

Effect of Methanolic Extract of *Arctium lappa* Root on Promastigotes and Amastigotes of *Leishmania major* in vitro

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ABSTRACT

Background and Objective: *Leishmania major* is a flagellate protozoan parasite causing cutaneous leishmaniasis. Although pentavalent antimony compounds are the first-line drugs for leishmaniasis, their application is often accompanied by numerous limitations and side effects. Therefore, it is necessary to seek drugs of herbal origin that have fast-acting benefits and few side effects without resistance. This study aimed to evaluate the in vitro effects of methanolic extract of *Arctium lappa* root on promastigotes and amastigotes of *L. major*.

Methods: This experimental study evaluated the effects of 10, 100, 500, and 1000 µg/ml of *A. lappa* root methanol extract on *L. major* promastigotes using direct cell counting and MTT assay. The mean number of amastigotes in infected macrophages was calculated after 24 and 48 hours.

Results: The half maximal inhibitory concentration (IC₅₀) of *A. lappa* root methanolic extract was 131.25 µg/ml after 24 hours. The mean number of amastigotes in macrophages after 24 hours in the control group and in the *A. lappa* group with concentrations of 500 and 1000 µg/ml were 3.52, 2.02, and 1.27, respectively.

Conclusion: The results show that the methanolic extract of *A. lappa* root has anti-leishmanial effects on the promastigotes and amastigotes of *L. major* in vitro.

Keywords: *Leishmania Major*, Amastigotes, Promastigotes, *Arctium*.

INTRODUCTION

Leishmaniasis is a complex disease shared between humans and animals, caused by the protozoan *Leishmania*. It is prevalent in 88 countries and considered 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 depending on the clinical manifestations. Sand flies in the genus *Phlebotomus* are vectors of the disease (1,2).

Pentavalent antimony compounds are considered first-line drugs in the treatment of cutaneous leishmaniasis. However, the use of these drugs have some disadvantages such as unavailability, high cost, painful injection into the wound, treatment duration, severe toxic effects on the heart and kidneys, recurrence of the disease, scarring, and drug resistance (3,4,5). Therefore, it is necessary to find herbal alternatives for these pharmaceutical compounds.

Arctium lappa is a medicinal plant belonging to the *Asteraceae* family. The main constituents of this plant are five powerful flavonoids, terpenoids, inulin, several polyphenols, tannins and polyactylene (6). Roots of *A. lappa* have numerous therapeutic effects, and have been used in traditional medicine for treatment of arthritis, myelination, eczema, acne, and dysphasia (7). Pharmacological studies and clinical trials also indicated that burdock roots have anti-inflammatory, free radical scavenging, anti-oxidant, anti-tumor, anti-viral, anti-bacterial, anti-fungal, cytotoxic and genotoxic activities (8-16). Due to the fewer side effects associated with herbal compounds compared to chemical agents, the effect of methanolic extract of *A. lappa* root was evaluated in this study.

MATERIAL AND METHODS

An Iranian strain of *Leishmania major* (MRHO/IR/75/ER) was used in this study. The parasites were acquired from the Department of Parasitology of Razi Institute and cultivated in RPMI 1640 medium with 10% FBS (its complement was disabled at 56°C for 30 minutes) at 23 ± 2 °C.

A. lappa was collected from Fandegloo region in Ardabil. After verification by a botanist, the aerial parts were cleaned, and the roots were shadow-dried. The roots were crushed, grounded and drenched in methanol for

extraction. The ground *A. lappa* root was immersed in 80% methanol at ratio of 1:5 (m/v) in the laboratory, away from the sunlight in lidded glass jars for 72 hours. The contents of the jars were filtered through sterile gauze and filter paper. They were turned into powder in vacuum at 40°C. The extract was refrigerated in opaque glasses until used for testing.

First, the powder was dissolved in saline. Then, 0.2 µl was filtered by syringe filters to determine extract dilution by filter size before the powder was applied and tested. After that, 100 µL of *L. major* promastigotes in logarithmic phase of the growth at density of 2×10^6 were added to the 96-well plates. Next, 100µl of prepared concentrations of scabies extract were added to the each well, and the plate was incubated at 23±2°C. Five wells containing medium without drugs were considered as controls. All tests were repeated four times. The effects of the herbal extract on the parasites were determined after 24, 48, and 72 hours by direct method and MTT assay. First, 50 mg of MTT powder were measured and placed into a Falcon tube. Then, 10 ml of buffer (pH 7.4) were added to the tube. The tube was shaken in the dark to achieve a uniform yellow solution. The solution was filtered under cell culture hood using a 0.2 micron filter into a sterile Falcon tube wrapped completely in tinfoil and was stored in the dark until testing.

Next, 100µL of *L. major* promastigotes in logarithmic phase was added to each well so that each well contained 2×10^6 parasites. *A. lappa* root extract at concentrations of 10, 100, 500, 1000 µg/ml was prepared. Then, 100 µl of Glucantime at concentrations of 10, 100, 1000, and 10000 µg/ml was added to the 96-well plates with four repetitions. Furthermore, 100 µl of medium containing parasites and 100 µl of RPMI with 10% FBS were added to five well of the plate, and then incubated for 24, 48, and 72 hours as controls. Next, 20 µl of the MTT solution (10% of each well volume, 5 mg/ml) was added to the wells after 24, 48, and 72 hours in the dark. After incubation for four hours at 23°C, 100 µl of dimethyl sulfoxide (DMSO) solution was added to each well. Formazan crystals dissolved after 15 to 30 minutes, and a purple color was observed. Optical density (OD) of the wells was read at the wavelength of 570 nm using an ELISA

plate reader. The percentage of parasite survival was calculated using the following formula (17):

Cell survival rate = $[AT-AB] / [AC-AB] \times 100$
 AB= Blank OD (RPMI), AT= the treatment plate light absorption of the light is absorbed, AC= control light is absorbed well
 IC₅₀ after 24 hours calculate by Microsoft Excel.

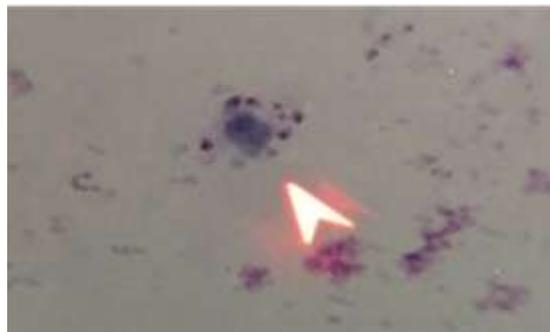
$\text{Log (IC50)} = \frac{\log(x1) + [y1-y0/2]}{(y1-y2)}$
 $[\log(x2) - \log(x1)]$.

Several 4-week-old BALB/C mice (due to their sensitivity to *Leishmania*) were euthanized with ether in a desiccator under sterile conditions. The mice were immersed in 70% alcohol and then placed on an expanded polystyrene foam. Abdominal skin was lifted using sterile forceps and notched with scissors in a longitudinal line without creating a hole in the peritoneum. Then, 5ml of sterile PBS solution (pH 7.4) was injected under the mouse's peritoneum by a syringe without contact with animal offal. Peritoneum fluid was collected by syringe in a 15cc sterile Falcon tube and centrifuged for 10 min at 1500 rpm. The contents of the tube were discarded under the hood and the sediment was washed with PBS. After discarding the supernatant, 4 ml of RPMI 1640 medium was added to the macrophages deposited. Finally, 20 µl of the fluid was removed, and cells were counted with a Neobar slide (17,18).

Cell cultures and round lamella were put at the bottom of a 12-well cell culture plate under

sterile conditions. Then, 200 µl of the tube contents containing 10⁴ macrophages and 200 µl of *L. major* promastigotes at stationary phase were added to each well to infect the macrophages. Overall, 10⁵ parasites were present in each well. After incubation at 37°C and 5% CO₂ for 24 hours (Or parasite/macrophage ratio of 10.1), a sterile sampler was used to remove the contents of plates containing un-adhered macrophages and parasites that did not penetrate into the macrophages. The RPMI 1640 medium and 10% FBS were added to the plates. The plates were investigated under inverted microscope to ensure that macrophages were infected (Figure 1) (17). After that, 200 µl of *A. lappa* root extract with concentrations of 500 and 1000 µg/ml was added to each well. In addition, 1000 and 10,000 µg/ml of Glucantime were added to each well, with four repetition. Each 5-ml ampoule of glucantime® contained 1.5 g meglumine antimoniate corresponding to 0.405 g of pentavalent antimony (Sanofi-Aventis, France). The two control wells only contained infected macrophages. Round lamellas were removed from the bottom of the 12-well plate after 24 and 48 hours of incubation at 37 °C. They were fixed with pure methanol and then stained by Giemsa. The number of amastigotes inside the macrophages was counted under a microscope using the 100x (oil immersion) objective lens, and the results were presented as percentages.

Figure 1- Macrophage infected with amastigotes



Statistical analysis was performed by SPSS software (version 16) using one-way analysis of variance (ANOVA) at confidence level of 95%.

RESULTS

As shown in Figure 2, different concentrations of the *A. lappa* extract had potent anti-leishmanial activity against promastigotes. This leishmanicidal effect had a positive correlation with time and concentration in comparison with the control group. The half maximal inhibitory concentration (IC₅₀) of *A. lappa* extract was 131.25 µg/ml after 24 hours. The survival rate of all groups treated with different concentrations of *A. lappa* root extract had significant difference from that of control group after 24, 48, and 72 hours (p<0.05).

The IC₅₀ of Glucantime was 221.5 µg/ml after 24 hours. All groups treated with different concentrations of Glucantime showed significant differences with each other and the control group at the tested times (P<0.05). The IC₅₀ of Glucantime was 221.5 µg/ml after 24 hours. All groups treated with different concentrations of Glucantime showed significant differences with each other and the control group at the tested times (P<0.05). The number of amastigotes in all groups treated with different concentrations of *A. lappa* and Glucantime had significant differences with the control group (P<0.05).

Figure 2- Survival rate (%) of *L. major* promastigotes under the influence of *A. lappa* root extract

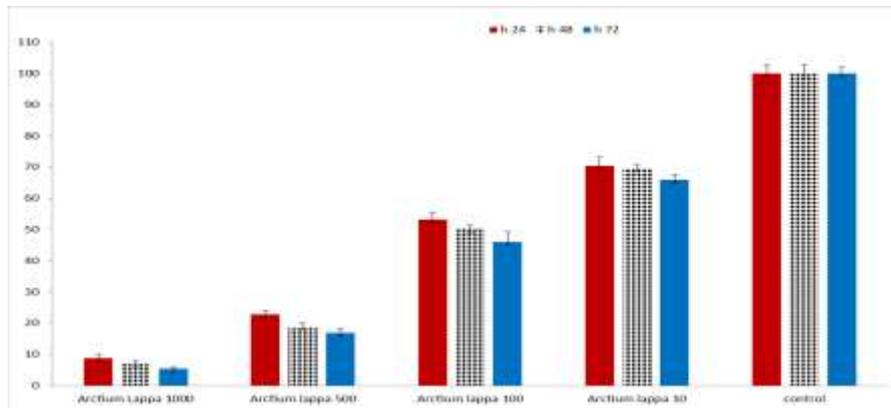
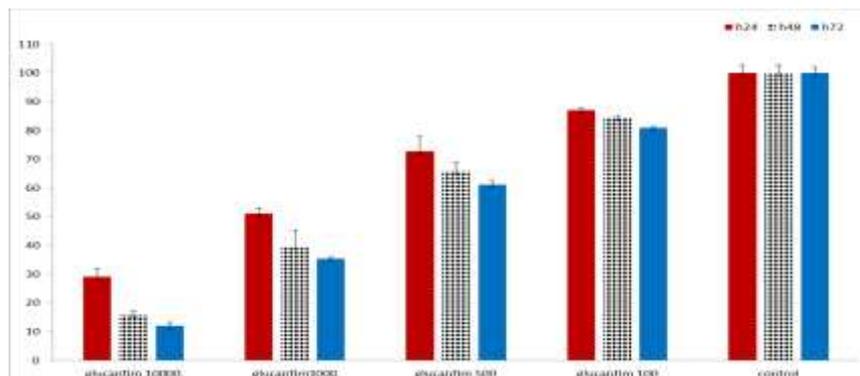


Table1- Average number of amastigotes within macrophages in the groups treated with different concentrations of *A. lappa* root extract and Glucantime

Time Group	24h	48h
Control	3.52±0.21	3.72±0.09
<i>Arctium lappa</i> 1000µg/ml	1.27±0.14	1± 0.10
<i>Arctium lappa</i> 500 µg/ml	2.2±0.19	1.6±0.11
Glucantime10000 µg/ml	0.85±0.14	0.42±0.05
Glucantime 1000 µg/ml	1.5±0.05	1.12±0.07

Figure 3-Survival rate (%) of *L. major* promastigotes under the influence of Glucantime



DISCUSSION

The results of this study showed that the anti-leishmanial effect of *A. lappa* root methanolic extract and Glucantime is dose-dependent, so that an increase in drug dose and duration reduced the parasite's survival rate. Significant differences were observed between all groups and the control group after 24, 48 and 72 hours. The effect of the methanolic extract of *A. lappa* on amastigotes within macrophages was observed after 24 and 48 hours. The anti-leishmanial effect of *A. lappa* was observed at concentration of 1000 µg/ml, resulting in a reduction in the number of amastigotes within macrophages. In some studies, the anti-bacterial, anti-fungal, anti-viral, anti-inflammatory, antioxidant, and anti-tumoral effects of *A. lappa* alcoholic extract have been demonstrated. However, no anti-leishmanial and anti-parasitic effect have been reported for the *A. lappa* extract. Several studies investigated the effects of plant extracts on promastigotes and amastigotes of *Leishmania*. Shirani et al. showed the efficiency of ethanolic extracts of thyme, yarrow and propolis on healing *L. major* wounds (19). Suzangar also found that Abu-Khesar and yarrow extracts have cytotoxic effects on promastigotes (20). In another study, Barati showed that the herbal extracts of Shirazi thyme and esfand have anti-leishmanial effects on the promastigotes of *L. major* (21). Asadi et al. revealed that the hydro-alcoholic extract of *Hypericum perforatum* and medlar leaves have anti-leishmanial effects on the promastigotes of *L. major* (22). Savia et al. demonstrated that propolis also has anti-leishmanial effects on the promastigotes of *L. major* (23). Moreover, Hosseini demonstrated that extracts of turmeric and licorice have cytotoxic effects on the promastigotes of *L. major* in vitro (24). Comparison of the in vitro effects of aqueous

extract of *Artemisia seiberi* and Artemisinin on *L. major* showed that the increased concentration of both agents hiegten the reduction rate in the number of parasites. Contrary to our study, their results showed that the effects of both drugs on uninfected and healthy macrophages were little (25). Another study investigated the effect of *Caparis spinose* root extract on promastigote and amastigotes of *L. major*, and found that the anti-protozoal activity of *Capris* was similar to Glucantime (as a gold standard drug). In the mentioned study, both *C. spinose* root extract and Glucantime were able to kill 97.8% of promastigotes after 72 hours. *C. spinosa* and its root extraction have positive effects on amastigote of *L. major* but at higher concentrations (26). A study conducted in Mazandaran showed that the extract of *Artemisia aucheri* inhibited the multiplication of promastigotes at concentrations of 150, 300 and 450 µg/ml after 48 and 72 hours of culture (27). However, no effect was observed against amastigotes. Nevertheless, the *A. lappa* extract seemed to be more effective.

CONCLUSION

The results of the present study show the in vitro anti-leishmanial effects of *A. lappa* methanolic extract against the promastigotes and amastigotes of *L. major*.

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CONFLICT OF INTEREST

All contributing authors declare no conflicts of interest.

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