Original Article

In Vitro Study of the Leishmanicidal Activity of *Perovskia Abrotanoides* Terpenoid-Rich Fractions Against *Leishmania Major* (MRHO/IR/75/ER)

Masoud Sadeghi Dinani[1], Seyed A. Emarati Noushabadi[1], Fatemeh Namdar[2], Parastoo Hassani Abharian[3], S. H. Hejazi[3], Zahra Sebghatollahi[3]

[1]Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran, [2]Noncommunicable Diseases Research Center, Fasa University of Medical Sciences, Fasa, Iran, [3]Skin Diseases and Leishmaniasis Research Center, Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: Cutaneous leishmaniasis (CL) is an ulcerative skin disease caused by some species of the genus *Leishmania*. Evidence shows that *Perovskia abrotanoides* is an important herbal medicine against *Leishmania.* This study was conducted to investigate the killing effect of terpenoid-rich fractions on promastigotes of *L. major* (MRHO/IR/75/ER).

Material and Method: The eluates of reverse phased medium pressure liquid chromatography (RP-MPLC) of the extract were subjected to thin-layer chromatography (TLC) and categorized into six final fractions. Primary proton nuclear magnetic resonance (H-NMR) spectroscopy confirmed fractions' nature. Fractions 4, 5, and 6 (F4, F5, F6) were identified as terpenoid-rich content. Two concentrations of 50 and 100 µg/ml were prepared to test leishmanicidal activity. Followed by treating promastigotes of *L. major* by the fractions in incubation times of 12, 24, and 48 hours, their viability was determined using a cell proliferation MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay.

Result: F4, F5, and F6 showed significant killing activity on promastigotes of *L. major* in a concentration-dependent manner. The viability of promastigotes was significantly reduced at a concentration of 100 µg/ml compared to 50 µg/ml (P-value <0.05). Also, over time a significant decreasing trend in the viability of promastigotes confirmed the time-dependent manner of the fractions (P-value <0.01). Furthermore, F5 had the highest leishmanicidal activity at the first incubation time compared with other fractions.

Conclusion: Terpenoid-rich fractions of the *P. abrotanoides* have a leishmanicidal activity that depends on time and concentration. Among them, F5 has the highest potency that may contain potent terpenoid constituents.

Keywords: *In vitro*, *Leishmania major*, leishmanicidal, *perovskia abrotanoides*, terpenoid-rich fraction

Address for correspondence: Mrs. Zahra Sebghatollahi, Skin Diseases and Leishmaniasis Research Center, Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.
E-mail: zahrasebghatollahi@gmail.com
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Introduction

Leishmaniasis is one of the most common infectious diseases in tropical countries which affects 600000 to 1 million people around the world annually.[1] While over 15 different species of *Leishmania* are known to cause leishmaniasis in humans, *L. major* and *L. tropica* are responsible for 70–90% of all cases in Asian, African, and South American countries.[2-4]

The administration of pentavalent antimony is a common treatment for leishmaniasis, and in addition to its low

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efficacy and numerous side effects, some resistances have been reported.[5] However, new drugs such as miltefosine, paromomycin, and liposomal amphotericin B, and some novel approaches associated with significant adverse effects and resistance, improved.[6-8] Nowadays, improving diagnostic methods, combination therapy, and treatment monitoring increases patient adaptation and enhances the rate of successful treatment.[9]

The low efficacy of current drugs and their unsafe side effects have aroused interest in traditional herbal medicines to discover new pharmaceutical agents for the treatment of leishmaniasis.[10] In Iranian traditional medicine, various medicinal plants such as garlic, wormwood, yarrow, brazambal, and thyme have been used to treat leishmaniasis. Among them, *P. abrotanoides* (Brazambal in Persian) is an effective and well-established leishmanicidal medicinal plant that is domesticated in the center of Iran.[11-13] Root and ground parts of the plant are traditionally used for topical treatment of CL. Recent studies have proven its ethnomedical properties through *in vitro* experiments.[14]

According to the approved leishmanicidal activity of *P. abrotanoides*, a total extract of aerial parts of the plant was fractionated and the leishmanicidal effects of three terpenoid-rich fractions were evaluated. In this study, the leishmanicidal effect of terpenoid-rich fractions was correctly proved directly related to incubation time and concentration, Also, F5 has known as the highest active terpenoid-rich fraction against promastigotes of *L. major*.

Materials and Methods

Aerial parts of *P. abrotanoides* were collected from Kashan (Isfahan, Iran), in May 2020 and identified by a botanist. A voucher specimen (No. 3538) was deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran. Air-dried aerial parts of the plant (1300 g) were crushed using a mill and extracted with 5 liters of ethanol: water (8:2), using the maceration method with stirring at room temperature. The whole extract was concentrated by a rotary evaporator and freeze-dried to make a fine powder. Then, the dried extract was subjected to fractionation by reverse phased chromatography using MPLC that was performed by the Buchi Gradient System C-605 through a glass column of RP-18 silica gel (36 × 460 mm; Lichroprep®, 25-40 µm) and the Buchi C-660 fraction collector. TLC was performed on silica gel plates (SiO_2 60F254) using C_4H_9OH: CH_3COOH: H_2O 4:1:5 (BAW) as a solvent system and cerium sulfate in 2 N H_2SO_4 as the reagent to visualize the spots. HNMR spectra were recorded by a Bruker 400 MHz spectrometer, using CD_3OD as the solvent and for signal calibration (δ_H = 3.31 ppm).

Cryopreserved *L. major* (MRHO/IR/75/ER) *was* obtained from the Department of Parasitology & Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran. The parasites were transferred to a modified medium of Nicole Novy Neal (N.N.N.) supplemented with 4% BHI, streptomycin (100 µg/ml), and penicillin (100 U/ml). After reaching a proper proliferation at 25°C, to mass production, the promastigotes were transferred to RPMI 1640 culture medium supplemented with FBS 10% v/v, L-glutamine 2 mM, penicillin 100 U/mL, and streptomycin 100 µg/ml.

After achieving a harvested yield of 6 × 10^6 cells/ml, the leishmanicidal activity of the prepared fractions was evaluated using Thomsen Bioassay techniques.[15] Briefly, *L. major* promastigotes were cultured in wells of microplates at the logarithmic phase (10^6/100 µL). Fixed predetermined concentrations of fractions were made in RPMI 1640 using 2% DMSO as a co-solvent and added to the wells to achieve final concentrations of 50 and 100 µg/ml. Amphotericin B at 25 µg/ml and RPMI 1640 were used as the positive and negative controls, respectively.[16]

After incubation times of 12, 24, and 48 hours, the microplates were centrifuged at 1500 rpm for 5 min, and the supernatants were discarded. Then, they were washed with sterile PBS and after removal of the PBS, the microplates were incubated at 25°C, and the viability of the treated promastigotes was evaluated by adding 20 µL of MTS solution and 80 µL of RPMI 1640. The viability of promastigotes was recorded by an ELISA (Enzyme-linked immunosorbent assay) microplate reader at an emission wavelength of 490 nm that could indicate a killing effect and vice versa (All experiments were done in triplicate).[16]

Finally, the mean percentage of promastigote viability was determined by Kolmogorov-Smirnov (KS) normality test and statistically analyzed by one-way Analysis of variance (ANOVA) and Tukey's post hoc test via a prism software.

Results

A dried total extract of 84.5 gr was extracted from 1300 gr of air-dried aerial parts of the plant. Fractionation of extract by RP-MPLC resulted in the preparation of six final fractions. The nature of fractions was evaluated and confirmed by TLC and primary H-NMR spectroscopy, among them, F4, F5, and F6 were determined to be rich in terpenoid compounds. Terpenoid-rich fractions were freeze-dried, to free them from any remaining solvent and form a fine powder. The concentrations of 50 and 100 µg/ml were prepared from the fractions, using RPMI 1640 and DMSO as a co-solvent.

After mass production of promastigotes, the yield of parasites was 6 × 10^6 cells/ml. The results of the MTS assay showed that all three terpenoid-rich fractions had significant leishmanicidal activity on *L. major* promastigotes. To elaborate more, the F4 at the concentration of 50 µg/ml, reduced the number of viable parasites to 43.33, 21.56, and 8.62% during 12, 24, and 48 hours of incubation, respectively [Figure 1]. Also, the leishmanicidal activity of the fractions was increased at 100 µg/ml concentration in a time-dependent manner. At

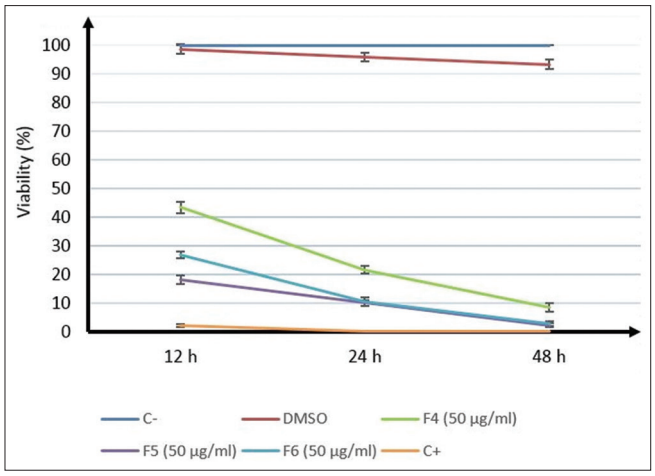

Figure 1: Mean viability percentage of promastigotes treated with three terpenoid-rich fractions (F4, F5, and F6) at a concentration of 50 μg/ml and at three incubation times (12 h, 24 h, and 48 h)

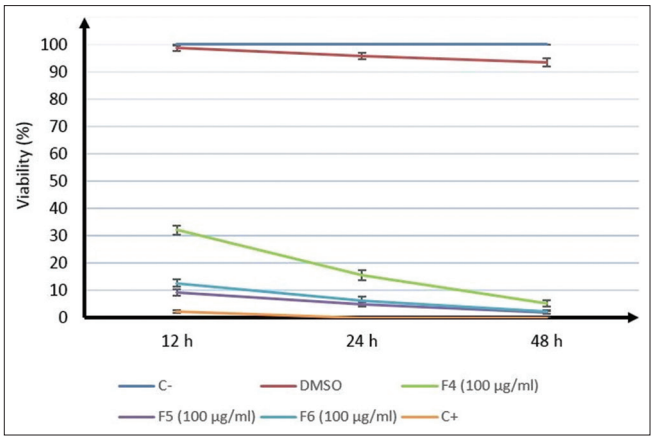

Figure 2: Mean viability percentage of promastigotes treated with three terpenoid-rich fractions (F4, F5, and F6) at a concentration of 100 μg/ml and at three incubation times (12 h, 24 h, and 48 h)

this concentration during 12, 24, and 48 hours of incubation, the number of viable parasites was significantly reduced to 32.11, 15.65, and 5.33%, respectively [Figure 2]. The last 48 hours incubation period showed a comparable reduction in promastigote viability without any significant difference between the two concentrations.

Both concentrations of F5 with the highest leishmanicidal activity showed a significant reduction in the viability of promastigotes. For example, the concentration of 50 µg/ml, reduced the number of viable parasites to 18.17, 10.21, and 2.2% during 12, 24, and 48 hours of incubation, respectively [Figure 1]. Also, the same trend of 9.17, 5.31, and 2.13% was determined during 12, 24, and 48 hours of incubation at 100 µg/ml concentration, respectively [Figure 2]. It is noteworthy that in 48 hours of incubation, F5 showed its maximum leishmanicidal activity without any significant differences between concentrations.

The F6 showed a significant reduction in the number of viable parasites, for instance, at a concentration of 50 µg/ml, the percentage of viable cells was decreased to 26.83, 10.58, and 2.78 during 12, 24, and 48 hours of incubation, respectively [Figure 1]. A similar decreasing trend of 12.67, 6.33, and 2.18% was shown during 12, 24, and 48 hours of incubation [Figure 2]. Similar to previous fractions, in 48 hours of incubation, F6 has shown its maximum leishmanicidal activity without any significant differences between concentrations.

The results of cell viability assays were determined for DMSO, positive control (amphotericin B), and negative control (culture medium) groups and showed 96, 2, and 100%, respectively.

The viability of promastigotes at two concentrations of fractions during three incubation times was recorded. The results showed a significant reduction in cell viability compared to the negative control and DMSO group (P-value <0.001), On the other hand, the results showed lower differences in cell viability for all fractions compared to the positive control at 12 and 24 hours of incubation (P-value <0.01). Also, at 48 hours of incubation F5 and F6 showed non-significant differences compare to positive control. This was concluded that they have leishmanicidal activity as the positive control at 48 hours of incubation. This similar potency in long-term incubation makes them logically alternative agents, just in the case of long-term incubation. All three fractions showed a significant difference of *P* value <0.05 in cell viability between the two concentrations, except at 48 hours of incubation. In summary, it can be noted that all fractions at 12 and 24 hours of incubation have a concentration-dependent manner on the viability of promastigotes. According to the time-dependent manner of the three terpenoid-rich fractions, decreasing trends of promastigotes viability have illustrated a significant difference among the incubation times (12, 24, and 48 hours) at both concentrations (P-value < 0.01).

Comparison of F4, F5, and F6 at the first incubation time (12 hours) at both concentrations showed lower parasite viability of F5 compare to F4, *P* value <0.01, and F6, *P* value < 0.05 simultaneously. F5 was proved to be the strongest active fraction against *L.major* promastigotes.

Discussion

Leishmaniasis is one of the most common parasitic diseases in different parts of Iran, which is more common in the cutaneous than visceral type.[17] In the treatment of disease, the low efficiency of approved drugs and their resistance induction, as well as hazardous side effects, are major problems.[5,18] Based on the evidence, changes in the clinical response to pentavalent antimonial, sodium stibogluconate (pentostam), and meglumine antimoniate (glucantime) in VL and CL have been a persistent problem for the past 50 years.[19] Although Pentamidine has been a second-line treatment of VL and CL for over 40 years. and its usage in the treatment of CL was re-evaluated by clinical trials of New World CL in the 1990s, it is not widely used as a leishmanicidal drug.[20,21]

Amphotericin B is a polyene antibiotic that has been used since the 1960s as a second-line treatment for leishmaniasis.[22] Recently, Miltefosine (hexadecylphosphocholine) has been added to the armory of leishmanicidal drugs.[23] In this regard, both promastigote and amastigote of *L. donovani*, *L. major*, *L. tropica*, *L. aethiopica*, *L. mexicana*, and *L. panamensis* were reduced in several parasite viability assays by treating *in vitro* examinations.[24] Also, edelfosine from the miltefosine family of phospholipid analogs (ET-18-OCH3) was used against leishmaniasis, although it was initially developed as an anticancer drug.[25] Paromomycin, an aminoglycoside-aminocyclitol antibiotic, has been used for the treatment of VL in a parenteral formulation with or without glucantime.[26]

The pathway of ergosterol biosynthesis, the main sterol of fungi, *Leishmania* spp, and *Trypanosoma cruzi* is the target of the most important antifungal and antileishmanial drugs.[27] Among them**,** allylamines and azoles drug families such as terbinafine which inhibits squalene epoxidase, and ketoconazole that inhibits C14-demethylase (CYP51), are the most interested leishmanicidal agents.[28,29] drug combination therapy has attracted the researcher's attention to reach a synergic effect on the disease, through herbal medicines.[30] In this respect, various medicinal plants including families of Fabaceae, Malvaceae, Lythracea, Berberidaceae, Agavaceae, and Asteraceae have been used to treat leishmaniasis by traditional methods.[31] For example, *Berberis vulgaris* L, *Vitis vinifera* L., honey, and *Allium* species especially garlic have shown leishmanicidal activity.[19] specifically, the leishmanicidal activity of some plant derivatives such as quinolone, 2-propenyl quinoline, and epoxypropyl quinolone, isolated from the *Galipea longiflora,* were proved.[32]

The *P. abrotanoides*, brazambal in traditional Iranian medicine, predominantly has been used in the treatment of leishmaniasis, especially in a topical form that is made of sesame oil and water.[33] The ethnomedical evaluation for the leishmanicidal activity of the *P. abrotanoides* has been recently confirmed by methanolic and ethanolic extract of its roots *in vitro*.[34] Also, its phytochemical evaluations showed the presence of terpenoids such as diterpenes, sesquiterpenes, and monoterpenes, e.g., camphene, 1, 8-Cineol, myrcene, pinene, camphor, caryophyllene, and humulene in plant extract.[35] On the other hand, the effect of terpenoid compounds against different species of *Leishmania* has been sufficiently strong to be considered as lead compounds for drug development.[36]

According to mentioned evidence, the leishmanicidal activity of the terpenoid-rich fractions isolated from the aerial parts of the *p. abrotanoides* was evaluated against the *L. major* promastigotes. The results showed that the robustness of their activity is highly dependent on time and concentration. As a result, F5 can be introduced as the most potent fraction with the highest leishmanicidal activity due to 70% and 90% of its leishmanicidal activity at the first incubation time (12 hours) for concentrations of 50 and 100 µg/ml, respectively.

Conclusion

Both concentrations significantly reduced the viability of *L. major* promastigotes after treatment with three prepared terpenoid-rich fractions, therefore they are categorized as leishmanicidal agents. Based on their time-dependent manner, terpenoid-rich fractions showed a significantly decreasing trend over time (P-value <0.01), and due to concentration dependency, they showed a significant decrease in promastigotes viability (P-value <0.05). It is noteworthy that F5 with the strongest leishmanicidal activity could be introduced as the most potent fraction. Therefore, it can be suggested that F5 may contain potent terpenoid constituents. So, further evaluation of its active ingredients by more accurate techniques is valuable and could clarify some of the potent specific terpenoid compounds with leishmanicidal activity.

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Conflicts of interest

There are no conflicts of interest.

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