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Original paper

In vivo anti-leishmanial activity of concocted herbal topical preparation against *Leishmania major* (MRHO/IR/75/ER)

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ABSTRACT. Cutaneous leishmaniosis (CL) is considered as one of the most important tropical diseases. Herbal therapy is the ideal treatment for CL because of the reduced injection pain, availability, lower cost and non-toxicity effects. The present study aimed to evaluate the *in vivo* antileishmanial activity of concocted herbal topical preparation (*Aloe vera*, *Perovskia abrotanoides*, *Nigella sativa*, propolis, lavender and olive oil) to evaluate its efficacy against *Leishmania major* (MRHO/IR/75/ER) in comparison to the gold standard treatment. Following the cause of cutaneous leishmaniosis, the BALB/c mice were divided into three groups, test group (ointment formulation), positive control (Glucantime) and negative control (untreated), respectively, which were treated twice a day for 28 consecutive days. The lesion size and parasite burden were evaluated for *in vivo* evaluation. The herbal topical ointment was able to significantly decline the lesion progression and reduce parasite burden in mice inoculated with *L. major* promastigotes in the test group compared with the negative control group (P=<0.001). In mice treated with the formulation, the number of amastigotes significantly decreased (P=<0.001), compared with that in the negative control group. Moreover, comparative features of both treatments showed there was no difference between the herbal-treated and glucantime-treat mice (P=0.63). The herbal topical ointment displayed significant *in vivo* antileishmanial activities. It may be that using ointment formulation beside other skin repair compounds can be used as an alternative medicine in the treatment and healing of human CL lesions. Further investigations are needed to study the pharmacologic and pharmacokinetics aspects of ointment formulation in the treatment and healing of human CL lesions.

Keywords: herbal traditional ointment, cutaneous leishmaniosis, *Leishmania major*, therapy

Introduction

Leishmaniosis is caused by the genus *Leishmania,* which is transmitted by the bite of infected female phlebotomine sandflies in tropical and sub-tropical areas [1]. Based on clinical manifestations, leishmaniosis is classified into three forms: cutaneous leishmaniosis (CL), muco-cutaneous leishmaniosis (MCL) and visceral leishmaniosis (VL) [2]. According to the World Health Organization (WHO) report, this disease is one of the six most important infectious diseases and about 0.7 to 1.2 million new CL cases and 0.2 to 0.4 million VL cases annually occur in the world [3]. The CL form is endemic in over 98 countries, with over 85% of new CL cases occurring in 10

countries, including: Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran, Iraq, Pakistan, the Syrian and Tunisia [4,5]. Iran has reported 20000 CL cases in 2012 [5] and *L. major* is the most common etiologic agent [6,7].

The currently available treatment for CL is pentavalent antimony (Glucantime) as the first line treatment, and amphotericin B, pentamidines or paromomycin as the second-line [8,9]. On the other hand, there are side effects presented by the current drugs, including high toxicity, high cost and parasite resistance [10]. Considering the fact that the lack of a safe vaccine for disease, the development of new therapeutic agents is required [11]. It should be noted that recently attention has been paid to medicinal plants for new therapeutic agents in the treatment of leishmaniosis [12,13].

Many previous published studies have confirmed the antileishmanial activity of *Aloe vera*, *Perovskia abrotanoides* (*P. abrotanoides*), *Nigella sativa* (*N. sativa*), propolis, lavender and olive oil [14–19]. Moreover, the results of a systematic review and meta-analysis of Iranian medicinal plants with anti-leishmanial activity revealed the *P. abrotanoides*, *N. sativa*, and propolis extracts have effective anti-leishmanial activity [19]. In addition, in other studies in Iran and other countries, *Aloe vera*, lavender and olive oil showed *in vitro* and *in vivo* activity against the *Leishmania* parasite [14,18,19].

The goal of this study was to show that a combination of medical plant and natural products (*Aloe vera*, *P. abrotanoides*, *N. sativa*, propolis, lavender, and olive oil) had *in vivo* antileishmanial activity against *L. major* (MRHO/IR/75/ER).

Materials and Methods

Plant and natural materials

The *P. abrotanoides* (Persian name: brazambel) was collected from Shirvan district, North Khorasan, Iran. *Aloe vera* (Persian name: Sabr-e-Zard) and lavender (Persian name: Ostokhodous) were collected from the Neka region, Mazandaran Province, Iran. Moreover, *N. sativa* (Persian name: siah daneh), propolis (Persian name: Baremoom), and olive oil were purchased from beekeepers in Shirvan district, North Khorasan, Iran. These plant specimens were characterized and deposited in the herbarium of Mazandaran University of Medical Sciences in the Department of Pharmacognosy, School of Pharmacy, Sari, Iran.

Preparation of ointment formulation

The ointment formulation consists of the leaves and flowers of *P. abrotanoides*, fresh *Aloe vera* leaves, *N. sativa*, propolis, lavender and olive oil, and honey wax. To prepare the ointment, dried *P. abrotanoides* leaves and flowers (400 g), dried lavender leaves and flowers (70 g), fresh *Aloe vera* gel (80 g), *N. sativa* (50 g), were powdered and mixed with olive oil (200 ml), and then kept for 15 days at room temperature. The honey wax (150 g) and propolis (50 g) were heated in a water bath (100°C), and then added to the plant mixture. In the next step, the mixture was cooled at room temperature until firmed and then kept at 4–8°C until use.

Parasite culture

The strain of *L. major* (MRHO/IR/75/ER) obtained from the School of Public Health, Tehran University of Medical Sciences and the strain was approved using PCR nagt-RFLP [20] and cultured in RPMI 1640 media (Gibco, Life Technologies GmbH, Germany), containing 10% heat-inactivated fetal bovine serum (Sigma, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, Germany). Briefly, promastigotes were sub-cultured at regular intervals (72–96h) after adjusting the inoculum density to 2×10^6 cells/ml at 25°C [18].

Mice

Thirty female BALB/c mice (6–8 weeks old, weighing about 20–25 g, procured from the Razi Vaccine and Serum Research Institute, Iran) were used for *in vivo* experiments. BALB/c mice were housed under standard laboratory conditions (light/dark cycles, controlled temperatures of 25±1°C) with free access to food and fresh drinking water.

In vivo experiments

L. major promastigotes (MHROM/IR/75/ER) at stationary phase (2×10^6) were injected sub-cutaneously into the base of the tail in BALB/c mice. After the injection, the animals were divided into three groups (n=10) including test group (ointment formulation), positive control (Glucantime) and negative control (untreated) groups. The treatment was initiated when the lesions appeared (about 4 weeks post-inoculation). Group 1 were treated topically with ointment formulation twice a day for 28 consecutive days as a group test. Group 2 included infected animals treated with 20 mg/kg/day

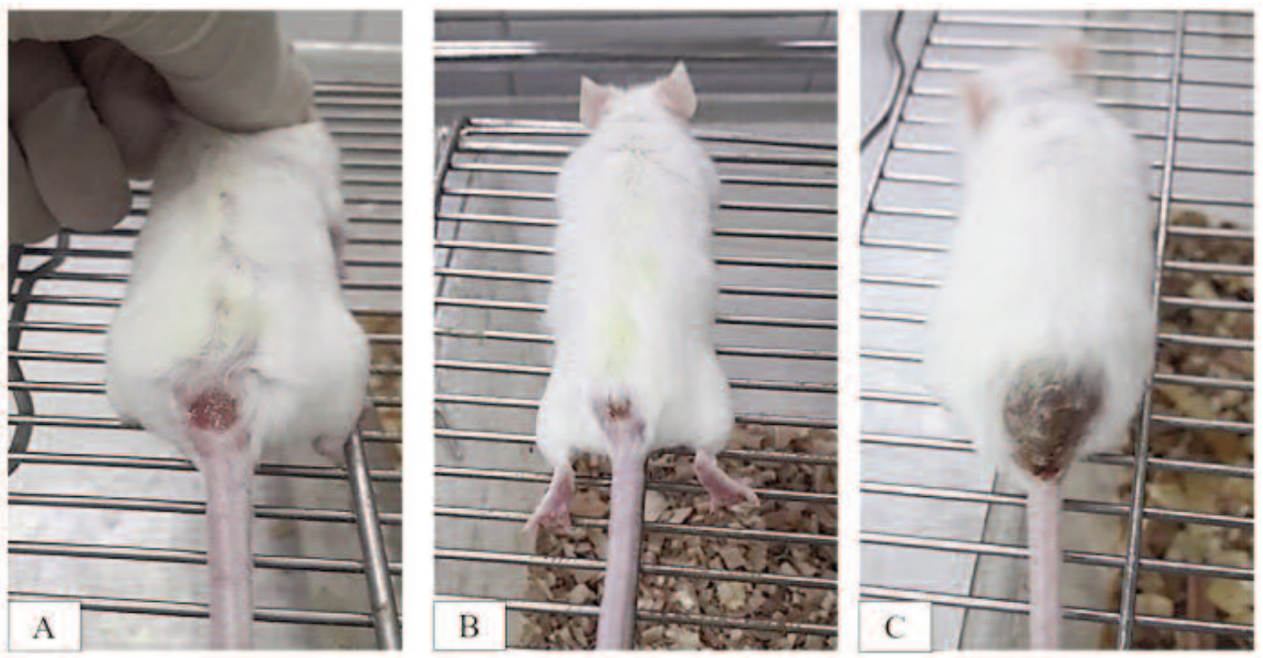

Figure 1. Lesions of BALB/c mice infected with *L. major* on the last days of the treatment. A: positive control (Glucantime); B: test group (ointment formulation); C: negative control (untreated) twice a day for 28 days

of glucantime administered by intramuscular route as a positive control group and group 3 included infected untreated mice as a negative control group. Animals were measured before and after treatment for 28 days using a dial micrometer in two diam (a and b) and the following formula [5]:

Amastigotes density

Smears were prepared from the margins of lesions and the exudate materials were then air-dried, fixed with absolute methanol, stained with Giemsa, and examined for the presence of the amastigotes under a light microscope under 1000× magnification. Grading of *Leishmania* parasites was done according to WHO protocols of average parasite density, as follows: 4+ (1–10 parasites/field), +3 (1–10 parasites/10 fields), 2+ (1–10 parasites/100 fields), and 1+ (1–10 parasites/1000 fields) [20].

Statistical analysis

Repeated measures analysis of variance was used to compare mean lesion diameters at four-evaluation time points (weeks). Statistical analysis was carried out using SPSS software v.16 (IBM Analytics, USA). P values < 0.05 were considered statistically significant.

Ethical statement

This study is ethically approved by the ethical committee of the Mazandaran University of Medical Sciences with the ethical code of: IR.MAZUMS.REC.1399.269

Results

Anti-leishmanial effects of ointment formulation and glucantime on the lesion sizes (mm^2) of test and control groups were described weekly in table 1.

The sizes of lesions in both the test and glucantime-treated (positive control) group were decreased. The decrease in the lesions size in receiving ointment formulation and positive control groups were statically significant during before and after treatments and also in comparison to the negative group (P=0.001) (Fig. 1)

The activity of the ointment formulation against *Leishmania* infections was verified by determination of the parasite burden in the lesion. In mice treated with ointment formulation, the number of amastigotes significantly decreased (P=0.001), compared with that in the negative control group (Tab. 2). In a group that received ointment formulation, few amastigotes as grade 2+ (1–10 parasites/100 fields) were observed. On the other hand, negative control displayed heavy parasite burden as grade 4+ (1–10 parasites/field). Moreover, comparative features of both treatments showed there was no difference between the herbal-treated and glucantime-treat mice (P=0.63).

Table 1. Comparative evaluation of lesion size (mm^2) of BALB/c mice, before and after treatment

Groups	Before treatment mean±SD	1-week mean±SD	2-week mean±SD	3-week mean±SD	4-week mean±SD	P-value
Herbal ointment	14.3±1.2	13.5±0.8	12.3±1.0	11.3±1.0	9.5±1.2	<0.001*
Glucantime®	14.5±2.3	12.9±0.1	10.8±0.8	9.8±0.7	8.8±0.3	<0.001*
NC	14.1±1.9	14.6±0.7	15.5±0.7	16.5±0.6	17.6±0.6	

Explanations: values are represented as mean±SD; *significant at P<0.05 compared with negative control, NC, negative control; size of the lesions was measured in triplicate

Table 2. Parasite burden decreases in BALB/c mice before and after treatment with traditional ointment formulation compared with control groups

Parasite burden Group	Before treatment	After treatment	P-value
Ointment formulation	4+	*2+	0.001
Glucantime	4+	*2+	0.001
NC	4+	4+	

Explanations: *significant at P<0.05 compared with negative control (NC)

Discussion

Treatment of diseases is one of the most important health problems in the world. Due to the resistance of microorganisms, the drugs used must be updated or changed over time [21]. Leishmaniosis is one of the main endemic parasitic infections worldwide, especially in developing countries [22]. According to WHO reports, leishmaniosis is a leading cause of death due to infectious diseases worldwide [10]. Pentavalent antimonials compounds, miltefosine, amphotericin B and paromomycin are the first-drugs used to treat leishmaniosis. However, there are many problems with the use of these drugs today, such as reduced effectiveness, high cost, unavailability, and high toxicity, so researchers concluded that they should look for an alternative drug to treat leishmaniosis

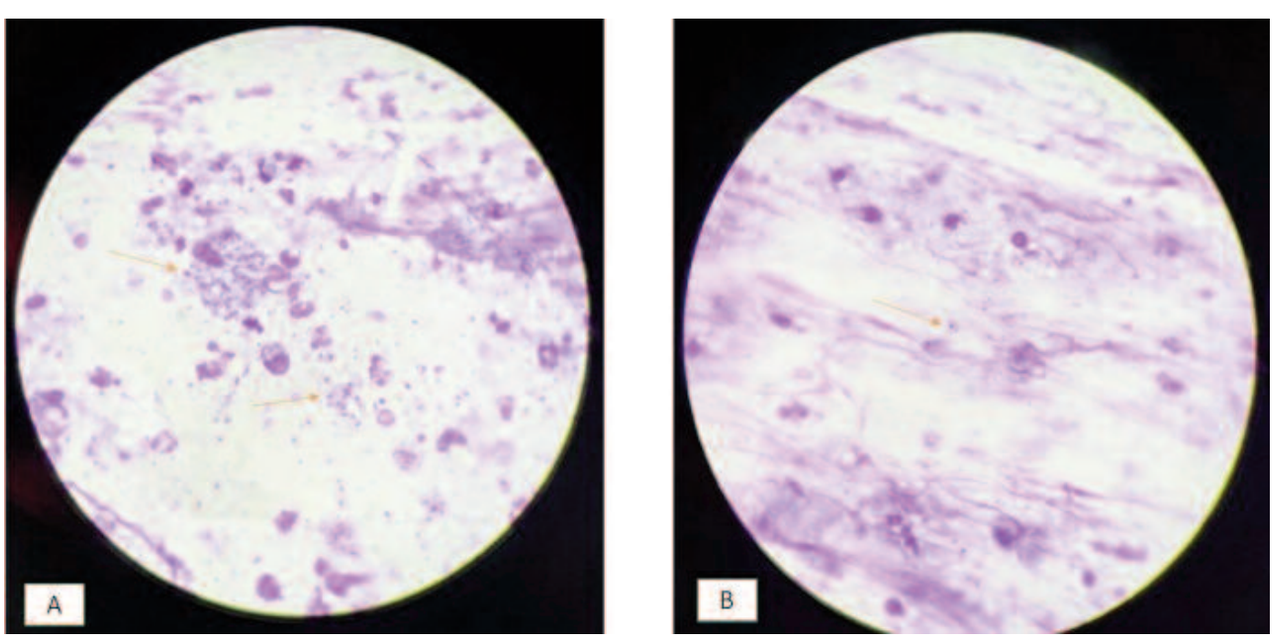

Figure 2. Parasite load through the treatment. A: negative group; B: concocted herbal topical ointment twice a day; arrows show *L. major* amastigotes

[23,24]. To date, many efforts have been made in this field and many drugs, both natural and synthetic drugs, have been evaluated [5,18]. The results of various studies have shown that among the drugs used, herbal medicines have shown better effects with less toxicity. The use of herbal medicines has also been recommended by the WHO, and the winning of the Nobel Prize in recent years by two antiparasitic and herbal medicines (ivermectin and artemisinin) shows the importance of this issue [25,26].

In this study, we evaluated the anti-leishmanial effect of six herbal plants and natural products in the form of ointment formulation in a mice model. Each of these herbal plants and natural products has already been studied individually, and their satisfactory results are briefly mentioned below.

A previous study reported that *Aloe vera* leaf exudates significantly decreased the size of ulcers in comparison to the control group [14]. In Iranian traditional medicine, *P. abrotanoides* dried root has been successfully used for the treatment of CL [15]. In another study, ethanolic and methanolic extracts of *P. abrotanoides* root were found to have antileishmanial activity and to kill *L. major* promastigotes at concentrations of 0.06, 0.12, 0.25, 0.5, and 1 mg/ml [27]. According to a study conducted by Fattahi Bafghi et al. [16], *N. sativa* can inhibit parasite growth and viability, which can be useful in the treatment of leishmaniosis. In addition, propolis hydroalcoholic extracts were significantly more effective in reduction of ulcer size as compared with glucantime [17]. In a study carried out on antileishmanial activity of *Lavandula angustifolia* on *L. major*, the researchers evaluated lavender essential oil significantly decreased the number of amastigotes in macrophages compared with control [18]. In addition, the result of a study conducted by Kheirandish et al. [19] showed olive leaf extract has potent therapeutic effects on CL. The findings from this study proved the good potential of ointment formulation in treatment of CL *in vivo* condition. The result from *in vivo* experiments demonstrated that ointment formulation significantly decreased the size of the *L. major* lesions in BALB/c mice, compared with the control group (P=0.001). In the test group treated with ointment formulation, the number of parasites in lesions was significantly decreased compared with that in the negative control group (P=0.001). These results were in agreement with those of the previous studies, indicating that medicinal herbs have the potential for the generation of novel drugs to be used as alternative or in combination with available drugs.

In conclusion, current research efforts have been directed toward the development of novel antileishmanial candidates. Our results revealed bright antileishmanial effects by mixed natural ointment formulation in *in vivo* experiments on lesion size in infected BALB/c mice with no adverse effects on lesions. In addition, significant decreases in the parasite load were shown in the infected tissue and organs. Further investigation is needed to study the pharmacologic and pharmacokinetics of ointment formulation in the treatment and healing of human CL lesions.

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