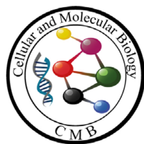

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# Susceptibility of *Leishmania major* to *Veronica persica* Poir. extracts - *In vitro* and *in vivo* assays

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**Abstract:** *Leishmania major* is an intracellular parasite generally responsible for cutaneous leishmaniasis (CL), one of the most encountered skin diseases especially in Pakistan, Iran, Iraq, and Saudi Arabia. Current treatment options are not ideal, due to unwanted side effects and increasing resistance and availability is often limited in developing countries. Medicinal plants continue to attract attention because of their beneficial effects in the prevention or/and accelerating the healing process of various diseases. In this study, *in vitro* and *in vivo* susceptibility of *L. major* to *Veronica persica* Poir. extract, a medicinal plant with many applications, has been evaluated. Antileishmanial activity of plant extract was investigated both on cultured *L. major* promastigotes and in mice challenged with *L. major*. Animals were divided into three groups including control (without any treatment), test (treated with plant extract) and glucantime (the reference drug) treated groups. After treatments, skin lesion sizes and body weights of animals were checked during 4 weeks. The potential of the plant extract in decreasing the number of parasites in spleen cells of animals as well as inducing the nitric oxide (NO) production by macrophage cells was also investigated. *In vitro* tests showed that the plant extract was able to reduce the survival time of promastigotes in a concentration-dependent manner. *In vivo* experiments also revealed a significant influence of *V. persica* extracts on accelerating the healing process as well as reducing the overall disease burden in animal model by inducing NO production in macrophage cells. Our findings indicated the promising potential of *V. persica* extract as an ideal candidate in the treatment of CL caused by *L. major*.

***Key words:*** Cutaneous leishmaniasis; Plant extract; Medicinal plants; Bioactive phytochemicals.

## Introduction

*Leishmania major* is an intracellular parasite responsible for human cutaneous leishmaniasis mostly recurring in Asia and Africa (1-3). In the life cycle, rodents and sand fly sequentially acts as the hosts and the vector of this pathogen (4-6). The main targets of *L. major* in the human body are dendritic cells and macrophages, which are two central agents of the immune system. After the *Leishmania* promastigotes enter the dermis, the responsibility of macrophages is to collect them from the environment before they can interact with intracellular matrix (7, 8). However, it is known that the entrance of promastigotes is an active process by their attachments to several receptors on macrophage cells. The cycle then continues after division and transformation of promastigotes into amastigotes, which can be phagocytosed again or transferred to sand fly vectors during feeding. The manifestation of this process is forming nodules and lesions on the place of the sandfly bite on the skin (9, 10). The cutaneous lesions can be small and self-healing or they can be severe lesions with no chance of healing (11).

The high incidence rate of leishmaniasis (1.5–2 million cases per year) has led to great efforts by many scientists to overcome this problem (12, 13). In developing countries, medicinal plants have received much attention because of their beneficial effects on the prevention or/and acceleration of the healing process of various diseases (14-18). Compared to common therapies which can lead to serious side effects and provide a time-consuming healing process, these plants can be ideal candidates with low prices and safe profiles in many situations (15, 19-31).

In this context, *Veronica* species are known as an effective traditional treatment with huge applications from wound healing to anti-cancer and anti-rheumatic activities (9, 32, 33). *Veronica persica* Poir. (also known as bird's-eye speedwell, common field-speedwell, Persian speedwell, large field speedwell, bird's-eye, or winter speedwell) from the Plantaginaceae family is one of the widespread *Veronica* species in Eurasia and eastern Asia (34-36). As with other *Veronica* species with a promising medical perspective, the investigation of *V. persica* therapeutic effects would be of utmost relevance. The aim of this study was to assess the susceptibility of *L. major* to *V. persica* extract in both *in vitro* and *in vivo* conditions. As far as we know, there is no information

regarding the therapeutic potential of *V. persica* in the treatment of cutaneous leishmaniasis.

## Materials and Methods

### Preparation of plants

Aerial parts (stems, leaves, and flowers) of *Veronica persica* Poir. were collected during the flowering period, April 2016, from wild plants in the mountains of Meymand, Firuzabad County, Fars Province, Iran. The plant was taxonomically identified by a botanist at the herbarium of the Zabol University of Iran. Plant parts were air-dried in the shade at ambient temperature (18-25 °C) for 3 days. The crude extracts were obtained by maceration of dried aerial parts (100 g) to which 100 mL methanol was added in a dark place at room temperature. The pure extracts were filtered (Whatman No. 2 filter paper), and the solvent removed from the filtrate under reduced pressure (rotary evaporator) at 35 °C. The concentrated extracts so obtained were then stored at -20 °C in labeled sterile bottles and kept until further evaluation. The extract concentrations were made based on dry weight of extract/volume.

### Culture of parasite

The Iranian strain of *Leishmania major* MRHO/IR/75/ER was used in this study. The parasite was cultured in Novy-MacNeal-Nicolle medium (Sigma-Aldrich Co., St. Louis, MO, USA) before being mass produced in RPMI-1640 (Sigma-Aldrich Co., St. Louis, MO, USA) containing 4.5 mg/mL glucose, 10% fetal bovine serum and 292 mg/mL L-glutamine as well as 100 IU/mL penicillin and 100 mg/mL streptomycin for decontamination of bacteria. The cultures were incubated at 25 ºC and the stationary phase of parasite growth was obtained after one week, they and used within 2 weeks post-incubation (37).

### *In vitro* assays

The anti-leishmanial activity of *V. persica* extract was tested on late log phase of *L. major* promastigotes inoculated in RPMI-1640 medium (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum at $10^6$ parasites/mL (38). The viability of parasites was evaluated in triplicate against different concentrations of 10, 50, 100, 150, 300, 500, 750, and 1000 µg/mL plant extracts. The viable promastigotes were then counted after 24, 48, and 72 h incubation period at 25ºC using a Neubauer chamber. The concentration of the extract able of inhibiting 50% of parasite growth ($IC_{50}$) was calculated. In this test, glucantime was used as a positive control for each set of experiments.

### Extract-based ointment preparation

In this study, the extract-based ointment was prepared as per the following formulation: dried plant extract (20%) was added to lanolin (10%) (to enhance the hydrophilicity of the preparation) and dimethyl sulfoxide (DMSO 12%, as penetration enhancer and was used to improve the drug absorption through the skin) before being integrated in soft paraffin (as a greasy ointment base to incorporate the abovementioned materials) (38). For the preparation of extract-based ointment, preservatives were not added and the ointment was stored at 4 ºC and used within 3 days after preparation.

### *In vivo* assays

All animal manipulations were carried out according to the Helsinki Convention. The protocol study received the approval from Ethics Shahid Beheshti University of Medical Sciences, Tehran, Iran (Approval code number: IR.SBMU.RETECH.REC.1396.586). The female BALB/c mice, aged 4–5 weeks and weighed 25–40 g, were obtained from Razi Vaccine and Serum Research Institute (Karaj, Iran), kept in stainless-steel cages in a well-ventilated room and allowed to adapt to their controlled environmental conditions (25 ± 3.5 ºC and 12:12 hours light-dark cycle) before and during the experiments. Animals had free access to food and water. Twenty-one mice randomly divided into 3 groups (N = 7) were used for the assay. An inoculum of *Leishmania* promastigotes ($10^6$) at stationary phase was intradermally injected in the tail base of each mouse. The groups of mice included the test groups, which received glucantime (gold standard) or the plant extract (1000 µg/mL), and the control group which received either the ointment vehicle without plant extract. After one month, the inoculated mice developed nodules and ulcers. Then, the infected mice were treated by applying the preparation twice daily at the ulcer site for 4 weeks. The concentration of 250 mg of each ointment was weighed and applied by a cotton applicator for each mouse on each day. Also, the glucantime at 25 mg/kg/day was injected intramuscularly. The lesions diameters of the treated mice were measured by Kulis Vernier, and were then weighed by a digital scale at weekly intervals (38). The mice were followed up for 30 days following the treatment.

### Determination of parasite burden

In order to determine the parasite burden, three mice from each group were sacrificed after one month of treatment to obtain spleen samples. Then, spleen samples were aseptically weighed and homogenized in 3 mL of Schneider's Drosophila medium (Sigma-Aldrich Co., St. Louis, MO, USA) including 0.1% gentamicin and 20% heat-inactivated fetal calf serum. Under sterile conditions, the homogenates were subjected to serial dilutions ($1.0\text{-}1.4 \times 10^{-4}$) in 96-well tissue culture plates. The plates were incubated at 25 ºC and evaluated for mobile promastigotes for 5 to 20-day post-incubation using an inverted microscope at 40× magnification. The parasite burden (cells/g tissue) was calculated by following the formula (39):

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

### Nitrite levels determination

The Griess reaction assay was used to determine nitrite levels (40). The peritoneal macrophages were harvested from mice exposed to plant extract (1000 µg/mL), glucantime, and from untreated mice. The harvested macrophages were cultured in the flask before the adherent cells were removed by gentle scraping and washed with warm medium (25± 2.5 ºC). Then, the macrophages were counted and their viability was determined. The viable cells were additionally cultured in 24-well plates before incubation in 5% $CO_2$ at 37 ºC for

**Table 1.** The mouse weights (g) in the test (plant extract and glucantime) and control group.

| Time | Plant extract (1000 µg/mL) | Glucantime (25 mg/kg/day) | Control |
|---|---|---|---|
| First week | 19.34 ± 0.54 Aa | 19.25 ± 0.24 Aa | 19.42 ± 0.11 Aa |
| Second week | 19.32 ± 0.93 Aa | 19.22 ± 0.44 Aa | 18.55 ± 0.52 Bb |
| Third week | 19.29 ± 0.11 Aa | 19.18 ±0.72 Aa | 14.32 ± 0.78 Bc |
| Forth week | 19.24 ± 0.22 Aa | 19.15 ± 0.92 Aa | 12.22 ± 1.22 Bd |

Note: Values are expressed as mean ± SD. The values with different capital letters within a row are significantly different among different groups ($P < 0.05$; LSD). The values with different lower-case letters within a column are significantly different among different weeks ($P < 0.05$; LSD).

20 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 nitrite synthase inhibitor. After 48 h, the supernatants were collected and nitrite accumulation was evaluated.

**Statistical analysis**

Data obtained were subjected to analysis of variance (ANOVA) following a completely random design to determine the least significant difference (LSD) at $P < 0.05$ by SPSS v. 11.5. All assays were carried out in triplicate.

## Results

After exposure of *L. major* promastigotes to the plant extract and glucantime in different concentrations and time intervals (24, 48, and 72 h), a dose-dependent and significant decrease was observed in survival time of the promastigotes compared to the control group ($P < 0.05$) (Figures 1-3). In addition, a decrease in promastigote survival time was observed after 72 h exposure with the same concentrations of plant extract and glucantime when compared to other time intervals (24 and 48 h).

*In vivo* inhibitory effects of plant extract in promastigote-inoculated mouse models were assessed by measuring the mouse weights and lesion sizes in different weeks. In the first week, there was no difference between mouse body weights in the test (treated with 1000 µg/mL plant extract) and the control groups ($P < 0.05$); however, during the next weeks, the weight of animals remained stable with a slight decrease in the test and glucantime groups. Conversely, animals in control group suffered from a remarkable decrease in their body weights ($P < 0.05$) (Table 1).

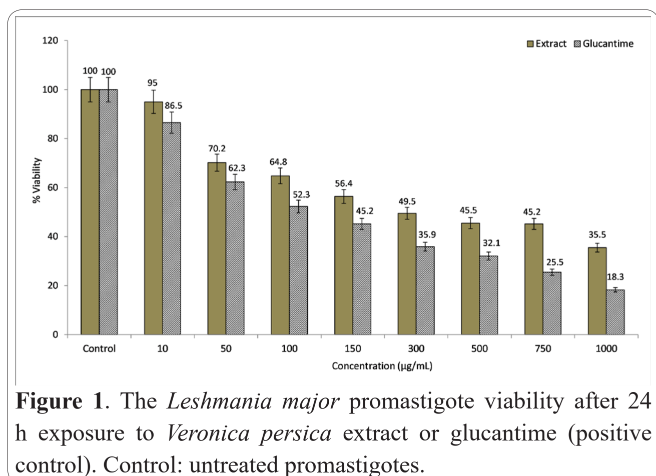


**Figure 1**. The *Leshmania major* promastigote viability after 24 h exposure to *Veronica persica* extract or glucantime (positive control). Control: untreated promastigotes.

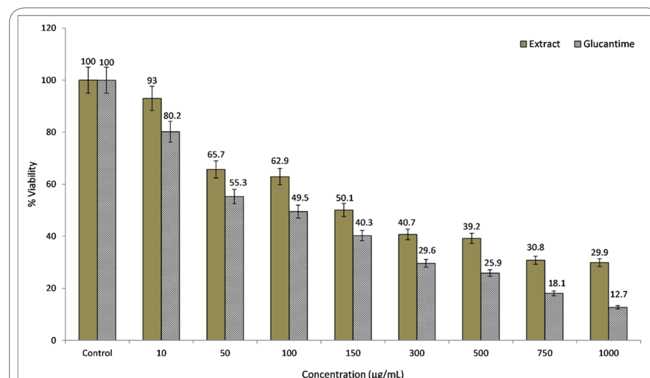


**Figure 2**. The *Leishmania major* promastigote viability after 48 h exposure to *Veronica persica* extract or glucantime (positive control). Control: untreated promastigotes.

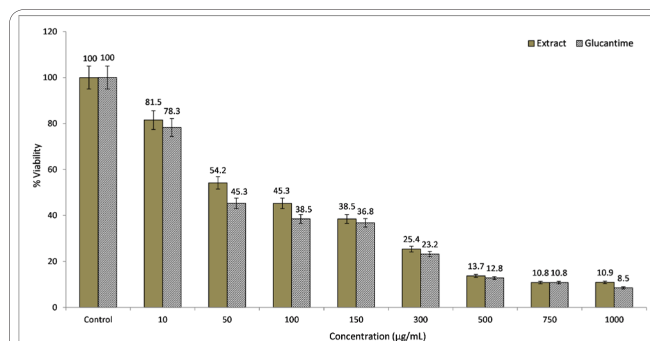


**Figure 3**. The *Leishmania major* promastigotes viability after 72 h exposure to *Veronica persica* extract or glucantime (positive control). Control: untreated promastigotes.

The lesion sizes (mm) increased over time in the three groups; however, the lesion growth rate was lower for the groups treated with plant extract and glucantime and faster for the control group. In the fourth week, the lesion sizes were around 1.94 ± 0.81, 1.99 ± 0.11 and 2.56 ± 0.65 mm for the plant extract, glucantime and control groups, respectively (Table 2).

After counting the parasites in spleen cells, a reduced

**Table 2.** The lesion sizes (mm) in the test (plant extract and glucantime) and control group.

| Time | Plant extract (1000 µg/mL) | Glucantime (25 mg/kg/day) | Control |
|---|---|---|---|
| First week | 1.44 ± 0.33 Aa | 1.62 ± 0.94 Aa | 1.42 ± 0.43 Aa |
| Second week | 1.42 ±0.42 Aa | 1.73 ± 0.45 Aa | 1.95 ± 0.22 Aa |
| Third week | 1.79 ± 0.63 Aa | 1.81 ± 0.32 Aa | 2.32 ± 0.16 Bb |
| Forth week | 1.94 ± 0.81 Aa | 1.99 ± 0.11 Aa | 2.56 ± 0.65 Bb |

Note: Values are expressed as mean ± SD. The values with different capital letters within a row are significantly different among different groups ($P < 0.05$; LSD). The values with different lower letters within a column are significantly different among different weeks ($P < 0.05$; LSD).

number of parasites was observed in the test groups when compared to the control group. The mean number of parasites (per mg of spleen tissue) in the plant extract (1000 µg/mL), glucantime and control groups were 6.11 ± 0.01, 5.99 ± 2.8 and 9.1 ± 0.7, respectively.

Nitric oxide (NO) level determination in the three groups showed an increased NO production by plant extract (1000 µg/mL) ointment and glucantime treatments. The NO levels in plant extract, glucantime and control groups were 8.7 µg/mL, 8.1 µg/mL and 4.4 µg/mL, respectively.

**Discussion**

About 350 million people are living in the area where leishmaniasis occurs (41). With about 1.5 to 2 million new cases per year, leishmaniasis is one of the notable medical problems in the affected regions (41, 42). The disease is caused by parasitic protozoa that are transmitted by sandflies. *L. major*, *L. infantum*, and *L. braziliensis* are three known species of this protozoa (43). Among these species, *L. major* is generally responsible for cutaneous leishmaniosis (CL) which is considered as the most encountered form of the disease especially in Pakistan, Iran, Iraq and Saudi Arabia (44). Despite many advances and achievements in the medical area, unfortunately, still there is no best treatment for the CL. Some chemotherapies plus surgical approaches have been suggested for treatment of CL. For instance, it has been reported that fluconazole can be used in CL patients for accelerating the healing process (45-47). However, chemotherapies can bring adversity to patients because of their various side effects. On the other side, resistance of the parasite to chemotherapeutics can make this problem more complicated. Nowadays, there is a growing interest in the application of natural products, especially plant extracts, as an ideal treatment choice in various situations (14-17, 24, 25, 48-60). In accordance with this, various investigations have been carried out to examine the probable effects of different plant extracts as a treatment option for leishmaniasis. For instance, in one experiment, effects of three plant extracts (*Hyssopus officinalis, Tussilago farfara, Carum copticum)* were evaluated on mouse models treated with *L. major* (39). Findings of this experiment revealed remarkable influence of all three extracts on reducing the lesion sizes and parasite load in the spleens of these animals. In another experiment, applying yarrow (*Achillea millefolium*) and thyme (*Thymus vulgaris*) extracts in leishmanial animal models exhibited a significant reduction in mean of ulcer sizes when compared with control groups (61). *In vitro* and *in vivo* effects of *Peganum harmala* on *L. major* were checked by another research team (37). *In vitro* results showed that the plant extract is able to reduce parasite count in a concentration-dependent manner ($IC_{50}$ = 59.4 mg/ml). Results of *in vivo* assay also exhibited a significant influence of plant extract in reducing the parasite count and lesion sizes in tested animals (37).

Our study was designed to check the therapeutic activity of *V. persica* as a medicinal plant for treatment of CL. In particular, *in vitro* and *in vivo* sensitivity of *L. major* to various concentrations of *V. persica* extracts was investigated. *In vitro* assays showed a high and dose-dependent inhibitory activity of the plant extract on *L. major* promastigote survival time. Based on our findings, over three time intervals, increasing the concentration of plant extract reduced the survival time of the promastigotes. However, a significant result was observed after 72 h exposure time, where the survival time of promastigotes decreased to 10 % by concentration of 750 µg/ml. In addition, compared to other exposure times, after 72 h, comparable effects were observed by the extracts and glucantime, especially in concentrations higher than 150 µg/mL. Measuring the body weights of the mice in three different groups, including controls, treated with plant extract and glucantime, was performed in different weeks. Although, in the first week, no significant effect was observed in the test group, in following weeks, our observations revealed a slight weight loss in this group. Similar results were observed in glucantime treated group. On the other hand, the weight loss in animals of control group was remarkable, as expected. In the control group, the body weight of animals decreased gradually from the first week until the fourth week. The lesion sizes in the control group also were significantly different than in treated groups with plant extract and glucantime. Infected animals that received plant extract-based ointment, showed a lower progression in lesion size when compared to the control group. Such results showed the potential of *V. persica* extract as active agent in reducing the lesion development by *L. major*. Counting the number of parasites in spleen cells also revealed the inhibitory effects of plant extract, which can ultimately result in reducing the burden of disease. Although the number of parasites was remarkably higher in the control group, there was a reduced amount of parasites in both plant extract and glucantime treated groups. Further evaluations to assess the effects of the *V. persica* extract on NO production of macrophages revealed a similar result with both *V. persica* extract and glucantime. Despite a lower NO production in the control group, there was an increase in NO production in both plant extract and glucantime treated groups. These findings indicated the promising potential of *V. persica* extracts to induce NO production by macrophage cells. These findings are in agreement with above-mentioned reports where various plant extracts were tested to find a therapeutic activity against leishmanial infections. Indeed, we demonstrated the *V. persica* extract as a potential effective therapeutic agent in reducing the lesion diameter in CL animal model. In addition, compared to the control group, this extract showed an ability to decrease significantly the overall burden of parasites in animals. Another interesting action of the *V. persica* extract was its incremental effects on NO production. NO production by macrophages is a known mechanism for removing intracellular pathogens such as *L. major*. Various studies demonstrated the significant antileishmanial activity of NO in mice macrophages (62, 63). In animals infected with these pathogens, NO production is decreased naturally because of amastigote activities (64, 65). Thus, restoration of NO production by plant extracts can be beneficial in diminishing the parasite burden.

In conclusion, our findings revealed the promising potential of *V. persica* extract as an ideal candidate in treatment of CL caused by *L. major*. However, additio-

nal investigations need to be carried out in animal models and humans to confirm the efficacy and the exact mechanism of action of this attractive natural product.

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**Conflict of Interest**
The authors declare no conflict of interest.

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