 Concetration of fraction	0.8 Concetration of fraction	Time
	Mean wound size ± SD (mm)	Mean wound size ± SD(mm)	Mean wound size ± SD (mm)	Mean wound size ± SD(mm)	Mean wound size ± SD (mm)	
0.99	18.6 ± 3.4	19.2 ± 5.3	19 ± 3.7	19.2 ± 2.7	18.9 ± 2.2	Before treat
0.63	18.2 ± 3.2	19.9 ± 5.5	18.5 ± 3.5	19.6 ± 2.5	20.3 ± 2.3	First week
0.04	17.3 ± 2.9	20.8 ± 5.6	17.9 ± 3	18.7 ± 1.9	20.3 ± 2.4	Second week
0.01	16.6 ± 2.8	22.1 ± 5.7	17.4 ± 3	18.7 ± 2.3	19.5 ± 2.8	Third week
0.02	15.5 ± 2.8	23.2 ± 7.7	16.6 ± 3.3	18.1 ± 2.6	19.3 ± 2.8	Forth week

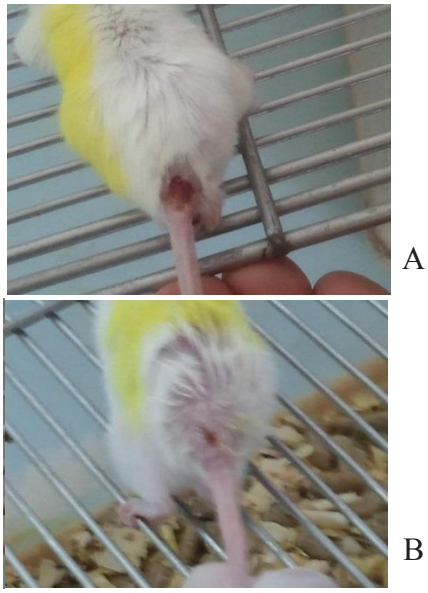

Figure 1. A: Leishmaniasis ulcers in mice before treatment, B: Leishmaniasis ulcers in mice after treatment with ointment containing terpenoid fraction in concentration of 3.2%

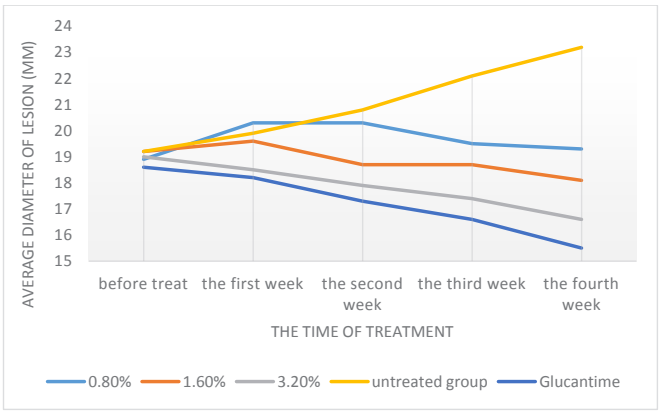

Figure 2. The average diameter of lesion (mm) in the period before the intervention until the end of the fourth week of the intervention

Also, the mean number of days of survival of mice was significantly different between the 5 groups, so that the average number of days of survival in the two groups of glucantime and ointment with a concentration of 3.2% was significantly higher than the group with a concentration of 1.6% and in the group with a concentration of 1.6% was higher than the two groups of ointment with a concentration of 0.8% and without medication ($p < 0.05$) (Table 2 & Figure 3).

Statistical tests showed that compared to the control group the parasitic burden decreased with increasing the concentration of fraction and in the treatment group with a concentration of 3.2% drug was similar to the group treated with glucantime (Figure 4).

Table 2. Mean number of days of survival of mice in different groups

P-value	Standard deviation	Days of survival of mice	Fraction concentration in ointment
< 0.001	2.8	9.2	0.8%
	4.9	16.5	1.6%
	7.7	25	3.2%*
	4.2	6.7	Without treatment
	7.03	25.2	Glucantime*

* groups with more survival from others in confirm of statistical analysis

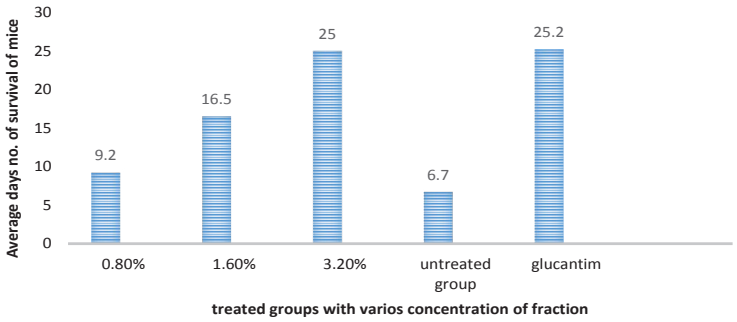

Figure 3. Mean number of days of survival of mice in different groups

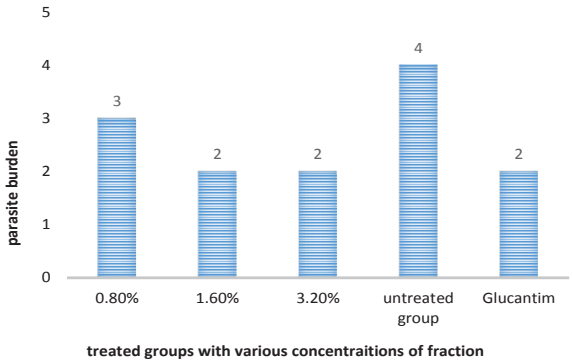

Figure 4. Amount of parasitic burden in different groups

Discussion

Many studies have been done on cutaneous leishmaniasis and now, the first line of treatment is pentavalent antimony salts. Other drugs such as antimalarial drugs (chloroquine and quinacrine), emethine, metronidazole and some antibiotics such as monomycin, tetracycline and rifampin are also used [15,16]. However, due to problems such as drug resistance and side effects of first-line drugs, observation of chronic cases of leishmaniasis, as well as the spread and increase of cases of leishmaniasis, further efforts are required to find new drugs that can be a good alternative to previous drugs [8]. Meanwhile, according to numerous studies on medicinal plants and their effects on the treatment of many parasitic diseases, the use of these plants is an important option for leishmaniasis. It is estimated that today 70% of the global community has an approach to the use of medicinal plants in various forms in daily life and the treatment of various diseases [17].

The leishmanicidal effect of terpenoid fraction of *P. abrotanoides* has been proven. Twenty-nine components have been detected in the essential oil of *P. abrotanoides*, representing 98.9% of the total oil composition. 1,8-Cineole (32.4%), myrcene (13.0%), α-pinene (10.2%), camphor (9.1%), β-caryophyllene (7.9%), α-humulene (6.4%), camphene (5.0%) and α-bisabolol (2.6%) were found to be the major constituents of the oil [7].

So in this study, the effect of terpenoid fraction of *P. abrotanoides* extract on zoonotic cutaneous Leishmaniasis was evaluated in a mouse model [18,19].

In the present study, the anti-leishmanial effect of the terpenoid fraction was confirmed after observing wound healing and reducing the mean wound diameter and a significant difference between wound sizes in the groups treated with topical ointment in comparison to positive and negative control groups. Ointment was prepared based on methanol at a percentage of 1%. This amount of methanol has no effect on the treatment process [20].

Also, *L. major* infection in mice is fatal; however, there was a significant difference between the survival time of treated mice and the negative control group. Also, since there was a significant difference between the parasite burden of treated leishmaniasis and control groups, the effectiveness of the terpenoid fraction of Barazambal plant in the treatment of leishmaniasis is proven. There are other methods for determining parasitic load such as real time PCR and culture microtitration, but due to the high cost and being time consuming, we chose the method of counting parasites in the spleen, which is cost-effective and provides acceptable results [14].

During the experimental stages, it was found that these effects were concentration-dependent, so that the effect of the 3.2% ointment, equivalent to 400 µg terpenoid fraction, was very similar to the effect of the first-line drug, glucantime. Jafari *et al*. showed that ethanolic extract of roots and leaves of this plant has anti-leishmanial and anti-parasitic effects due to its terpenoid compounds including monoterpenes and sesquiterpenes like 1, 8-cineole, myrcene, pinene, camphor, caryophyllene, humulene, camphene and

bisabolol and also phenolic compounds [21]. Kayser et al. named *P. abrotanoides* extract as one of the important medicinal plants for the treatment of parasites, intestinal worms and leishmaniasis [22]. Obame et al. also attributed the antiparasitic action of the essential oil of this plant to the presence of terpenes, as well as phenolic compounds and anthocyanins in the extracts of flowering branches [23]. Vardar *et al*. also introduced thymol and γ-terpinene compounds as the most important antimicrobial compounds in the flowering branches of this plant, as well as phenolic compounds as anti-inflammatory, antiseptic and antibacterial ingredients [24].

Conclusion

The results of the present study showed that the terpenoid fraction of *P. abrotanoides* plant has significant anti-leishmanial effects on *L. major* parasite. This effect is through improving and reducing wound size and also increasing the lifespan of treated mice. According to the history of traditional use of plants of this genus in the treatment of leishmaniasis and the results obtained in this study, additional studies including the effects of leishmaniasis in different doses and with other animal models and also isolation of compounds in the active fraction of this plant are recommended.

Conflict of Interests

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

Acknowledgment

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