

Antiulcer Activity after Oral Administration of the Wormwood Ethanol Extract on Lesions due to *Leishmania major* Parasites in BALB/C Mice

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Abstract

Herbal extracts were used to investigate the *in vivo* efficacy of *Artemisia absinthium* on the treatment of cutaneous leishmaniasis in susceptible mice. A total of 40 BALB/c mice were subjected to assays. In each, $3\text{-}5 \times 10^3$ amastigotes of standard *Leishmania major* strain were inoculated subcutaneously into the tail base of mice. Groups of mice were assigned as: I-negative control, II-positive control, III-Glucantime®, IV-ointment twice a day, V-ointment with oral medicine, VI-oral medicine on parasite injection, VII-oral medicine once ulcer develops, and VIII-ointment-based crème on ulcer. The gold standard of clinical infection control was based on ulcer size measurement using a Vernier scale weekly during 4 weeks Post-Ulcer Development (PUD). The mean ulcer sizes in different groups were compared using the post hoc Dunnett's 3 statistical analyses. There was a significant difference between the two groups of ointment with medicine (V) and medicine on parasite inoculation (VI) ($P \leq 0.027$). Antiulcer activity and healing was noted after oral treatment with aqueous extract on parasite injection. There was a significant difference between data from positive control group and local ointment with oral medicine ($P \leq 0.045$) indicating that ointment use facilitated ulcer growth. There was also a significant difference between data from Glucantime® use and ointment with medicine group ($P \leq 0.039$) which showed the deteriorating effect of oil-based ointment use. The oral administration of extract had an effect similar to Glucantime® use and led to the repair of ulcer. *A. absinthium* extract as oral feeder appeared to cause modulation of host responses, ulcer size reduction and tissue repair.

Keywords: Artemisia, Balb/c mice, Glucantime®, Leishmania major, Treatment

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1. Introduction

Cutaneous Leishmaniasis (CL), as ulcerative skin lesions caused by the flagellate protozoan parasites in the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), represent widespread neglected zoonotic and public health diseases being endemic in most developing countries of Africa, Latin America, and Asia including Iran⁴². CL is the second most important vector-borne protozoan disease in Iran, after malaria²⁸. The national incidence rate of CL disease has risen from 0.002 to 1.337 over the first decade of this century²². CL transmission is, however, anthroponotic or zoonotic based on whether the main reservoir host is human or other mammals³⁴. Among 15 well-defined *Leishmania* species known to cause parasitic infection in humans, 13 have a zoonotic natural history¹⁶. Most transmission cycles of Iranian CL cases are zoonoses²⁷. The parasite is transmitted by the female blood-sucking phlebotomine sand flies (Diptera: Psychodidae) often feeding on rodent reservoir hosts prior to biting humans^{2,3}.

Chemotherapy with pentavalent antimonial drugs, like meglumine antimoniate (Glucantime®), is still the mainstay activity in endemic areas of Iran¹⁷. CL treatment is often impeded by the development of drug resistance¹¹, side effects like cardiac and renal toxicities, variable efficacy, necessity to endure long courses of parenteral administration, the absence of an effective vaccine, as well as the formidable task of developing a simple, cheap, sustainable and swift drug-evaluating system globally effective on the different *Leishmania* species/strains infecting humans; though this being too optimistic²⁰. Attempts to identify alternative search strategies are thus conspicuously essential²⁵. About 80% of the world's inhabitants depend on folk medicines for their healthcare⁹. Traditional treatment of CL includes the administration of natural products from medicinal herbs such as wormwood, *Artemisia*, in some parts of the world^{6,35}.

Medicinal herbs are normally rich sources of various types of bioactive organic compounds. The medicinal plant genus *Artemisia* is one of the largest and most widespread members of the daisy family in the world which includes about 500 species growing mostly in temperate regions of Asia, Europe and North America⁵. One of these botanicals, known commonly as the absinthe wormwood *Artemisia absinthium* L. (Asteraceae/Compositae), is an aromatic perennial herb with silvery-green foliage growing naturally native on dry open slopes in Fars

province, south Iran. They are characterized to have multiple diverse effects. Their efficacy could be due to the composition and biochemical effects of various volatile Essential Oils (EOs) which are reported to have antifungal⁴⁰, antimicrobial^{12,31}, and antiparasitic^{1,39} effects against *Leishmania* species. Additionally, certain EOs from *A. absinthium* has been reported to bear acaricidal⁸, insecticidal²³, and repellent activities against fleas, flies, gnats, and ticks²¹.

Much of the studies on the detection of natural medicinal products with anti-*Leishmania* activity have been performed using the promastigote (flagellate) or sand fly stage of the parasite as it is more amenable to maintain under *in vitro* conditions. However, since the extracellular promastigote is not the pathogenic form of the parasite in vertebrate hosts, assays carried out with promastigotes have solely an indicative value of the likely anti-*Leishmania* activity of the herbal extract or its metabolite tested. An initial evaluation using promastigotes have thus to be complemented with an assay on intracellular amastigotes within parasitophorous vacuoles of vertebrate host's macrophages⁷. Consequently, a recent study was conducted on the *in vitro* activities of *A. absinthium* on *Leishmania major* parasites, using a series of synchronous cell sensitivity and flow cytometry assays⁴. A growth inhibitory concentration (IC₅₀) and a lethal concentration (LC₅₀) of 16.6 and 101 μgml⁻¹ were thus recorded, respectively. In the present study, the main aim was to unravel the *in vivo* antiulcer efficacy of *A. absinthium* medicinal herb ethanol extract on the treatment of CL lesions caused by *L. major* parasites in susceptible BALB/c mice. To the best of authors' knowledge, there appear to have been no previous reports on the *in vivo* effects of absinthe wormwood against lesions induced by *L. major* amastigotes parasites within the scope of this investigation.

2. Materials and Methods

Plant identification, preparation, and isolation of its extracts as well as parasite culture were previously described⁴. The hydro alcoholic extract obtained from *A. absinthium* was then delivered to pharmaceutical Department of the School of Pharmacy for the preparation of 90% ointment. The creme based formulation comprised 18 g liquid paraffin, 7 g Vaseline, 5 g Cetostearyl alcohol, 3 g Cetomacrogol, 0.5 g Carbomer in 70 g distilled water, and 5 ml of one normal sodium hydroxide (NaOH).

2.1 Laboratory Animals

All experimental animals were susceptible BALB/c male mice between 4-6 weeks old and weighing 18-24 g which were supplied from the Institute of Pasteur at the Iranian capital city of Tehran. These mice were grouped into eight batches of five mice as follows:

- Group I: Naïve uninfected mice but oral treatment with drug (negative control).
- Group II: Untreated but infected with amastigote parasites (positive control).
- Group III: Intramuscular Glucantime® injection 3 weeks Post-Ulcer Development (PUD).
- Group IV: Local ointment twice (A.M. and P.M.) a day PUD.
- Group V: Local ointment twice daily with oral medicine PUD.
- Group VI: Aqueous oral medicine upon parasite inoculation.
- Group VII: Aqueous oral medicine once ulcer appears.
- Group VIII: Local application of ointment-based crème once ulcer appears.

2.2 Parasite Inoculation into Susceptible BALB/c Mice

Experimental infections of susceptible BALB/c mice were performed using *Leishmania major* parasitic amastigotes. To inoculate parasites, the lesion clots from 5 *L.*

major-infected mice were removed after detachment. The serosal fluids from below lesion clots were taken up and transferred to physiological sera. A suspension of parasites, physiological serum and antibiotics (100 µg/ml streptomycin, and penicillin 100 U/ml) were thus prepared. Using a Neobar Chamber slide on an inverted compound microscope, parasite counts were examined by taking samples from this suspension. In addition, a drop of amastigote parasites suspension was poured onto a microscope slide, air-dried, methanol-fixed, and stained in Giemsa for direct observation. All susceptible BALB/c mice, except those in the first group, were subcutaneously inoculated at their tail base with equal volumes of $3-5 \times 10^3$ amastigotes of standard Iranian *L. major* parasite strain MRHO/IR/75/ER. After three weeks of parasite inoculation, a raised nodule was felt at the site of entry which resulted in an open ulcer.

2.3 Treatments and Measurements

The first naïve group was solely treated with oral medicine. Those groups which were treated with oral medicine received 1.4 g/kg drug daily. The second group (positive control) did not receive any treatment as it was solely inoculated with parasites. The groups of I, II, IV, V, and VIII were followed up and except group II received treatments for 4 weeks. The groups of VI and VII were treated for 5 weeks. The mice in group III received daily intramuscular (*i.m.*) inoculation of 0.6 mg/kg Glucantime® for a period of 21 days (Figure 1).



Figure 1. A BALB/c mouse is being injected with Glucantime® drug into its calf muscle.

Prior to treatments, the ulcer areas on mice in each experimental group were recorded following measurements. They were also re-measured during the course of treatments once a week and the data on ulcer areas were noted. The length and width of each ulcer were directly measured with a Collis Vernier caliper scale, and its area was calculated using the formula of: $S = a \cdot b / 2 \cdot \pi$; where 'a' variable was the length and 'b' was the width of each ulcer.

2.4 Data Analysis

In order to compare the mean size of ulcers between different mice groups, the statistical test of Analysis of Variance (ANOVA) was used. Data analysis by ANOVA was significant, but the presumption of equal variances was not established. Therefore, the post hoc Dunnett's T3 statistical analyses were used to determine the efficacy of different treatment regimes. In addition, *P*-values of 0.05 or less (or a confidence interval of 95%) were considered significant. Data analysis was done using SPSS software version 16.

3. Results

The data analysis revealed that there was a significant difference between ulcer sizes in the two groups of V and VI ($P \leq 0.027$); that is oral medicine upon parasite inoculation and ointment twice daily with oral medicine post-ulcer development; PUD (Table 1). This ameliorative response of lesion growth retardation was similar to that of the oral administration of Glucantime® PUD which culminated in lesion repair and resolution. The outcome of positive control (untreated infected) group

with those of fifth (ointment with oral medicine) group were also significantly different ($P \leq 0.045$); such that local ointment application accompanied by oral medicine administration led to the exacerbation of lesion growth (Table 2). The Glucantime®-treated and the ointment with oral medicine groups also showed a significant difference ($P \leq 0.039$) reflecting that the oil-based ointment caused an aggravating effect on lesion size. In contrast, oral administration of herbal medicine upon parasite inoculation and once ulcer develops did not exhibit any significant statistical difference with the Glucantime® group ($P \geq 0.05$). These oral protocols led to a repair and reduction of lesion size.

In the present study, an ulcer developed on average about 35 day post-inoculation of amastigote parasites into the mice. As indicated in Table 1, the biggest ulcer size had a mean area of about 141.86 mm² due to the local application of ointment twice daily with oral medicine PUD, so that there was a 5-fold increase in the mean ulcer size from the start to the end of this treatment. The largest ulcer growth rate was noted in group IV using local ointment twice (A.M. and P.M.) a day PUD without any oral administration, such that there was an 8.87-fold increase in the mean ulcer size from the start to the end of treatment. On the other hand, the smallest ulcer size with a final mean area of about 14.64 mm² was recorded in that group of mice receiving aqueous oral medicine upon parasite inoculation. The mean ulcer size from the start to the end of this treatment grew by only 1.03-fold. Likewise, the mean ulcer size in Glucantime® group grew by 1.6-fold which indicated that oral drug administrations have had meaningful effects on the prevention of lesion growth due to the intracellular proliferation of *L. major* amastigote parasites within tissue macrophages.

Table 1. Average lesion size (mm²) and its growth rate among different treatment groups of mice during a five-week period

Groups [†] / Weeks	1	2	3	4	5	Lesion growth rate
I: -ve. Control	-	-	-	-	-	-
II: +ve. Control	6.76	16.76	20.78	22.84	-	3.37
III: Glucantime PUD*	11.07	18.38	17.82	-	-	1.60
IV: Oint. PUD	10.17	44.44	59.19	90.23	-	8.87
V: Oint./Oral PUD	28.16	68.84	81.74	141.86	-	5.03
VI: Oral on Inject.	10.14	8.75	8.24	10.92	14.64	1.44
VII: Oral on Ulcer	12.80	16.99	22.93	29.68	29.23	2.28
VIII: Oint.-base crème	12.90	19.59	42.02	47.67	-	3.69

[†]Numbers I-VIII refer to different treatment protocols, *PUD = post ulcer development.

Table 2. Statistical analyses from multiple comparisons of oral and local wormwood extract administrations under *in vivo* conditions on BALB/c mice using pair wise Dunnett's T3 test to determine the significant differences between different variables.

Variable [†] (I)	Variable [†] (J)	Mean difference (I-J)	Standard error	Significance level	95% Confidence interval	
					Lower limit	Upper limit
II	III	1.02400	8.68868	1.000	-36.1334	38.1814
	IV	-34.22000	20.07622	0.788	-137.1351	68.6951
	V	-63.37600*	12.94557	0.045	-125.0324	-1.7196
	VI	6.24800	7.80993	1.000	-26.2494	38.7454
	VII	-5.54400	12.90244	1.000	-66.9482	55.8602
	VIII	-13.76000	14.01266	0.993	-81.6496	54.1296
III	II	-1.02400	8.68868	1.000	-38.1814	36.1334
	IV	-35.24400	20.82109	0.797	-135.5404	64.9624
	V	-64.40000*	14.07307	0.039	-125.5228	-3.2772
	VI	5.22400	9.56338	1.000	-34.0709	44.5189
	VII	-6.56800	14.03339	1.000	-67.4690	54.3330
	VIII	-14.78400	15.06047	0.994	-81.4910	51.9230
IV	II	34.22000	20.07622	0.788	-68.6951	137.1351
	III	35.24400	20.82109	0.797	-64.9624	135.4504
	V	-29.15600	22.92624	0.959	-128.2830	69.9710
	VI	40.46800	20.46997	0.659	-60.8072	141.7432
	VII	28.67600	22.90191	0.963	-70.4252	127.7772
	VIII	20.46000	23.54526	0.999	-79.5388	120.4588
V	II	63.37600*	12.94557	0.045	1.7196	125.0324
	III	64.40000*	14.07307	0.039	3.2772	125.5228
	IV	29.15600	22.92624	0.959	-69.9710	128.2830
	VI	69.62400*	13.54817	0.027	8.6822	130.5658
	VII	57.83200	17.00091	0.121	-11.5144	127.1784
	VIII	49.61600	17.85812	0.271	-23.3993	122.6313
VI	II	-6.24800	7.80993	1.000	-38.7454	26.2494
	III	-5.22400	9.56338	1.000	-44.5189	34.0709
	IV	-40.46800	20.46997	0.659	-141.7432	60.8072
	V	-69.62400*	13.54817	0.027	-130.5658	-8.6822
	VII	-11.79200	13.50696	0.998	-72.4962	48.9122
	VIII	-20.00800	14.57119	0.927	-86.8773	46.8613
VII	II	5.54400	12.90244	1.000	-55.8602	66.9482
	III	6.56800	14.03339	1.000	-54.3330	67.4690
	IV	-28.67600	22.90191	0.963	-127.7772	70.4252
	V	-57.83200	17.00091	0.121	-127.1784	11.5144
	VI	11.79200	13.50696	0.998	-48.9122	72.4962
	VIII	-8.21600	17.82687	1.000	-81.1185	64.6865
VIII	II	13.76000	14.01266	0.993	-54.1296	81.6496
	III	14.78400	15.06047	0.994	-51.9230	81.4910
	IV	-20.46000	23.54526	0.999	-120.4588	79.5388
	V	-49.61600	17.85812	0.271	-122.6313	23.3993
	VI	20.00800	14.57119	0.927	-46.8613	86.8773
	VII	8.21600	17.82687	1.000	-64.6865	81.1185

†Numbers II-VIII refer to different treatment protocols, *The mean difference is significant at the 0.05 level.

4. Discussion

Cutaneous leishmaniasis mostly caused by the intracellular *Leishmania major* amastigote parasites remains a formidable therapeutic challenge and a serious public health concern in most tropical countries, including Iran^{2,3}, though occasionally other infectious and non-infectious diseases could also be superimposed^{13,18,29}. Although the pentavalent antimony compounds such as sodium stibogluconate (Pentostam®; Glaxco Wellcome, UK) and meglumine antimoniate (Glucantime®; Aventis, France) are the first-line drugs used in the treatment of all forms of leishmaniasis, the report of cases of resistance to these drugs and the fact that they have to be administered parenterally represent the main hurdles for the chemotherapy of leishmaniasis¹¹. Unfortunately, no ideal treatments for the leishmaniasis have yet been advocated⁹.

There appears to be a new herbalism trend in search of folk medicines to treat human infectious diseases with increasing frequency⁴¹. Novel approaches including the oral administration of natural pharmaceutical products from medicinal herbs such as wormwood, *Artemisia*, have been proposed³³. The currently well-known anti-malaria compound, artemisinin (a sesquiterpene with an endoperoxide bridge), is derived from this plant^{24,36}. In the *in vivo* test, this compound in micromolar concentrations has been reported to reduce the severity of lesions caused by *Leishmania amazonensis* in BALB/c mice¹⁴. The toxicity of artemisinin derivatives for laboratory animals and its safe use in man have also been reviewed¹⁵. The rapid reduction of parasitaemia and short elimination half-life (with an almost immediate effect) of these compounds after oral administration are elucidated in this review. The toxicity resulting from the oil-based formulations is also implicated, which is compatible with the data from the present study. Orally administered chemotherapeutic agents (e.g., as salt) have the privilege of minimizing socioeconomic problems associated with populations living in endemic areas where the infrastructure of health care facilities is rudimentary³⁰.

The leaf and seed extracts of the annual or sweet wormwood, *Artemisia annua*, have been shown to mediate apoptosis in *Leishmania donovani*, the cause of visceral leishmaniasis (Kala-azar) in the Indian subcontinent¹⁹. Furthermore, the *in vivo* studies have indicated that a 70% ethanol extract from the commonly used vegetable, known in Iran as Tarkhoon (*Aretemisia dracunculus*), had

potent anti-inflammatory (or antiulcer) activity³². Other studies have confirmed that the leaves from this medicinal herb have inhibitory effects on blood platelet adhesion and aggregation in rats³⁷. This could mean swift flow of blood from the site of lesion and carriage of *Leishmania*-infected macrophages to remote vessels where they can readily be cleared by the host's immune responses.

The main aim of using an animal model such as mice is to deplore a drug that can be administered orally, be effective in a short period (<10 days) and have minimal sign of side-effects at the highest testable doses (100 mg/kg). According to recent reports^{10,26}, the BALB/c mouse – *L. major* model has extensively been used in drug discovery studies but it is an exceptionally rigorous non-cure model in which the most active drugs have any efficacy and absolute cure is scarce. The treatment of CL is difficult due to the intracellular (within macrophages) location and replication of the infectious *Leishmania* amastigote parasites. The main observation from the present study was that oral *in vivo* treatment with an aqueous extract of *A. absinthium* was found to be effective in experimental murine models of CL. As oral administration of this herbal extract provided a prolonged slow release of active ingredients in mice which probably potentiated the host immune responses, the lesion growth rate was thus minimized with this route of administration compared with other choices. In contrast, the dermal application of an ointment-based crème formulation culminated in increased lesion growth rate indicating that the oil-based moiety prevented lesion resolution. This finding was in accordance with other studies¹⁵. The present finding, that oral herbal extract administration was also slightly more effective than Glucantime® *i.m.* use, was supported by the data reported from another similar *in vivo* model system using *Artemisia auchery*³⁸.

One of the main limitations of this study was that the determination of drug efficacy by scoring 3-dimensional changes in lesion size during and after the course of treatment could be biased as much of the lesion consists of inflammatory cells such as macrophages filled with amastigotes limited to the dermal layer of the skin. Culturing parasites from skin biopsies gives an alternative quantitative score of activity¹⁰. This was accomplished in a recent publication⁴.

The lack of a suitable association between the data acquired by the *in vitro* bioassays and those from the *in vivo* evaluations, as well as the necessity to have rapid and simple evaluation procedures in which all species and

strains of *Leishmania* could be incorporated, have stimulated the search for novel bioassay techniques to confirm leishmanicidal activity⁷. This is exemplified by the follow up and uptake by parasites of radioactive trace elements such as the use of tritiated hydrogen [³H] atom in medicinal herbs.

It was thus concluded from the present data that oral administration of *A. absinthium* herbal extract due to its pharmaceutical chemical entities had the most therapeutic effect on lesions due to the *L. major* amastigote parasites in susceptible BALB/c mice. It seems that *A. absinthium* extract as an oral feeder caused modulation of host responses, ulcer size reduction and tissue repair. In contrast, ointment formulation from this extract had an inverse effect on ulcer size and its diameter increased possibly due to its impregnation with an oil moiety.

5. Conflict of Interest

The authors declare that there is no conflict of interests.

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