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Cytotoxic Effects of *Artemisia Dracunculus* L. and *Heracleum Persicum* Desf. Extracts on *Leishmania major* and *Leishmania infantum* Promastigotes Using MTT Assay

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Abstract

Background: Leishmaniasis is a parasitic disease that occurs in subtropical and tropical regions with approximately 350 million people worldwide and 2 million new cases annually. The annual increase in cutaneous leishmaniasis (CL) is observed, especially in endemic areas such as Iran. Since there is no effective vaccine, the detection of natural anti-leishmanial products is essential.
Objective: The purpose of this study was to evaluate the *in vitro* anti-leishmanial activity of two herbal medicine including *Artemisia dracunculus* L. and *Heracleum persicum* Desf. (Golpar).
Materials and Methods: The extracts of selected plants were obtained by maceration, and *in vitro* anti-leishmanial activity was assayed on *Leishmania major* and *Leishmania infantum* promastigotes using colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] assay in comparison with glucantime as a reference.
Results: Based on the results, 50% inhibitory concentration (IC50) values of selected plants and glucantime solutions were determined at 24, 48, and 72 hours incubation. Further, the anti-leishmanial activity of the leaf extract of *A. dracunculus* with IC50 values of 1.85 and 3.5 µg/mL and the fruit extract of *H. persicum* with values of 31.32 and 11.7 µg/mL were evaluated against *L. major* and *L. infantum* promastigotes, respectively.
Conclusion: These results revealed anti-leishmania properties of the above-mentioned plants and the need to study the effects of these extracts on the *Leishmania* genus in animal models and *in vivo* assay in the future.

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Background

Leishmaniasis with different clinical manifestations has been identified as one of the most common known skin diseases.[1] The most common forms of leishmaniasis are cutaneous and visceral forms. The disease is occurring in 88 native countries, most of which are reported in developing countries. Cutaneous leishmaniasis (CL) is one of the most important endemic diseases in Iran and the second parasitic disease transmitted by arthropods after malaria in the urban and rural areas.[2]

Although CL can be treated without treatment, it can cause serious problems.[3] Therefore, low-cost and low-toxicity anti-leishmanial compounds are needed.[4] On the other hand, the use of herbal medicines for the treatment of CL is recommended by the World Health Organization.[5] Since the herbal medicine has fewer side effects and is also available and inexpensive, it is, therefore, necessary that the native plants of each region be identified as a rich source of anti-leishmanial drugs.[6] "Tarkhun" (*Artemisia dracunculus* L.) belongs to the Asteraceae family. The genus *Artemisia* L. has 30 species in Iran. These species are annual herbs or small shrubs containing compounds such as artemisinin with the anti-parasitic properties. Furthermore, *Artemisia* species contain chemical compositions such as flavonoids, alkaloids, glycosides, coumarins, monoterpenes, sesquiterpenes, sesquiterpene, lactones, sterols, and polyacetylenes which manifest antibiotic activity.[7] Tarkhun is traditionally used as a flavoring agent in foods and for preparing different kinds of sauces.[8] The *Artemisia* species possess biological activities such as insecticidal, antifungal, and cytotoxic,[9-11] along with anti-inflammatory effects.[12] Alizadeh Behbahani et al determined the antimicrobial properties of *A. dracunculus* extract, reporting high antimicrobial properties against

Candida albicans with the inhibition zone of 14.70 mm, and all tested bacteria (except for *Streptococcus pyogenes* and *Staphylococcus aureus*) were more resistant than *C. albicans*.[7] The previous studies reported the leishmanicidal properties of extract and their natural compounds of different *Artemisia* species.[13] Another study reported the anti-leishmanial effects of *Satureja hortensis* (*S. hortensis)* and *A. dracunculus* extracts on *L. major* promastigotes. The results of this study showed the significant anti-leishmanial effects of these plants ($P<0.05$).[14] Another herb selected in this study was *Heracleum persicum* Desf. (Family Apiaceae), an annual plant known as "Golpar" in Iran. In traditional Iranian medicine, *H. persicum* is used as an antisepticherbal remedy for carminative and pain relief.[15] Although some researchers have worked on the antibacterial effects of *H. persicum* against some pathogenic microorganisms, to the best of the researchers' knowledge, no studies have been carried out on the effects of *H. persicum* extract on the growth of *L. major* and *L. infantum* promastigotes. Therefore, the aim of this study was to evaluate the anti-leishmanial activity of two hydroethanolic extracts of *A. dracunculus* and *H. persicum* with different concentrations (0.075-156 mg/mL) on *L. major* and *L. infuntum* promastigotes.

Materials and Methods

Plant Materials

Two herbal medicines (i.e., *A. dracunculus* L. and *H. persicum* Desf.) were prepared from a local market in Ahvaz, Iran.

Extraction

To prepare the hydroethanolic extract, add 10 g of selected plant powder in 100 mL of ethanol alcohol 85% (Merck) solution and soak it on a rotary shaker for 72 hours, then filter through Whatman No. 1 and keep it in incubator 37°C until it is evaporated,[16] and extraction should be kept in the refrigerator until the test.

Parasite Culture

Parasites L. major (MRHO/IR/75/ER) and *L. infantum* (MCAN/IR/96/LONDON 49) promastigotes strains were prepared from School of Health, Tehran University of Medical Sciences, Iran. Then they were kept in RPMI-1640 (Sigma, Chemical Company) medium and completed by 10% fetal calf serum Sigma, 100 IU/mL Penicillin, and 100 µg/mL Streptomycin. All promastigotes (10^6 parasites/mL) were incubated at 26°C for 24, 48, and 72 hours in fresh RPMI-1640.[17]

Antileishmanial Evaluation

The antileishmanial activity of selected plants was carried out by MTT assay based on the reduction of the tetrazolium salt soluble to insoluble formazan crystal by the mitochondrial enzymes in viable parasites.[17] For the preparation of MTT reagent (Sigma Chemical Company), 5 mg MTT powder was dissolved in 1 mL PBS sterile solution (5 mg/mL). Next, 96-well microplate was used for the MTT assay. After adding 100 µL 1×10^6 promastigotes per well, 10 µL ethanolic extracts of *A. dracunculus* leaf and *H. persicum* fruit in different increasing concentrations (0-156 µg/mL) and 20 µL MTT reagent were added per well to treat the parasites. Microplates were incubated in 25±1°C after 24, 48, and 72 hours, and promastigotes viability was tested using MTT assay. Then, optical density at wavelength 540 nm was measured using the ELISA reader device,[18] and the IC_{50} was determined as well.

Statistical Analysis

In vitro anti-leishmania activity was determined as IC_{50} (50% inhibitory concentration) by linear regression analysis. Data were described as the means ± SD. To compare the two groups, *P*-values were calculated using paired t-test of two student sequences. In all cases, $P<0.05$ was considered as statistically significant.

Results

Cytotoxic activity of *A. dracunculus* leaf and *H. persicum* fruit extracts were assayed by MTT colorimetric assay. Anti-leishmania effects of the leaf extracts of *A. dracunculus* with 50% inhibitory concentrations (IC_{50} values) for the hydroethanolic extracts of *A. dracunculus* leaf at 24, 48, and 72 hours for *L. major* promastigotes and *L. infantum* promastigotes were 14.4, 4.2, and 1.85 µg/mL, as well as 10.5, 5.1, and 3.5 µg/mL, respectively. However, these values for *H. persicum* fruit extract at 24, 48, and 72 hours were found to be < 156, 70.2, 31.32 µg/mL and < 156, 33.5, 11.7 µg/mL for *L. major* promastigotes and *L. major* promastigotes, respectively. The results indicated that the hydroethanolic extracts of *A. dracunculus* leaf and *H. persicum* fruit had potent anti-leishmanial activity against the forms of *L. major* and *L. infantum* after 24, 48, and 72 hours of incubation ($P<0.05$) promastigotes *in vitro*. These results also revealed that the hydroethanolic extracts of *A. dracunculus* leaf extract had significantly ($P<0.05$) higher leishmanicidal effect on the promastigotes of *L. major* and *L. infantum* compared to the extract of *H. persicum* fruit since it exhibited lower IC_{50} values for the tested promastigotes. Table 1 and Figure 1 present the anti-leishmanial activity of the leaf extract of *A. dracunculus* against the forms of *L. major* and *L. infantum* promastigotes with various concentrations and at different hours (24, 48, and 72 hours). Likewise, Table 1 and Figure 2 illustrate the anti-leishmanial activity of the fruit extract of *H. persicum* on *L. major* and *L. infantum* promastigotes with different concentrations and at various hours (24, 48, and 72 hours). Glucantime drug also exhibited IC_{50} values of 40.2 and 18.5 µg/mL for *L. major* and *L. infantum* promastigotes after 72 hours

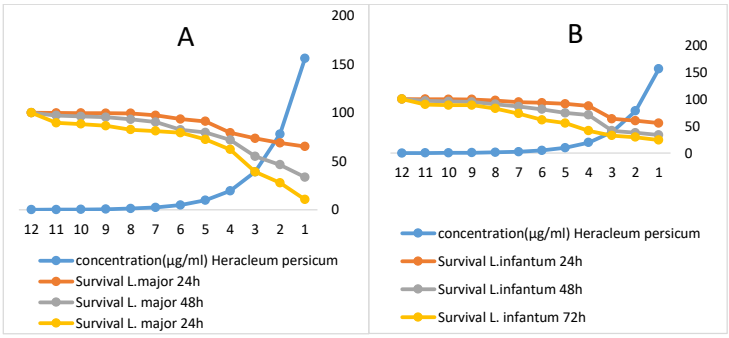

Figure 1. Viability of *L. major* and *L. infantum* Promastigotes With Various Concentrations of *H. persicum* After 24, 48, and 72 hours

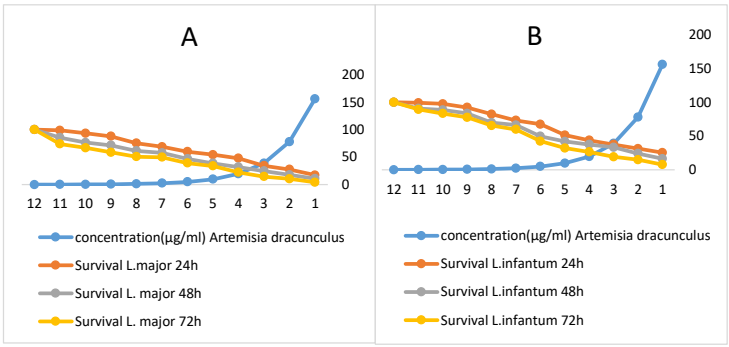

Figure 2. Viability of *L. major* and *L. infantum promastigotes* in Various Concentrations of *A. dracunculus* After 24, 48, and 72 Hours.

of incubation, respectively. The comparison of means of the anti-leishmanial activity of *A. dracunculus* leaf, *H. persicum* fruit extracts, and glucantime drug against the forms of *L. major* and *L. infantum* promastigotes was found to be significant ($P<0.05$) after 72 hours. *P* values for each plant and drug are presented in Table 2.

Discussion

Unfortunately, despite the significant prevalence of CL in Iran, there has not been a proper prevention, control, and treatment method yet.[19] Nowadays, there are cheap and effective chemotherapeutic agents for the treatment of leishmaniasis, but the application of these chemical agents manifested drug resistance and side effects. Numerous studies were conducted on the effects of different species of *Artemisia* on *L. major* promastigote form.[20,21] However, based on the previous literature, no studies have been carried out on the effects of *A. dracunculus* and *H. persicum* plant extracts on the *in vitro* growth of *L. infantum* promastigotes by MMT assay. In the present study, the hydroethanolic extracts of *A. dracunculus* and *H. persicum* significantly ($P<0.05$) inhibited the growth of promastigotes forms of *L. major* and *L. infantum*. The results also revealed that the hydroethanolic extract of *A. dracunculus* was more sensitive to *L. major* compared to *L. infantum* promastigotes after 72 hours of incubation, while the hydroethanolic extract of *H. persicum* and glucantime drug as the control were more sensitive to *L. infantum* than *L. major* promastigotes after 72 hours of incubation. Previous study reported that the hydroethanolic extracts of *A. dracunculus* did not show anti-leishmanial efficacy against *L. major* promastigotes after 24 and 48 hours, but they could significantly reduce the number of promastigotes after 72 hours with an effect more than 50% at concentrations of 10 µg/mL ($P<0.01$), 20 µg/mL ($P<0.001$), and 25 µg/mL ($P<0.0001$).[20] There is no consistency between the results of the above- mentioned study and the present research after 24 and 48 hours. Furthermore, based on the findings of the present study,

Table 1. IC_{50} of *A. dracunculus* Leaf, *H. persicum* Fruit Extracts and Glucantime Against *L. major* and *L. infantum* Promastigotes After 24, 48, and 72 hours of Incubation

Compounds	IC_{50} (µg/mL)		
	24 h	48 h	72 h
H. percicum on *L. major*	<156	70.2	11.7
H. percicum on *L. infantum*	<156	33.5	5.5
A. dracunculus on *L. major*	14.4	4.2	1.85
A. dracunculus on *L. infantum*	10.3	5.1	3.5
Glucantime on *L. major*	104.45	61.4	40.2
Glucantime on *L. infantum*	99.7	45.6	18.5

Table 2. Results of Comparison of Paired Samples *T* test for Each Plant and Drug Against Tested Promastigotes After 72 Hours of Incubation

Plant and Drug	Mean ± SD	*P* Value (2-Tailed)
A. dracunculus -L. major, and L. infantum	40.91±29.80[a]	0.001*
H. persicum, L. major, and *L. infantum*	64.58±27.00	0.001*
Glucantime - *L. major,* and *L. infantum*	60.15±34.36	0.008*

Note. SD: Standard deviation.
a: µg/ml
* Significance level < 0.05.

the anti-leishmanial efficacy of the *A. dracunculus* extract was significantly higher than that of the *H. percicum* extract. Numerous phytochemical screening reported active ingredients in many *Artemisia* species, including monoterpenes, terpenes, monoterpenes, terpene lactones, flavonoids, coumarin, sterols, and polyacetylenes.[22] It seems that higher anti-leishmanial activity of the *A. dracunculus* extract compared to the *H. persicum* extract is related to its compounds, and those anti-leishmanial properties have been already reported by Iranshahi et al.[22] The literature indicates that some researchers have worked on the antifungal and antibacterial effects of *H. percicum* extracts against some pathogenic microorganisms.[23-25] For example, Sadeghi-Nejad et al carried out anti-candida activity of the hydroalcoholic extracts of *H. persicum* fruit against pathogenic candida species such as *C. albicans, Candida glabrata*, and *Candida tropicalis*. The minimum inhibitory concentration values were 0.625-20 µg/mL, 0.625-40 µg/mL, and 5.0-20 µg/mL for *C. albicans*, *C. glabrata*, and *C. tropicalis*, respectively. The results of this study revealed that *H. persicum* fruit extract had the potential for anti-candida activity.[24] However, to the best of our knowledge, no studies have been carried out on the effects of *H. percicum* extracts against the *in vitro* growth of *L. major* and *L. infantum* promastigotes yet. Hence, this study was carried out for the first time on the anti-leishmanial activity of the hydroethanolic extracts of the *H. percicum* fruit by MMT assay.

Conclusion

Further clinical research is needed to confirm the effective and safe medicinal plant therapy. It is necessary to find their active components and potential cytotoxicity effects for the replacement of safe drugs for leishmaniasis.

Authors' Contribution

Sk: Study, concept, and design. KE: Performing all laboratory tests and data collection. BSN: Selection and extraction of the selected herbal medicine, manuscript writing, statistical analysis, and data interpretation. SYN: Preparation and authentication of the selected herbal medicine.

Conflict of Interest Disclosures

The authors declare that there was no conflict of interests to publish this study.

Ethical Approval

The protocol of this study was approved by the Ethics Committee of Abadan University of Medical Sciences, Abadan, Iran. The University Ethics Committee code number was 95U-1100.

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