Evaluation of leishmanicidal effect of *Perovskia abrotanoides Karel. root* extract by in vitro leishmanicidal assay using promastigotes of *Leishmania major*

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ABSTRACT

P. abrotanoides dried root has been successfully used for the treatment of Cutaneous leishmaniasis in Iranian traditional medicine as a poultice. To provide a scientific reason for ethnomedicinal use of *P. abrotanoides*, in this study the leishmanicidal effect of *Perovskia abrotanoides* extracts was evaluated on promastigotes of *L. major* in vitro.

In this study, the antileishmanicidal effect of different extract of *Perovskia abrotanoides* root was evaluated on the promastigotes of *Leishmania major* in vitro. The dried and ground root of the plant was extracted using either maceration in 80% ethanol or Soxhlet in methanol. Then, 5 different concentrations (0.06, 0.12, 0.25, 0.5 and 1 mg/ml) of each extract, one positive control (Amphotericin B, 0.5 mg/ml), one negative control (culture medium), and one solvent control (DMSO) were prepared and were placed in a 24-well plates containing 50,000 parasites/well. The plates were incubated at 25^{o}C for six days and the number of parasites in each well was determined on days 2, 4, and 6 of experiment microscopically using Neubauer chamber. It was observed that amphotericin B and both macerated and Soxhlet extracts at concentration of 1 mg/ml killed all the parasites. Lower doses exhibited a dose-dependent antileishmanial activity. The average of IC_{50} for macerated extract in DMSO was 4.03×10^{-2} mg/ml and for Soxhlet extract in DMSO was 7.33×10^{-2} mg/ml. The control solvents had no significant effect on the *L. major* promastigotes. These results indicated that both macerated and Soxhlet extracts of *Perovskia abrotanoides* have favorable leishmanicidal activity.

Key words: *Perovskia abrotanoides*; Antileishamanicidal activity; Cutaneous leishmaniasis; *Leishmania major*

INTRODUCTION

Leishmaniasis is a protozoal disease of man that occurs in most parts of the world. This disease affect approximately 12 million people world wide with 1.5-2 million new cases occurring each year [1]. Cutaneous leishmaniasis (CL), which caused by the different species of *Leishmania*, produces a skin ulcer that heals spontaneously in most cases, leaving an unsightly scar [2]. Control strategies are not always effective and available drugs for the treatment of leishmaniasis are either toxic, have limited efficacy or both and emerging drug resistance is a significant concern. The effects of available topical antileishmanial products are

also minimal [3]. Therefore, there is a great and urgent need for the development of new, effective and safe drugs for the treatment of leishmaniasis [4]. One strategy to discover new drug leads is to investigate natural products from medicinally used plants. Most people in areas where leishmaniasis is endemic depend largely on traditional medicine.

Perovskia genus, Lamiaceae family, has seven species such as *P. abrotanoides, P. atriplicifolia and P. hybrida. Perovskia abrotanoides* with vernacular name of "Berazamble", "Domou" and "Gevereh" is a perennial herb growing wild in Iran, Afghanistan, Pakistan and Turkmenistan [5]. There are few scientific reports about *P. abrotanoides.* It has some pharmacological effects such as leishmanicidal (some constituents of plant), antiplasmodial and cytotoxic activity [6], as well as antinociceptive and anti-inflammatory effects [7-8].

P. abrotanoides dried root has been successfully used for the treatment of CL in Iranian traditional medicine as a poultice [6]. To provide a scientific reason for ethnomedicinal use of *P. abrotanoides,* in this study the leishmanicidal effect of ethanolic macerated and methanolic Soxhlet extracts of *P. abrotanoides* was evaluated on promastigotes of *L. major* in vitro.

MATERIALS AND METHODS

Plant material

Perovskia abrotanoides was collected from near Chenaran (Mashhad Province, Iran). The root of the plant was dried and powdered. It was identified in the Herbarium of Ferdowsi University and voucher samples were preserved for reference at the Herbarium of the Mashhad School of Pharmacy with reference number of (152-120-1002).

Preparation of extract

Soxhlet methanolic extract: The plant powder (50 g) was extracted with methanol (200 ml) for 12 hours using Soxhlet apparatus. The methanol was removed under reduced pressure and dried. The extract was kept in refrigerator until use.

Macerated ethanolic extract: The powdered plant (50 g) was extracted with ethanol (1000 ml, 80%, v/v) by maceration. The extract was collected every 24 h for three days. The combined extracts were dried under reduced pressure. The dried extract was kept in refrigerator for further testing.

Leishmania parasites

Leishmania major strain MRHO/IR/75/ER was maintained with passage in BALB/c mice. The amastigotes were isolated from the lesions of infected BALB/c mice and transformed to promastigotes on NNN medium then subcultured in RPMI 1640 (Sigma) containing 10% v/v heat inactivated FCS, 2 mM glutamine, 100 U/ml of penicillin and 100 mg/ml of streptomycin sulfate (RPMI-FCS) at 25 °C. Antileishmanial assays were conducted using stationary-phase promastigotes.

Assay for leishmanicidal activity

The assay was performed according to Atta-ur Rahaman *et al*., [9]. Briefly, *L. major* promastigotes in stationary phase were seeded at 50,000 parasite/400 µl/well in 24-well plate in RPMI-FCS. The extract were dissolved in DMSO and added in further 400 µl/well to give final concentrations of 1 mg/ml and serial two-fold dilutions thereof. Promastigotes were incubated over a period of 6 days at 25 °C and the number of the viable parasites in each well determined on days 2, 4 and 6 of experiment using Neubauer chamber under a microscope. Amphotericin B (0.5 mg/ml) was used as positive control, culture media was used as negative control, and DMSO alone was used as solvent control.

Statistical Analysis

Statistical analysis was carried out using one-way ANOVA and multiple comparison Tukey-Kramer test was used to compare the means of different treatment groups. The IC_{50} was determined by Litchfield and Wilcoxon method.

RESULTS

Antileishmanial activity of macerated ethanolic extract of Perovskia abrotanoides in DMSO

Amphotericin B (0.5 mg/ml) and macerated ethanolic extract of *Perovskia abrotanoides* (1 mg/ml) in DMSO killed all of the *L. major* promastigotes (Figure 1, 2, 3) and lower doses of macerated ethanolic extract of *Perovskia abrotanoides* in DMSO killed *L. major* promastigotes dose-dependently while DMSO did not have any effect on the *L. major* promastigotes. The IC_{50} for macerated ethanolic extract of *Perovskia abrotanoides* in DMSO was 0.213, 0.652 and 0.343 mg/ml after 2, 4 and 6 days of incubation, respectively.

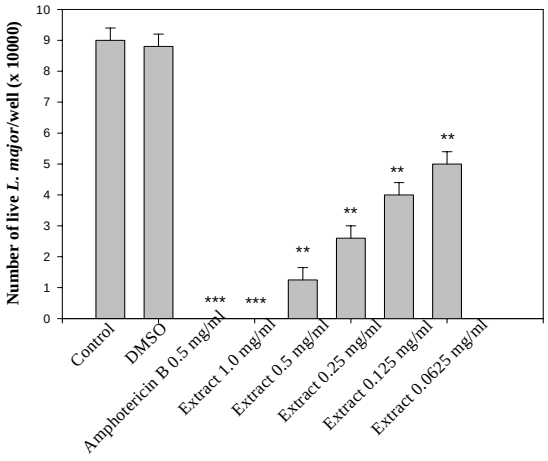

Figure 1. Effect of different concentrations of *P. abrotanoides* macerated extracts against *L. major* promastigotes after 2 days of incubation. Each bar represents the mean + S.E.M. of the number of promastigotes in 4 wells. **p<0.01, ***p<0.001, Tukey-Kramer test.

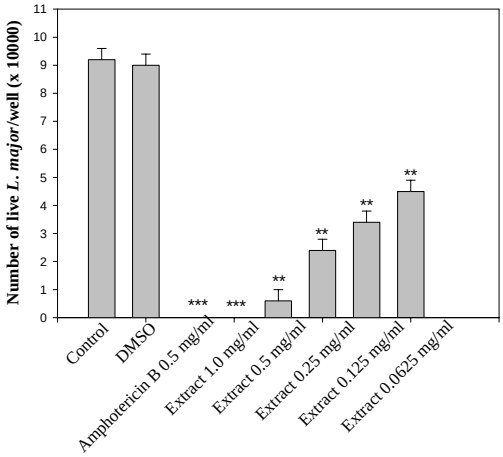

Figure 2. Effect of different concentrations of *P. abrotanoides* macerated extracts against *L. major* promastigotes after 4 days of incubation. Each bar represents the mean + S.E.M. of the number of promastigotes in 4 wells. **p<0.01, ***p<0.001, Tukey-Kramer test.

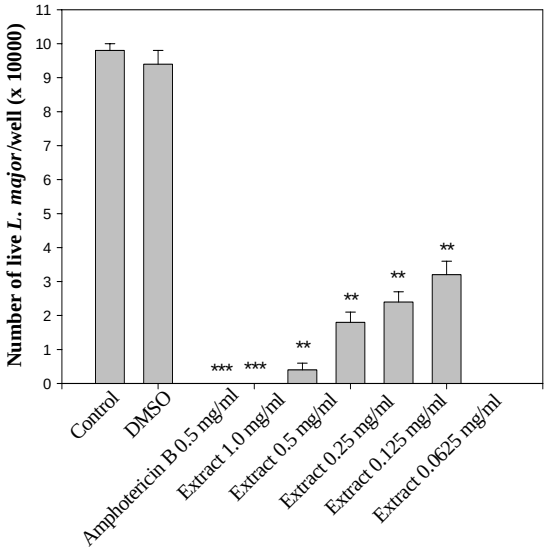

Figure 3. Effect of different concentrations of *P. abrotanoides* macerated extracts against *L. major* promastigotes after 6 days of incubation. Each bar represents the mean + S.E.M. of the number of promastigotes in 4 wells. **p<0.01, ***p<0.001, Tukey-Kramer test.

Antileishmanial activity of Soxhlet methanolic extract of Perovskia abrotanoides in DMSO

Different concentrations of Soxhlet methanolic extract of *Perovskia abrotanoides* in DMSO killed parasites dose-dependently (Figure 4, 5, 6). The IC_{50} for Soxhlet methanolic extract of *Perovskia abrotanoides* in DMSO was 0.926, 0.723 and 0.550 mg/ml after 2, 4 and 6 days of incubation, respectively.

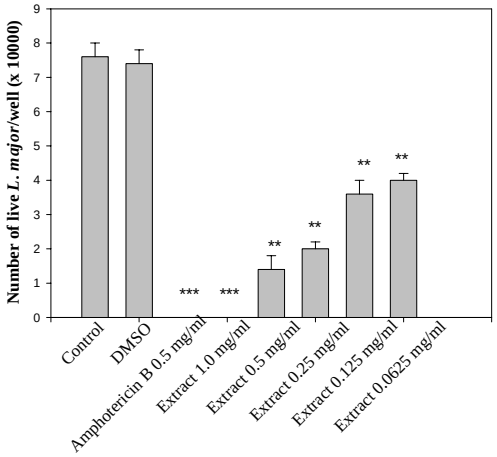

Figure 4. Effect of different concentrations of *P. abrotanoides* soxhelt extracts against *L. major* promastigotes after 2 days of incubation. Each bar represents the mean + S.E.M. of the number of

promastigotes in 4 wells. **p<0.01, ***p<0.001, Tukey-Kramer test.

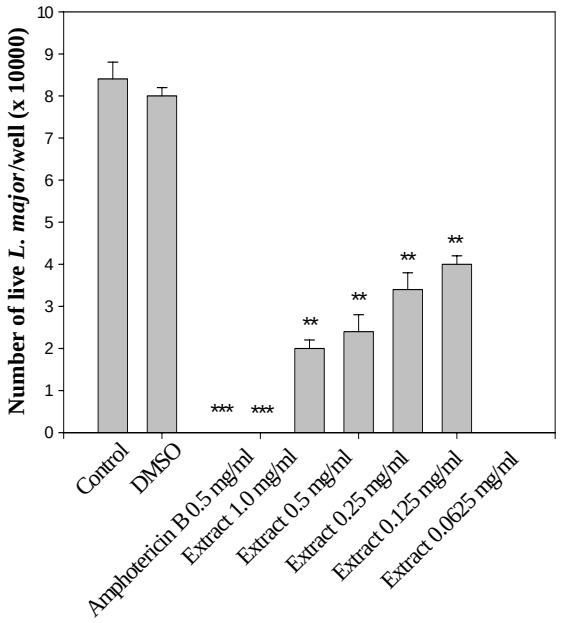

Figure 5. Effect of different concentrations of *P. abrotanoides* soxhelt extracts against *L. major* promastigotes after 4 days of incubation. Each bar represents the mean + S.E.M. of the number of promastigotes in 4 wells. **p<0.01, ***p<0.001, Tukey-Kramer test.

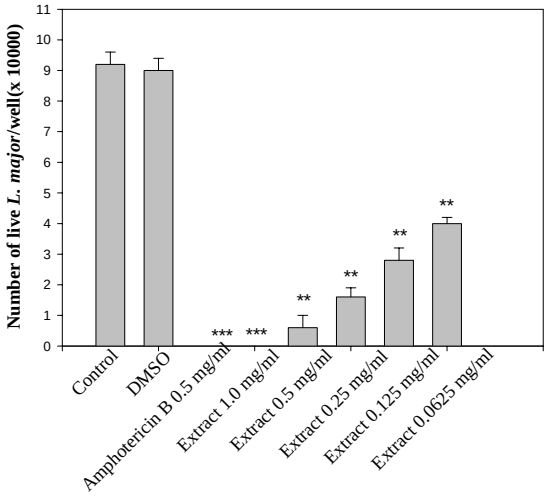

Figure 6. Effect of different concentrations of *P. abrotanoides* soxhelt extracts against *L. major* promastigotes after 6 days of incubation. Each bar represents the mean + S.E.M. of the number of promastigotes in 4 wells. **p<0.01, ***p<0.001, Tukey-Kramer test.

DISCUSSION

People customarily use the plant(s)/plant-derived preparations and consider them to be efficacious against cutaneaous leishmanisis without any scientific base to explain the action of such plants. Since cutaneous leishmaniasis has become one of the major health issue in Iran and chemotherapy is somewhat ineffective and painful, people are using medicinal plants sold on the local market as a remedy to cure their wounds. *P. abrotanoides* root has been used successfully in the treatment of cutaneous leishmaniasis in different part of Iran. Traditionally, the dried root of this plant is crushed and grinded. Then it is mixed with water, sesame oil and wax in a proper formula. The final product is like a paste. Then this remedy is applied on the lesions of the cutaneous leishmaniasis. The current study was therefore carried out on *L. major* promastigotes to evaluate its acclaimed efficacy using in vitro assay based toxicity. *L. major* and *L. tropica* are the major cause of cutaneous leishmaniasis in Iran [10-11]. Both macerated and Soxhlet extracts of the root of *P. abrotanoides* were prepared and tested against promastigotes of *Leishmania major*. All tested concentrations of both extracts exhibited antileishmanicidal activity after 2, 4, and 6 days of incubation. The average of IC_{50} for macerated extract in DMSO was 4.03×10^{-2} mg/ml and for Soxhlet extract in DMSO was 7.33×10^{-2} mg/ml. This difference could be due to the effect of heat on the constituent of extract during the Soxhlet process.

Recently the root of *P. abrotanoides* has been extracted by ethyl acetate and four active compounds cryptotanshione, 1β-hydroxycryptotanshione, 1-oxocryptotanshione and 1-oxomiltirone has been isolated [6]. These compounds are all diterpenes and constitute 0.8, 0.67, 0.01 and 0.0018% of the extract. The IC_{50} of these compounds against L. major promastigotes have been 5.45×10^{-3},

14.6×10^{-3}, 7.9×10^{-3} and 5.28×10^{-3} mg/ml, respectively. These IC_{50} approximately are 10 times less than the IC_{50} of total extract of *P. abrotanoides* that we acquired in our studies. Therefore, the leishmaniacidal effect of total extract not only depends to these compounds, but also in the extract could be other active components that might have leishmaniacidal effect in very low concentration. Further fractionation of the *P. abrotanoides* is required to characterize other antileishmanial constituents.

The phytochemical screening of aerial parts of *P. abrotanoides* has shown the presence of high content of monoterpenes and sesquiterpenes like 1, 8-Cineolo, myrcene, pinene, camphor, caryophyllene, humulene, camphene and bisabolol [11-12]. Beside the diterpenoides that present in the root of *P. abrotanoides* [6], these monoterpenes and sesquiterpenes might be also in the root of *P. abrotanoides* and involved in the leishmanicidal activity of the extracts of *Perovskia abrotanoides* root. Antileishmanial activity of terpenoides has also been reported by others [13].

These results indicate that the ethanolic macerated and methanolic Soxhlet extracts of *Perovskia abrotanoides* root have favorable leishmanicidal activity and kill the *L. major* promastigotes in a dose-dependent manner. Furthermore, *Perovskia abrotanoides* extraxt posses antinociceptive and anti-inflammatory effects [7-8]. Therefore, *Perovskia abrotanoides* root extraxt could be suitable topical treatment candidate for the treatment of cutaneous leishmaniasis.

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