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In vitro and *in vivo* susceptibility of *Leishmania major* to some medicinal plants

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ABSTRACT

Objective: To evaluate the efficacy of some medicinal plants and systemic glucantime in a comparative manner against the causative agent of cutaneous leishmaniasis both *in vitro* and in BALB/c mice.

Methods: For *in vivo* testing, inbred mice were challenged with *Leishmania major* parasites and the resultant ulcers were treated with extract based-ointments applied topically two times per day for a period of 20 days. A group of 56 mice were randomly divided into 7 subgroups. The control group received the ointment void of extracts, whereas the reference group received glucantime only. The efficacy of treatments was evaluated by measuring ulcer diameter, parasite burden and NO production.

Results: Our results indicated that plant extract based-ointments were effective in reducing ulcer size and parasite burden in spleens, but their effects did not differ significantly from that of glucantime. The plant extracts tested in this study were able to increase NO production that helped parasite suppression.

Conclusions: Our findings indicate that the tested plant extracts are effective against *Leishmania major* both during *in vitro* and *in vivo* experiments, but further researches are required to recommend a potential plant extract as an alternative drug.

1. Introduction

As a vector-borne parasitic disease, leishmaniasis has a spectrum of clinical manifestations from self-healing skin ulcers, mucosal damages to serious visceral infections [1]. According to the World Health Organization, leishmaniasis is one of the most neglected re-emerging and uncontrolled tropical diseases. The HIV and *Leishmania* coinfection is quickly growing in number in countries where *Leishmania* species are endemic. The disease is reported from many parts of the world with an estimated prevalence of about 12 million cases. However, the incidence of cutaneous leishmaniasis (CL) and visceral leishmaniasis is estimated to be about 1.5–2 million and 500 000 new cases each year, respectively [2,3]. Upon inoculation in the dermis, the leishmanial promastigotes are phagocytosed by macrophages before interacting with extracellular matrix components to produce variable clinical syndromes [4]. In developing countries, the medicinal plants have long been used for disease treatment because they are safe and available at low price. Given the various beneficial drugs derived from medicinal plants, discovering new sources of drugs against *Leishmania* infection would be of high significance [5–7]. This research was undertaken in order to respond to aggravating situation of leishmaniasis in Iran, manifested in high prevalence, drug resistance, costly and lengthy treatment and

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serious side effects of pentavalent antimony compounds as first-line therapies [8,9]. In this study, we examined the efficacy of a number of plant extracts against *Leishmania major* (*L. major*) parasite both *in vivo* using infected BALB/c mice and *in vitro* using parasite cultures. The plant extracts were derived from *Carthamus tinctorius* (Family: Asteraceae) (*C. tinctorius*) or safflower, *Pimpinella anisum* (Family: Apiaceae) (*P. anisum*), *Cuminum cyminum* (Family: Apiaceae) (*C. cyminum*), *Cinnamomum verum* (Family: Lauraceae) (*C. verum*) and *Alhagi persarum* (Family: Fabaceae) (*A. persarum*) [10–15]. Due to its diversity of climate, Iran is home for aforementioned plants where they grow profusely. The plants have already shown many therapeutic effects against helminths, bacteria, fungi and viruses. Also, the plants are used in traditional medicine as health maintaining supplements and useful drugs against diarrhea in children [16,17]. It is worth noting that the World Health Organization has accorded high priority and significance to investigation of medicinal plants as traditional drugs for leishmaniasis treatment [18,19].

2. Materials and methods

2.1. Medicinal plants preparation

Seeds of *C. tinctorius*, *P. anisum*, *C. cyminum*, juice and stem of *C. verum* and bark of *A. persarum* which were endemic to Iran, were obtained from Research Institute for Islamic and Complementary Medicine in Iran (Tehran), in July 2014, during their flowering stages. The plants were taxonomically verified by botanists from the herbarium of the above-mentioned institute in Tehran, Iran and preserved under voucher specimens No. 2338, 1135, 1172, 528 and 2110, respectively.

These green plants are cultivated in different parts of Iran such as Lordegan Region in Chaharmahal and Bakhtiari Province as well as in Kerman, Shiraz, Ardabil Provinces.

The crude extracts were obtained by maceration of 100 g of plants to which 100 mL methanol was added in a dark place at room temperature. After 7 days, the pure extracts were filtered and the solvents were removed using a rotary evaporator. The dried extracts were kept in dark amber colored containers. All concentrations of the extracts were made on the basis of dry weight of extract per volume.

2.2. Anti-leishmanial drug

There are two common therapies containing antimony (known as pentavalent antimonial), meglumine antimoniate (glucantime) and sodium stibogluconate (Pentostam). We used meglumine antimoniate purchased from Sigma (Sigma Chemical Co., St Louis, MO). Glucantime was injected intramuscularly at 20 mg/kg/day for 20 days.

2.3. Parasite culture

The standard strain of *L. major* (MRHO/IR/75/ER) was obtained from the School of Public Health (Tehran University of Medical Sciences, Iran). The parasite was cultured in Novy-MacNeal-Nicolle medium before being mass produced in RPMI-1640 containing 10% fetal bovine serum, 292 μg/mL L-glutamine and 4.5 mg/mL glucose as well as 100 IU/mL penicillin and 100 μg/mL streptomycin for bacterial decontamination (all chemicals obtained from Sigma). The cultures were incubated at 25 °C and the stationary phase of parasite growth was obtained after 6 days and used within 14 days post-incubation [20].

2.4. Concentrations of plant extracts

The plant extracts and anti-leishmanial drug were dissolved in phosphate-buffered saline and prepared for biological testing at the following concentrations: 0.05, 0.1, 0.2 and 0.4 μg/mL. To test the anti-leishmanial activity of concentrated plant extracts, solutions were prepared by dissolving the powdered extracts in 20% dimethyl sulfoxide (DMSO) followed by 5 min of sonication to make a stock solution of 100 mg/mL. The solutions were then stored at −20 °C before being filtered through 0.2 μm filter at the time of application. The concentration of DMSO in the solutions was kept at $\leq$ 1%.

2.5. In vitro experiments

The anti-leishmanial activities of plant extracts were tested on late log phase of *L. major* promastigotes inoculated in RPMI medium supplemented with 10% fetal calf serum at 10^6 parasites/mL. The viability of parasites was assayed in duplicate against ascending concentrations of plant extracts. The viable promastigotes were then counted after 24 h incubation period at 25 °C using a Neubauer chamber. The IC_{50} of the extracts was calculated as a concentration capable of inhibiting 50% of parasite growth. A negative and a positive control were added to each set of experiment using phosphate-buffered saline and glucantime as growth inhibitors, respectively. The protocol of *in vivo* experiments was approved by the Institutional Ethics Committee.

2.6. Experimental animals

BALB/c inbred females, aged 4–5 weeks and weighed 30–40 g were obtained from Razi Vaccine and Serum Research Institute (Karaj, Iran) and kept in standard boxes under controlled light and temperature conditions. Fifty-six BALB/c female mice, randomly divided into seven groups, were used for experimentation. Both experimental and control mice were followed up for 1 more month at the end of treatment periods. The approval for procedures using experimental animals was sought from Ethics Committee of Research of Iran University of Medical Sciences (Tehran, Iran).

2.7. Preparation of extract based-ointments

The extract based-ointments were prepared as per the following formulation: dried plant extract (20%) was added to lanolin (10%) and DMSO (12%) before being integrated in soft paraffin. The white soft paraffin served as a greasy ointment base to incorporate the abovementioned materials. Lanolin was added to enhance the hydrophilicity of the preparations. DMSO was also used as penetration enhancer and was used to improve the drug absorption through the skin. To economize, the extract concentrations for *in vivo* experiments were prepared based on their best results during *in vitro* experiments. No preservative was added and the ointments were kept at 4 °C and used within 7 days after preparation.

2.8. In vivo experiments

To initiate *Leishmania* infection, an inoculum of at least 10^6 stationary phase promastigotes was intradermally injected in the tail base of each mouse. The mice were then distributed into the following groups: the test group which received glucantime (gold standard) and plant extracts and the control group which received the ointment void of plant extracts. The inoculated mice developed nodules and ulcers after 30 days. Upon developing ulcers, the infected mice were treated by applying the preparations twice daily at the ulcer site (early morning and late afternoon) over a 20-day period. To fix the applied dose, 200 mg of each ointment was weighed and applied per mouse per day using a cotton applicator. Glucantime was injected intramuscularly at 20 mg/kg/day. A Kulis Vernier was used to measure lesions diameters of the treated mice which were then weighed by a digital scale at weekly intervals. At the end of the treatment, the mice were followed up for 1 month.

2.9. Parasite burden determination

To determine the parasite burden, 3 mice from each group were sacrificed after 4 weeks of treatment to obtain spleen samples. The spleen samples were aseptically weighed and homogenized in 2 mL of Schneider's *Drosophila* medium containing 20% heat-inactivated fetal calf serum and 0.1% gentamicin. The homogenates were subjected to serial dilutions ranging from 1 to $1/4 \times 10^{-4}$ in 96-well tissue culture plates under sterile conditions. The plates were incubated at 26 °C and examined for mobile promastigotes at 7 and 15-day post-incubation using an inverted microscope at 40× magnification. The dilution was made so that the last titer (well) contained at least one parasite. The parasite burden (cell/g tissue) was quantified as per following equation [21]:

Parasite burden = −log10 (Parasite dilution/Tissue weight)

2.10. Determination of NO

Griess reaction assay was employed for nitrite levels determination. To this end, peritoneal macrophages were harvested from both untreated mice and those exposed to various doses of plant extracts or glucantime. The harvested macrophages were cultured in flask before the adherent cells were removed by gentle scraping and washed with warm medium (25 °C). The macrophages were then counted and their viability was determined. The viable cells were further cultured in 24-well plates before incubation in 5% CO_2 at 37 °C for 18 h. After the removal of non-adherent cells, the plant extracts were added to wells either with or without 0.2 mmol/L L-NG-monomethyl arginine as NO synthase inhibitor. The supernatants were collected after 48 h and nitrite accumulation was assayed using Griess reagent [22].

2.11. Statistical analysis

Two-way ANOVA and student's *t*-test were used to analyze mean values. The experimental data were summarized using mean ± SEM. The statistical significant level for differences between mean values was accepted at $P < 0.05$. SPSS software version 12 was employed for statistical analyses.

3. Results

The plant extracts and glucantime inhibited 50% of the promastigote growth *in vitro* after 72 h (IC_{50}). The results depicted the *in vitro* inhibitory effect of plant extracts and the drug against *L. major* promastigotes. *In vitro* effects of the plant extracts on *L. major* promastigotes showed that death parasites in *P. anisum*, *C. verum*, *C. tinctorius*, *C. cyminum*, and *A. persarum* extracts were 80.08%, 74.16%, 70.14%, 60.75% and 36.58%, respectively. The plant extracts yielded the best results at 0.4 μg/mL concentration during *in vivo* experimentations. *P. anisum*, *C. verum*, *C. tinctorius*, *C. cyminum*, and *A. persarum* extracts and glucantime had an IC_{50} of (15.00 ± 0.65), (17.00 ± 0.91), (23.00 ± 0.59), (31.00 ± 0.71), (45.00 ± 0.61) and (20.00 ± 0.82) μg/mL, respectively.

Upon injection of the parasite into the mice tail bases, nodules developed 3–4 weeks later. The nodules then changed into ulcers after 1–2 weeks and increased in size. Anti-leishmanial activity of plant extracts was evaluated by measuring the lesion sizes and mice weights.

Our results showed that the plant extracts had no significant positive effect on the mice weights in the first week. However, slight weight losses occurred in the following weeks until the fourth week. In the control group, however, weight losses were considerable. The weight losses of mice in the test groups were not significantly different from those in glucantime treated group but differed from the control group.

The ulcer sizes in the test groups (treated with extracts) were significantly smaller than those in the control group, but they were not significantly different from glucantime treated group. The diameters of ulcers treated with *C. cyminum* and *P. anisum* extracts were almost similar to those treated with glucantime. Also, *A. persarum*, *C. verum* and *C. tinctorius* extracts caused reduction of the ulcers diameters in treated mice compared with control group which exhibited ulcers of significantly growing sizes. However, none of treatments could result in complete healing of the lesions as shown in Tables 1 and 2 ($P \leq 0.05$).

The parasite counts in the spleen cells showed significant reduction in the test group in comparison with the control group. The mean number of parasites (per mg of spleen tissue) in *C. cyminum*, *P. anisum*, *C. verum*, *C. tinctorius*, *A. persarum*, glucantime and control was 4.9 ± 1.1, 4.2 ± 1.9, 4.4 ± 2.3, 4.7 ± 3.2, 5.8 ± 1.8, 3.9 ± 2.8 and 7.0 ± 3.1, respectively. However, no significant difference was observed between extract- and glucantime-treated groups in terms of parasite counts.

On the other hand, extracts based-ointments and glucantime have induced statistically similar level of NO production, though *P. anisum* resulted in the highest amount of NO amongst all treatments. Our results showed that the plant extracts used in this research can promote NO production by murine macrophages. NO production was induced by plant

Table 1

The mice weights (g) in each test group compared with those in control and glucantime-treated groups.

Time	*C. cyminum*	*P. anisum*	*C. verum*	*C. tinctorius*	*A. persarum*	Glucantime	Control
Week 0	21.00 ± 1.16	21.40 ± 1.45	20.80 ± 0.89	21.80 ± 0.92	21.00 ± 0.78	21.40 ± 0.83	21.50 ± 1.00
Week 2	21.00 ± 1.51	21.50 ± 1.50	20.00 ± 0.95	21.70 ± 0.77	20.00 ± 0.86	21.47 ± 0.79	20.00 ± 1.10
Week 3	21.10 ± 0.91	21.60 ± 1.20	19.00 ± 0.99	21.60 ± 0.80	20.00 ± 0.90	21.59 ± 0.91	18.50 ± 1.30
Week 4	21.00 ± 0.87	21.70 ± 1.13	18.20 ± 0.97	20.80 ± 0.88	19.10 ± 1.13	21.80 ± 1.20	15.00 ± 1.12

Values are expressed as mean ± SD.

Table 2

Lesion sizes (mm) in each test group compared with those in control and glucantime-treated groups.

Time	*C. cyminum*	*P. anisum*	*C. verum*	*C. tinctorius*	*A. persarum*	Glucantime	Control
Week 0	1.30 ± 0.23	1.10 ± 0.20	1.20 ± 0.65	1.20 ± 0.49	1.20 ± 0.29	1.30 ± 0.68	1.10 ± 0.49
Week 2	1.30 ± 0.30	1.10 ± 0.10	1.20 ± 0.22	1.20 ± 0.59	1.30 ± 0.54	1.30 ± 0.33	1.80 ± 0.50
Week 3	1.50 ± 0.32	1.40 ± 0.48	1.40 ± 0.39	1.50 ± 0.42	1.80 ± 0.61	1.60 ± 0.53	2.00 ± 0.52
Week 4	1.60 ± 0.40	1.50 ± 0.71	1.70 ± 0.55	1.90 ± 0.11	2.00 ± 0.67	1.70 ± 0.58	2.30 ± 0.25

Values are expressed as mean ± SD.

extracts (*P. anisum*: 10.8 μm/mL, *C. verum*: 10.1 μm/mL, *C. tinctorius*: 9.9 μm/mL, *C. cyminum*: 9.1 μm/mL and *A. persarum*: 8.4 μm/mL) and glucantime (10.5 μm/mL) compared with control group (6.1 μm/mL).

4. Discussion

Amongst leishmaniasis forms, CL is the most commonly encountered one. Iran and its neighboring countries, Afghanistan, Pakistan, Iraq and Saudi Arabia, accommodate almost 90% of CL cases [23]. A number of chemical, physical and surgical therapies have been recommended for the treatment of CL. However, drug resistance by parasite to chemotherapeutic agents remains a serious obstacle in the way of treating leishmaniasis. Pentavalent antimonial therapy constitutes the first-line treatment for CL worldwide since 1945 [24,25]. In a bid to escape disadvantages of chemotherapeutic agents including drug resistance, this study has been conducted to evaluate the *in vitro* and *in vivo* effects of plant extracts obtained from *C. tinctorius*, *P. anisum*, *C. cyminum*, *C. verum* and *A. persarum* on *L. major* in comparison with glucantime. The necessity of the study was emphasized by the growing interest in natural products including medicinal plants as alternative therapies for CL. Several studies have already tackled the screening of plant extracts against leishmaniasis. This study, however, clearly indicated high efficacy of some medicinal herbs in inhibiting promastigotes growth during *in vitro* and *in vivo* experimentations. The medicinal plants examined in this research have a long history of use in Chinese medicine. Being common table vegetable available all over Iran, these plants have antiviral and anti-parasitic effects [26–28]. Given the lack of data about the natural ingredients of these plants and their effects against leishmaniasis, this study aimed to investigate their therapeutic effects on leishmanial lesions inflicted on BALB/c mice compared with glucantime effects.

Our results showed that the plant extracts produced significant decrease in parasite burden and lesion size in all treated mice groups compared with the control group ($P \leq 0.05$).

Various *Artemisia* concentrations (1%, 3% and 5%) failed to reduce diameters of CL lesions after 30 days of treatment compared with control group, whereas extracts of *Thymus vulgaris*, *Achillea millefolium* and propolis were more effective in reducing ulcer than glucantime [29,30]. Bafghi *et al.* found that the mean lesion sizes of mice receiving *Rubia tinctorum* extracts at 40%, 60% and 80% concentrations were not significantly different from those in control group ($P > 0.05$) [31]. The abovementioned studies are in agreement with our results. However, Akhlaghi *et al.* examined the effect of *Hyssopus officinalis*, *Tussilago farfara*, *Carum copticum* extracts on mice infected with *L. major* and showed that plants ointments were effective in reducing ulcer size and burden parasite in spleen [21]. Fata *et al.* have stated that, upon 2 weeks application, the ethanolic extract of *Berberis vulgaris* considerably decreases the lesion size caused by *L. major* in BALB/c mice [32]. Hejazi *et al.* showed significant difference between mean of lesion sizes among treated and untreated mice using yarrow (Bomadaran) and thyme (Avishan) extracts, although the plant extracts were as effective as glucantime [33]. Rahimi-Moghaddam *et al.* examined the *in vitro* effect of *Peganum harmala* against *L. major* and found a concentration-dependent decrease of parasite count, recording an IC_{50} value of 59.4 μg/mL. Conducting *in vivo* studies, they, also, demonstrated a significant post-treatment decrease in the lesion size and parasite count in infected animals, compared to placebo and control groups [20]. The abovementioned findings support our results. We showed that despite the fact that *P. anisum* extract produces comparable weight loss in mice, it is more effective than other extracts in reducing ulcer diameters and enhancing NO production. In fact, *P. anisum* extract produced almost similar anti-leishmanial activities as glucantime ($P < 0.05$). Therefore, *P. anisum* extract may be recommended as an appropriate drug for treating CL. The lowest parasite load in the spleen was observed in mice treated with *P. anisum* followed by those treated with *C. verum*, *C. tinctorius*, *C. cyminum* and *A. persarum* in a descending order of efficacy. We, also, observed that within test groups treated with *P. anisum* and *C. cyminum* extracts, 2 out of 8 mice had their lesions completely healed. Despite the notion that, in *L. major* infected BALB/c mice, the NO production decreases naturally due to amastigote actions, our results indicated that the plant extracts may restore NO production as a mechanism of parasite elimination. Some authors have already shown that artemisinin can cause 50% reduction in parasite burden of macrophages infected with *Leishmania donovani* due to NO production [34]. This is to the contrary of the effect of *Scrophularia striata* ethanolic extract which suppressed the ability of murine peritoneal macrophages to produce NO during *in vitro* and *ex vivo* experiments [35]. On the other hand, using RT-PCR technique, Gharavi *et al.* found that garlic extract promotes interferon gamma and inducible nitric oxide synthase genes expression in *L. major* infected J774 cells which can serve as an indication for more NO production [36]. However, the pathways underlying this kind of mechanism by plant extracts remain to be deeply understood.

It has been shown that NO production is part of microbicidal activity of macrophages to eliminate infectious intracellular pathogens such as *L. major*, *Toxoplasma gondii* and *Trypanosoma cruzi*. NO acts as an anti-leishmanial agent in mice macrophages by reducing parasite number within lesions. It seems that the development of intracellular amastigotes may be inhibited by both endogenous and exogenous NO. This finding is confirmed by *in vitro* and *in vivo* immunological studies in which NO radical within leishmanial lesions was responsible for parasite count reduction. The decrease in parasite burden can be due to stimulation of natural killer cells activator release by macrophages of both nitric acid and tumor necrosis factor [37,38].

Our study revealed that the tested plant extracts have a promising chemotherapeutic activity against *L. major* with *P. anisum* extract being of the potential to be recommended as a candidate for leishmaniasis treatment. In general, medicinal plants can offer promising drugs against CL. However, based on our data, further researches are required to elucidate the therapeutic mechanism of plant extracts against *L. major* both in animal models and human if we want to recommend a plant extract as a drug.

Conflict of interest statement

We declare that we have no conflict of interest.

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