

In Vitro Effect of Methanolic Extract of *Quercus infectoria* Galls on Promastigotes and Amastigotes of *Leishmania major* (MRHO/IR/75/ER)

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ABSTRACT

Background and objectives: *Leishmania major* is a flagellated parasitic protozoan that causes cutaneous leishmaniasis. Pentavalent antimony compounds are considered the first-line drugs in the treatment of cutaneous leishmaniasis. However, the use of these drugs is associated with numerous limitations and side effects. Therefore, there is a need for herbal and natural alternatives for these compounds with fewer side effects. In this study, we evaluated the in vitro activity of methanol extract of *Quercus infectoria* (oak galls) against promastigotes and amastigotes of *L. major*.

Methods: In this experimental study, the effect of 10, 100, 500 and 1000 µg/ml of methanolic extract of oak galls and 100, 500, 1000 and 10000 µg/ml of Glucantime was evaluated against *L. major* promastigotes using direct cell counting and MTT assay. Moreover, the effect of different concentrations of the extract and Glucantime was investigated on the mean number of amastigotes in macrophages after 24 and 48 hours. Data were analyzed using SPSS 16 and one-way analysis of variance.

Results: The half-maximal inhibitory concentration of the oak gall extract and Glucantime was 75 µg/ml and 221 µg/ml after 24 hours, respectively. After 24 hours, the mean number of amastigotes per macrophage was lowest at concentrations of 1000 µg/ml of the extract (0.9) and 10000 µg/ml of Glucantime (0.85).

Conclusion: Considering the inhibition of intracellular and extracellular growth of *L. major*, the oak gall extract might be used as an efficient and safe agent for treatment of cutaneous leishmaniasis.

KEYWORDS: *Leishmaniasis, Cutaneous, Quercus.*

INTRODUCTION

Leishmaniasis is a complex disease caused by various species of the protozoan *Leishmania*, an intracellular parasite of humans and animals. It is prevalent in 88 countries and a common problem in tropical and subtropical regions with 1.5 million new cases reported every year. Cutaneous leishmaniasis is divided into urban dry and rural wet forms based on clinical manifestations. The parasite is transmitted by the bite of infected sand flies from the *Phlebotomus* genus (1, 2).

Pentavalent antimony compounds are considered the first-line drugs in the treatment of cutaneous leishmaniasis. However, the use of these drugs is associated with several disadvantages, including unavailability, high cost, painful injection, long-term treatment, severe toxic effects on the heart and kidneys, recurrence of the disease, scarring and drug resistance (3-5). Therefore, there is a need for herbal and natural alternatives for these compounds with fewer side effects.

Quercus infectoria (oak) is a tree of average height native to the Zagros Mountains in Iran. Scabies is a mass caused by the abnormal growth of plant tissues in response to the larval secretions of gall wasps, including *Andricus sternlichti*. In fact, bees lay eggs on lateral buds and at the end of the tree branches, but gall wasps appear proximal to the eggs. Scabies contains various tannins that have been used in traditional medicine and different industries including textiles and paint manufacture (6). Oak galls have astringent, antiseptic, antioxidant and blood coagulating properties, and its decoction has wound and burn healing effects (7). The alcoholic extract of oak galls exerts anti-inflammatory effects by inhibiting the formation of inflammatory intermediates (8, 9). Wound healing acceleration (10), antibacterial and antifungal properties of scabies have also been reported (11-15). In this study, we evaluated the in vitro activity of methanol extract of *Q. infectoria* (oak galls) against promastigotes and amastigotes of *L. major*.

MATERIAL AND METHODS

L. major, an Iranian strain of MRHO/IR/75/ER, was acquired from the Department of Parasitology of Razi Institute and cultivated in RPMI 1640 medium with 10% fetal bovine serum (FBS, complement

inactivation conditions: 56 °C for 30 minutes) at 23 ± 2 °C. Oak galls were collected from *Q. infectoria* trees in the Baneh region of Kurdistan (Iran) and later approved by a botanist. After being crushed by an electric mill, the galls were grounded and drenched in methanol for extraction. The ground gall was immersed in 80% methanol (1:5 m/v) and kept in lidded glass jars away from sunlight for 72 hours. The contents of the jars were filtered through sterile gauze and then filter paper (16, 17). The samples were powdered in a vacuum at 40 °C. The extract was refrigerated in opaque glasses until used for testing. The powder was dissolved in saline, and 0.2 µl of the solution was filtered by syringe filters to determine dilution of the extract. Then, 100 µL of *L. major* promastigotes in the logarithmic phase of growth (at a volume of 5×10^6) were added to the culture medium in the 96-well plates (NEST Scientific, USA). Then, 100 µl of each prepared concentration of scabies extract were added to the medium in a 96-well plate, and five wells containing only the medium were considered as controls. The plate was incubated at 23 ± 2 °C. All tests were repeated four times. The effects of the herbal extract on the parasites were assessed after 24, 48, and 72 hours by direct count method and MTT assay. Fifty mg of MTT powder was placed in a Falcon tube. Then, 10 ml of buffer (pH 7.4) was added and the tube was shaken in the dark. A uniform yellow solution was obtained, which was later filtered under a cell culture hood using a 0.2 micron filter into a sterile Falcon tube wrapped completely in tin foil. The tube was stored in the dark until used. Next, 5×10^6 promastigotes of *L. major* in the logarithmic phase was added to the 96-well plate. The oak gall extract at concentrations of 10, 100, 500, 1000 µg/ml was prepared and 100, 500, 1000, and 10000 µg/ml of Glucantime were added to the wells. Each 5-ml ampule of Glucantime® contained 1.5 g meglumine antimonite equivalent to 0.405 g of pentavalent antimony (Sanofi-Aventis, France). Furthermore, 100 µl of medium containing parasites and 100 µl of RPMI with 10% FBS were added to five wells of the plate as a control. The plate was incubated at 23 ± 2 °C for 24, 48 and 72 hours. The in vitro survival of promastigotes was assessed by MTT assay. First, 20 µl of MTT solution (10% of total volume, 5 mg/ml) was added to each

well in the dark. After incubating the plate at 23 °C for 4 hours, 100 µl of dimethyl sulfoxide were added to each well. Formazan crystals were dissolved after 15-30 minutes, and a purple color appeared. The optical density of the wells was read at 570 NM using an ELISA reader (BioTek ELx800, USA) (16, 17). Percentage of parasite survival was calculated using the following formula: Cell survival rate = $[A_T - A_B] / [A_C - A_B] \times 100$

A_T =Absorbance of treated cells, A_B =Absorbance of blank, A_C = Absorbance of control (untreated cells).

Percentage of cytotoxicity was calculated by subtracting the cell survival percentage from 100. We also determined the half-maximal inhibitory concentration (IC50) at which each agent was able to inhibit growth of 50% of cell population.

Under sterile conditions, all equipment including the anatomy set and medium were transferred under a hood, and several 4-week-old BALB/C mice were euthanized with ether and immersed in 70% alcohol. The fixed mice were put on expanded polystyrene foam and abdominal skin was notched with scissors. Five ml PBS (pH 7.4) were injected under peritoneum of mice. Peritoneum fluid was collected by a syringe in a falcon tube. The solution was centrifuged for 10 minutes at 1500 RPM, the supernatant was discarded, and 5ml of RPMI containing 10% FBS and 0.5% gentamicin were added to the sedimented macrophages. Macrophages were counted with a Neobar slide. Ten parasites were calculated for each macrophage (17). Cell cultures were

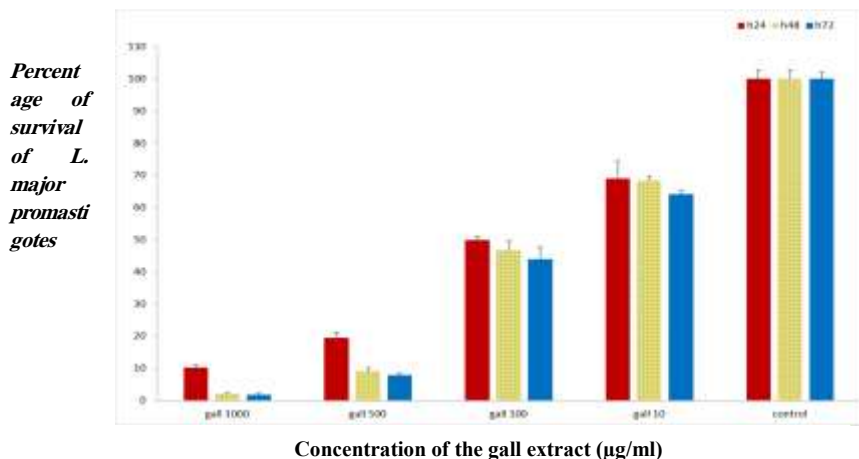
placed under the hood and round lamelles were put at the bottom of a NEST-12-well plate (NEST Scientific USA). Next, 10^5 macrophages were added to each well and the plates were incubated at 37 °C and in 5% CO₂ for 24 hours. Then, 10^6 promastigotes of *L. major* at stationary phase were added to the wells. The plates were incubated for 24 hours at 37 °C, extracellular parasites were removed by washing, and fresh medium was added to the plates. Oak gall extract at concentrations of 75, 500, 1000 µg/ml and Glucantime at concentrations of 221, 1000, 10000 µg/ml were added to each well. Only infected macrophages were put in the two control wells. After 24 and 48 hours, round lamellas were removed, fixed with methanol and then stained with Giemsa. The number of amastigotes inside the macrophages was counted under a microscope using the 100x (oil immersion) objective lens (16, 17).

Statistical analysis was conducted in SPSS 16 using one-way analysis of variance. P-values less than 0.5 were considered statistically significant.

RESULTS

As shown in figure 1, the oak gall extract had potent anti-leishmanial activity against promastigotes in a time- and concentration-dependent manner compared with the control. After 24 hours, the IC50 of the extract was 75.5 µg/ml. All concentrations of the extract significantly affected the survival of promastigotes after 24, 48, and 72 hours compared with the control ($P < 0.05$).

Figure 1- Percentage of survival of *L. major* promastigotes after treatment with different concentrations of the gall extract



The cytotoxic effect of different concentrations of Glucantime on promastigotes after 24, 48 and 72 hours are shown in figure 2. The IC50 of Glucantime was 221.5 µg/ml after 24 hours. All concentrations of Glucantime significantly affected the survival of promastigotes compared with the control (P<0.05). The leishmanicidal activity of the oak gall extract was more significant than that of

Glucantime. Different concentrations of the extract and Glucantime significantly affected the survival rate of amastigotes in macrophages compared with the control (Table 1). The leishmanicidal activity of the oak gall extract was more significant than that of Glucantime. Different concentrations of the extract and Glucantime significantly affected the survival rate of amastigotes in macrophages compared with the control (Table 1).

Figure 2- Percentage of survival of *L. major* promastigotes after treatment with different

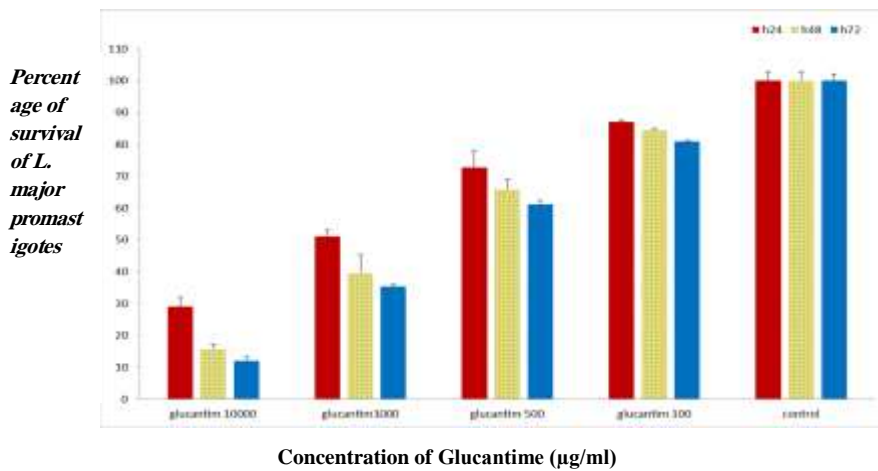


Table1- Average number of amastigotes in macrophages in the control group and under influence of different concentrations of oak gall extract and Glucantime

Group	24h	48h
Control	3.52±0.21	3.72±0.09
Gall 1000 µg/ml	0.9±0.11	0.35±0.07
Gall 500 µg/ml	1.57±0.14	1.15±0.09
Gall 75 µg/ml	2.63±0.08	2.24±0.06
Glucantime 10000 µg/ml	0.85±0.14	0.42±0.05
Glucantime 1000 µg/ml	1.5±0.05	1.12±0.07
Glucantime 221 µg/ml	2.71±0.11	2.53±0.07

DISCUSSION

Leishmaniasis is a complex disease caused by various species of the protozoan *Leishmania*, an intracellular parasite of humans and animals. It is prevalent in 88 countries and a common problem in tropical and subtropical regions with 1.5 million new cases reported every year (1,2). Pentavalent antimony compounds are considered the first-line drugs in the treatment of cutaneous leishmaniasis. However, the use of these drugs is associated with several disadvantages, including unavailability, high cost and drug resistance (3-5). Therefore, there is a need for herbal and natural alternatives for these compounds with fewer side effects.

In this study, we investigated the antileishmanial effect of methanolic extract of oak galls. Our results revealed that both the extract of oak galls and Glucantime exert antileishmanial effects in a dose- and time-dependent manner. However, the effect of the extraction on promastigotes was more notable compared to that of Glucantime, as the IC₅₀ for the extraction and Glucantime was 75 µg/ml and 221 µg/ml after 24 hours, respectively. After 72 hours, the cytotoxic effect of 1000 µg/ml of the extract and 10000 µg/ml of Glucantime was 98.2% and 87.95%, respectively (Figures 1 and 2).

After 48 hours, the mean number of macrophages in amastigotes was 0.35 and 0.42 when treated with 1000 µg/ml of the extract and 10000 µg/ml of Glucantime, respectively (Figure 3). In addition, 1000 µg/ml of the oak gall extract had toxic effects on the macrophages. However, the oak gall extract demonstrated better efficacy against promastigotes and amastigotes of *L. major* in vitro.

In some studies, the antibacterial, antifungal, anti-inflammatory, antioxidant and antiseptic properties of alcoholic extract of scabies have been demonstrated (11-15). Several studies investigated the effects of plant extracts on promastigotes and amastigotes of *Leishmania*. Shirani et al. showed the positive effects of ethanolic extracts of thyme, yarrow, and propolis on the healing of wounds caused by *L. major* (18). In another study, gallic acid and ellagic acid demonstrated great potential as

growth inhibitors of promastigote of *L. major* (19).

In study of Sadeghi-Nejad, the IC₅₀ of *Allium cepa* extract, *Ixora brachiata* root extract and Glucantime against *L. major* promastigotes was 0.078, 1.25 and 21.25 mg/ml, respectively (20). In another study, Barati showed that the extract of Shiraz thyme and espond have antileishmanial activity against promastigotes of *L. major* (21). Shemshadi et al. stated that the effect of 0.9 mg/ml of Caparis root extract on the reduction of lesion size was similar to that of 120 mg/ml of Glucantime, and both compounds were able to kill 97.8% of promastigotes after 72 hours (22). In a study by Yosefi et al., 40 µg/ml of *Peganum harmala* and 200 µg/ml of *Alkanna tinctoria* could inhibit the growth of promastigotes of *L. major*. In addition, the mean number of amastigotes in macrophages treated with *P. harmala*, *Alkanna tinctoria* and control agent was 0.7, 0.7 and 2.3, respectively (23).

In another study, Esmaeili et al. showed that the effective dose 50 of miltefosine and Glucantime against *L. major* amastigotes was 2.20 µM and 7.2 µM after 72 hours, respectively (24).

In a study by Kheirandish et al., concentrations of 10 and 20 mg/Kg of oak significantly reduced the mean number of parasites and the mean diameter of lesions in mice (25).

CONCLUSION

Considering the inhibition of intracellular and extracellular growth of *L. major*, the oak gall extract might be used as an efficient and safe agent for treatment of cutaneous leishmaniasis.

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CONFLICT OF INTEREST

All contributing authors declare that there is no conflict of interest.

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