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Antileishmanial Activity of *Hyssopus officinalis*, *Tussilage farfara*, *Carum copticum* extracts in Mice Infected with *Leishmania major*

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Abstract
Leishmaniasis is a parasitic disease transported by sand flies. In this study, we evaluated the efficacy of herbals and compared with systemic glucantime against cutaneous leishmaniasis in Balb/c mice. The in vivo studies were carried out on cutaneous leishmaniasis in inbred mice to evaluate the effects of topical application of the ointment-based extracts two times daily for 20 days.45 mice were randomised into five groups [placebo group received the ointment base without the extract ,systemic glucantime as a reference group ,Test groups including plants [nine mice]. The ulcers diameter were measured by Kulis Vernier. Burden parasite in spleen was assayed. The results were suggestive that plants ointments were effective in reduction of ulcer size and burden parasite in spleen but were not significantly more effective in reduction of ulcer size and burden parasite in spleen as compared with glucantime [p=0.006, 0.002 and0.008, respectively]. Our results are suggestive that plants are effective for treatment of cutaneous leishmaniasis in mice.

Keywords: Balb/c mice –Glucantime – Leishmaniasis – *Carum copticum.*

1. Introduction
Leishmaniasis are transferred by sand flies belonging to the genus *Phlebotomus*. It is found in most tropical and subtropical countries, but 90% of the estimated 1.5 million new cases each year happen in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria, where it is often associated with destitution [1, 2] Considering the prevalence of illness in Iran and many side effects associated with pentavalent antimony compounds use in its treatment, this study was designed. The disadvantages of the antimonials are their requirement for intramuscular or intravenous injection each day for 20-28 days, their toxicity, and the new and late development of resistance in same regions such as India. Traditional treatment of CL is a common custom of natives in many endemic areas including many parts of Iran [3, 4]. Recent investigations focused on plants have shown an alternative way to get a potentially rich source of drug candidates against leishmaniasis, in which effective alkaloids, quinones, iridoids, terpenes, indole analogues have been found. Many compounds, including alkaloid, quinones, iridoids, terpenes, indole analogues have been documented to have anti-leishmania activity *in vitro*, Moreover, topical treatment of CL is attractive compared with the systemic treatment because of the easy application, particularly in remote areas [5, 6].

1.1 Medicinal uses of plants used in this research:
Hyssopus officinalis or *Hyssop* [Family: Lamiaceae] has soothing, expectorant, and cough suppressant properties. The plant also includes the chemicals thujone and phenol, which give it antiseptic properties. Its high concentrations of thujone and chemicals that stimulate the central nervous system can provoke epileptic reactions when taken in high enough doses. The oil of hyssop can cause seizures and even low doses [2–3 drops] can cause convulsions in children. It has been also used in the formulation of eye drops and mouthwash. Herb hyssop has also been observed to stimulate the gastrointestinal system. Antimicrobial, antifungal, antiprotozoal and anticancer effects of *Hyssop* extract have been reported. *Tussilago farfara* or coltsfoot [Family: Asteraceae] has been used in herbal medicine and has been consumed as a food product with some confectionery products, such as Coltsfoot Rock. *Tussilago farfara* leaves have been used in the traditional Austrian medicine internally [as tea or syrup] or externally [directly applied] for treatment of disorders of the respiratory tract, skin, locomotor system,

viral infections, flu, colds, fever, rheumatism and gout. *Carum copticum* or *Trachyspermum ammi* [Family**:** Apiaceae**]** is administered in flatulence, atonic dyspepsia and diarrhea, and often recommended for cholera. In the Unani system, the herb is used as a drug to enhance the body's resistance, and is prescribed in amoebiasis, a parasitic infection of the intestines. It is a potent antimicrobial agent. The principal constituents of Bishop's Weed oil are the phenols, mainly thymol and some carvacrol. Thymol is a powerful antiseptic and antifungal agent. It is an ingredient in deodorant, mouthwashes and toothpastes. The aqueous portion, left after the separation of the essential oil, is known as omum-water and is prescribed in flatulence and gripe, especially in children. The herb is administered in gastrointestinal disorders [7-11].

2. Materials and Methods

2.1 Preparation of herbals

The plants were collected from around Tehran province. The *Carum copticum* seeds and *Hyssopus officinalis* and *Tussilage farfara* leaves were air dried at room temperature and kept in a dark amber-colored bottle until processed.

2.2 Preparation of herbals extract

The crude extracts were obtained by maceration of 100 g dried leaves and seeds in 200 ml solvent [55 ml double distilled water, 45 ml ethyl acetate and 100 ml 95% ethanol] in a dark place at room temperature. After one week, the pure extracts were filtered. The solvents were evaporated in vacuo. Dried extracts were stored in a dark amber- colored bottle. All the concentrations of the extracts were based on dry weight of the extracts.

2.3 Parasite preparation

The *L. major* used in this study was the standard strain MRHO/IR/75/ER. The infectivity of the parasites was maintained by regular passage in susceptible Balb/c mice. The parasites were cultured in the RPMI 1640 medium supplemented with 10% fetal bovine serum [FBS], 292 µg/ml l-glutamine and 4.5 mg/ml glucose [all supplied by Sigma]. Under these culture conditions, the stationary phase of parasite growth was obtained in 6 days. The culture was incubated at 25 °C and used within 2 weeks of cultivation [12, 13].

2.4 Topical formulation and ointment regimen design

A dried extract was prepared in the ointment base with the following formulation: plants extract 10%, lanolin 10% and DMSO 12%, all incorporated in white soft paraffin. No preservative was added and the ointments were kept at 4 °C and tested within 1 week after preparation.

2.5 Subjects

In this study, we used outbred; female Balb/c mice aged 4–6 weeks and weighted 30–40 grams. The mice provided by Iran Pasteur Institute were randomized into five groups each including nine mice. The test groups were compared with those of placebo and control groups. Control group received glucantime was supplied by Sigma [Sigma Chemical Co., St Louis, Mo. treatment]. **A**nd placebo group received the ointment base without the extract. This study was considered ethically approved by ethic committee of deputy of research of Iran University of Medical Sciences. About 6–8 week-old female Balb/c mice were then infected with 10^6 viable stationary-phase promastigotes through intradermal injection of parasites at the base of tail. After 30 days of inoculation of the *leishmania* promastigotes, the treatment was started at the nodule site. Once a well-developed lesion was observed, the ointment was applied twice daily [early in the morning and late afternoon] for a period of 20 days. To determine the amount of the ointment applied to the lesion, the ointment was weighted before application. It was found that a 200-mg amount of ointment was used per mouse per day using cotton applicator. The increase or decrease of diameters of each lesion was measured at the start of the study and weekly intervals by metric caliber. After the end of the treatment period, mice in the experimental and placebo groups were followed for 1 month.

2.6 Determination of parasite burden

Three mice from each group were sacrificed 4weeks after treatment and parasite burden was determined as follows: A piece of spleen was excised, weighed and then homogenized with a tissue grinder in 2 ml of Schneider's Drosophila medium supplemented with 20%heat-inactivated fetal calf serum and Gentamicin [0.1%].Under sterile conditions, the serial dilutions ranging from 1 to 1/4 x10-4 were prepared in wells of 96 well micro titration plates. After 7 and 15 days of incubation at 26° C, the plates were examined with an inverted microscope at a magnification of 40x. The presence or absence of mobile promastigotes was recorded in each well. The final titer was the last dilution for which the well contained at least one parasite. The number of parasite per gram was calculated in the following way: Parasite burden= -log10 [parasite dilution/tissue weight]

2.7 Statistical analysis

The data are expressed as mean ± SEM [standard error of mean] of animals. Mean values were analyzed with a two way analysis of variance [ANOVA] and Student's *t*-test. Differences between mean values were accepted as significant when $p < 0.05$. All statistical analyses were done using SPSS software, version 11.5 [14].

3. Results

A nodule developed 3 to 4 weeks after the inoculation of 10^6 promastigotes of *L major* into the base of the tail of a mouse. one to Two weeks later, the nodule transformed into an ulcer that was increased in size. Antileishmanial activity of plants extract was evaluated. After 20 days of treatment, twice daily, with the ointment base of plants extract the lesion size was studied up to the completion of the study. The lesion size in the test groups [10% extract] were decreased statistically significant in comparison with placebo group but not significantly with control group [Glucantime]. The weight of mice in the test groups [10% extract] were not increased statistically significant in comparison with placebo group but significantly with control group [Glucantime] were increased. The Parasite quantitation in the spleen cells in test groups was diminished significantly in comparison with placebo group but not significantly with control group. Treatments could not disappear completely the diameter lesions [p = 0.008, 0.006 and 0.002, respectively].

The Effects of a 20-day period of topical treatment with ointment-based extract of herbals extract on cutaneous leishmaniasis Inbred mice infected with *Leishmania major* were shown in figures 1-3.

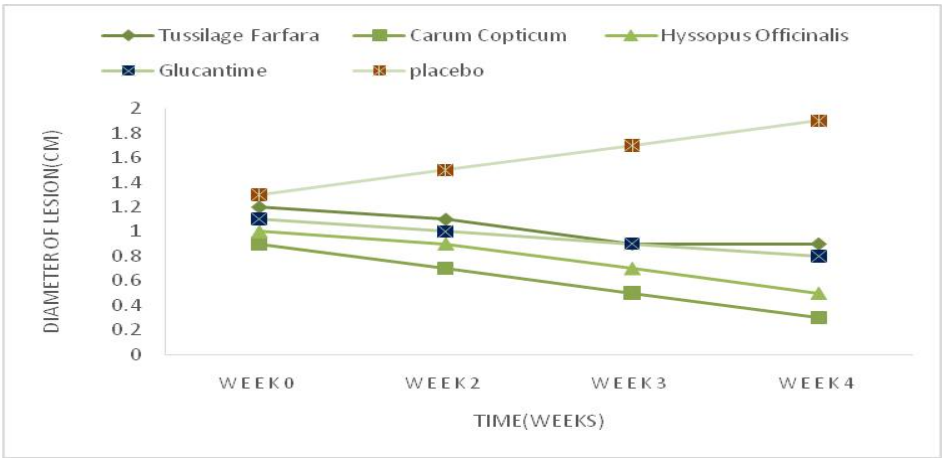

Fig 1: The Lesion size in each test group is compared with those of control and placebo groups at the beginning of [week 0], just after [week 2] and 3 weeks after completion of [week4] the treatment. The values represent the mean ± SEM. *p < 0.05; **p < 0.001

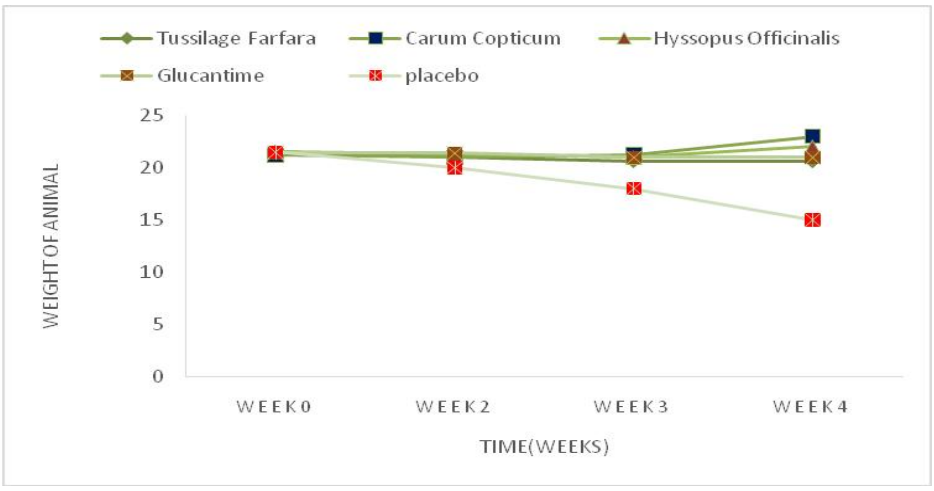

Fig 2: The weight of mice in each test group is compared with those of control and placebo groups at the beginning of [week 0], just after [week 2] and 3 weeks after completion of [week4] the treatment. The values represent the mean ± SEM. *p < 0.05; **p < 0.00

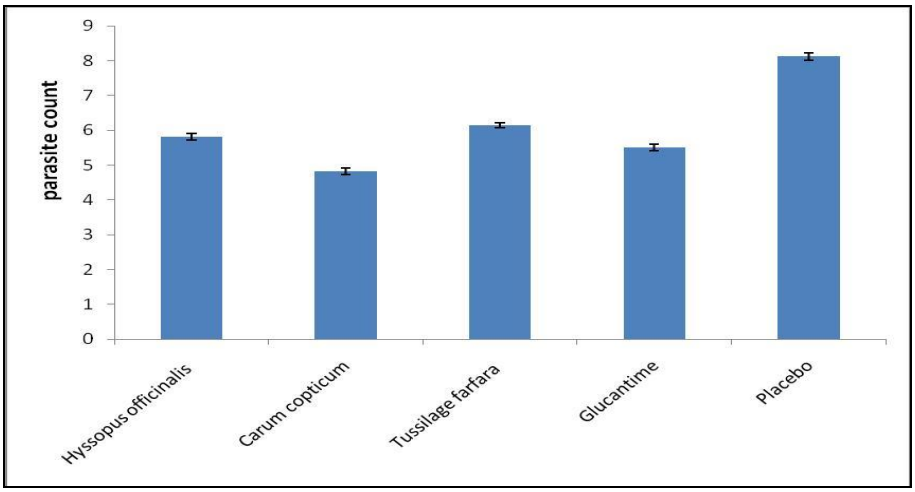

Fig 3: The Parasite count in each test group is compared with those of control and placebo groups at the beginning of [week 0], just after [week 2] and after completion of [week4] the treatment. The values represent the mean ± SEM. **p< 0.001

4. Discussion

Leishmaniasis is a group of tropical diseases caused by a number of species of protozoan parasites. It is also regarded that presently there exists a population of 350 million of people under risk of infection. Many therapeutic modalities have been used for the treatment of cutaneous leishmaniasis.

Pentavalent antimonials such as sodium stibogluconate, have been the base for therapy in the endemic regions because of its efficacy and cost effectiveness. The disadvantages of the antimonials are their requirement for intramuscular or intravenous injection each day for 20-28 days, their toxicity and the growing incidence of resistance in endemic and non-endemic areas. Due to the limited availability of effective pharmaceutical products, most people in areas where

leishmaniasis is endemic depend largely on popular treatments and traditional medicines to alleviate the symptoms. In addition to the various methods already mentioned, the treatment of leishmaniasis following the traditional medical practices of different cultures depends heavily on the use of native plants [15, 16]. To develop a suitable semisolid antileishmanial preparation, an ointment base of the extract was prepared. White soft paraffin [petrolatum] was selected as a typical oleaginous ointment base in view of its widespread use for many pharmaceutical ointments and lanolin was added to increase the hydrophilicity of the vehicle. DMSO is a well known penetration enhancer and was used to increase percutaneous absorption of the drug. Our study showed high efficacy of herbals against leishmaniasis *in vivo.* This result is consistent with the results of some previous studies. The results researchers were suggestive that *Thymus vulgaris*, *Achillea millefolium* and propolis extracts were effective for treatment of cutaneous leishmaniasis in mice with reduction of ulcer size also glucantime was not effective for the treatment of CL in mice but our study showed glucantime was effective for the treatment of CL in mice [14, 17] .

The plants used in this research containing plants are used medicinally in virtually all traditional medical systems, and have a history of usage in Chinese medicine dating back to thousands years.

These plants have antiviral and antiparasitic activity such as essential oil of these against *L. major*. On the other hand, these plants are common table vegetable all over Iran and abundant in Kashan as well as desert areas and access to these plants are very cheap and available.

In other study After 30 days, diameter of CL lesions increased in 1%, 3% and 5% *Artemisia* concentrations and the control groups. Ulcers got bigger with the more concentration. Treatments could not reduce the diameter or caused small lesions. The results of the studies mentioned above are not in agreement with the results of our study [18]. In our study discontinuation of treatment did not lead to significant increase in the lesion size or parasite count during a 4-week period. In each test groups [10%], the lesions in 2 animals, out of 10, disappeared completely. The possible mechanism for this effect is through release of nitric acid and tumor necrosis factor from macrophages.

In another study, peganine exhibited *in vitro* activity against both extracellular promastigotes as well as intracellular amastigotes residing within murine macrophages in *L. donovani* then *P harmala* seeds extract showed significant *in vitro* and in vivo antileishmanial activities [14].

The results of this study mentioned above is in agreement with the results of our study. In this regard, medicinal plants seem to be promising candidates for drug discovery against leishmaniasis.

The present study was an attempt to develop a topical treatment for CL. In conclusion, to find the effective concentration and the mechanism of the effectiveness of plants, further investigations and extensive study with various concentrates of these plants essence are recommended.

5. Conflict of Interests

Authors have no conflict of interests

6. Acknowledgments

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