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Assessment of Spring Leaf Extract of *Artemisia aucheri* Effects on *Leishmania tropica/infantum*

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Abstract

Background: *Leishmania* infection is a serious worldwide health concern that is caused by various types of *Leishmania* parasite genus. Current therapies for this disease, including pentavalent antimonial complexes, are not safe and do not have enough efficacy. The *Artemisia aucheri* spring leaf extract has been reported to have antimicrobial effects on a variety of pathogens.

Objective: This study aimed to find how a spring leaf extract of *A. aucheri* affects *Leishmania tropica* and *infantum*.

Materials and Methods: After culturing macrophages and *L. tropica* and *infantum* in enriched the Roswell Park Memorial Institute (RPMI) 1640 culture medium with 10% fetal bovine serum (FBS), the impacts of different dosages of *A. aucheri* spring extracts on *L. tropica*, as well as *L. infantum* promastigotes were evaluated *in vitro*. In addition, 2, 5-diphenyltetrazolium bromide was used to assess the cytotoxicity of the extract on parasites and macrophages. Eventually, flow cytometry with annexin staining was performed to evaluate the potential of apoptosis induction in *L. tropica/infantum*.

Results: The flow cytometry test results showed the amount of apoptosis was 22.28% for *L. tropica* and *L. infantum* parasites. The spring leaf extract of *A. aucheri* inhibitory concentration was 15.17 and 9.13 for *L. tropica* and *L. infantum*, respectively.

Conclusion: Overall, A. *aucheri* spring extracts revealed a good *in-vitro* anti-*leishmania* effect on *L. tropica* and *L. infantum* promastigotes.

Keywords: *Artemisia aucheri*, *Leishmania tropica*, *Leishmania infantum*, Spring leaf extract

Background

Leishmaniasis persists as an important public health issue, caused by the parasite *Leishmania* and transmitted by the biting of female *Phlebotomine* sandflies in tropical and subtropical climatic zones.[1-3] According to clinical manifestations caused by *Leishmania* parasites, there are at least three different clinical forms of the disease, including cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis, and visceral leishmaniasis (VL).[4] On ordinary, 350 million individuals are thought to be at serious risk of *Leishmania* infection, and 12 million persons become infected with *Leishmania* worldwide. According to the World Health Organization reports, among people in both developed and developing nations, approximately one million and 500 000 new cases are annually infected with CL and VL, respectively.[5-7] *Leishmania tropica* usually induces CL and *L. Infantum* usually induces VL.[8,9] Macrophages are immune cells that have various functions such as killing pathogens or damaged cells or repairing them. According to these functions, macrophages are divided into two subgroups; one of them is classically activated macrophage, which is also known as M1 phenotype, and the other is alternatively activated macrophage, which is also known as M2. The M1 phenotype has an inflammatory effect and kills pathogens, while the M2 phenotype has an anti-inflammatory and repairing effect and inhibits the killing of pathogens.[10-13] Some pathogens, including *Leishmania* spp., seek to induce the M2 phenotype and escape from the immune system.[14] Despite scientific attempts, there is currently no efficient leishmaniasis prevention and therapeutic intervention approach in this regard. Chemotherapy has been the most prominent cure for leishmaniasis for the last 70 years, and antimony compounds, including sodium stibogluconate also known as Pentostam, as well as meglumine antimoniate also known as Glucantime, have been the most effective leishmaniasis cures.[15-17] It has also been revealed that these medications can inhibit the manufacture of adenosine triphosphate by interfering with the phosphokinase enzyme action.[17] The medications used to treat leishmaniasis infection have not been entirely effective so far, and have numerous drawbacks such as minimal responsiveness, non-specificity, toxic effects, economic cost, long-term

treatment, drug resistance, annoying injections, and tissue damage.[9,17-19] Proper therapy strategies and effective *anti-leishmania* substances must be discovered and developed since antiparasitic treatments have undesirable side effects or might lead to important issues.[20] *Artemisia* is a member of the *Asteraceae* family, which includes several herbal medicines with antibacterial properties.[21] *Artemisia* mixtures such as artemisinin and its derivative products have been recently used by scientists for their anti-parasitic, anti-microbial, anti-oxidant, anti-tumor, and anti-inflammatory properties.[22-32] This plant, also renowned as Sage Bush or Worm Wood, is found in several geographical regions such as Asia, South America, and Africa.[33] In Iran, 34 of the approximately 500 *Artemisia* species found in warmer regions, dry areas, or semiarid areas are prevalent. *Artemisia aucheri* Boiss, also known as *Dermaneh Koohi*, is a popular herbal medicine in Iran that is used for the therapy of numerous illnesses, including *Leishmania* infection. This plant includes flavonoids, coumarin, and santonin.[34]

The purpose of this study was to uncover how a spring leaf extract of *A. aucheri* can affect *L. tropica* and *infantum*.

Materials and Methods

Aqueous Extract Preparation

Artemisia aucheri was gathered in the springtime (March). *A. aucheri* was initially completely dry in the best possible situations such as shade, room temperature, and adequate moisture. Then, 50 g of a leaf was ground and soaked in water for 48 hours in 500 milliliters of distilled water. Afterward, it was softly warmed (30 minutes), filtered, and thickened in Bain-marie (60°C). Next, the plant extract was dried in an oven,[35,36] and different extract concentrations (i.e., 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100, and 200 µg/mL) were added to the Roswell Park Memorial Institute (RPMI) 1640 culture medium. The final solutions were then kept at 4°C and utilized in cytotoxicity tests and *in vitro* assays.[37]

Use of the 2, 5-Diphenyl Tetrazolium Bromide (MTT) Assay to Determine the Cytotoxicity of Aqueous Extracts on Macrophages

A murine macrophage cell line (RAW 264.7) was utilized for the MTT assay in this study. The MTT (Sigma Aldrich) test was used to assess the influence of the extracts on macrophage cells. Accordingly, 5 mg of MTT powder (Sigma Chemical Company, Germany) and 1 mL of phosphate-buffered saline (PBS) were mixed to create an MTT solution. Raw.264.7 macrophage cells were trypsinized and implanted in 96-well microplates with 100 µL per well at 5×10^5 cells/well in the Dulbecco's Modified Eagle medium containing 10% fetal calf serum, followed by a 24-hour incubation at 37°C and 5% CO_2. Subsequently, 100 µL of the extract at various concentrations (i.e., 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100, and 200 µg/mL), with the RPMI culture medium were separately added to the wells, and then the plate was incubated at 37°C for 5 hours in a dark area after adding 20 µL MTT to each well after 72 hours to permit the cells to convert the tetrazolium to an insoluble formazan. After draining the supernatant from the wells, 100 µL of *dimethyl sulfoxide* (DMSO) was added to each well. The optical density was then assessed using an enzyme-linked immunosorbent assay (ELISA) plate reader instrument (Stat Fax, USA) set to 540 nm. Finally, the cell viability rates as a percentage in the exposed and control groups were calculated using the following formula[24,35,38,39]:

$$Viable\ (Live)\ macrophages\ (percentage) = (AT\text{-}AB) / (AC\text{-}AB) \times 100$$

AT: Macrophage absorbance when exposed
AC: Untouched macrophage absorption
AB: The absorbency of the blank

Determine of Cytotoxicity of Aqueous Extracts on the Promastigotes of Parasites by MMT Assay

In a darkened room, 5 mg of MTT powder (tetrazolium salt) was dissolved in 1 mL of the PBS solution to make the MTT solution. In addition, 5×10^5 promastigotes per mL were added to 96-well culture plates with various doses of the extract. After a 72-hour incubation in the dark, 20 microliters of the prepared MTT solution were placed in each well. Then, the cells were centrifuged, and their supernatant was removed. The incubation was repeated for 5 hours at room temperature, and then the wells of the plate received 100 µL of DMSO, and the plate was placed into an ELISA reader with a wavelength of 540 nm to determine the absorbance of each well.[40,41]

Determine the Apoptotic Status of Cells by Flow Cytometry

The annexin V-FITC apoptosis detection kit (BioVision, Palo Alto, USA) was used to recognize apoptotic and necrotic cells. All test steps were performed by the company's protocol. In brief, 2×10^6 of *L. tropica* and *L. infantum* promastigotes were exposed to 50 µg/mL of *A. aucheri* spring and incubated for 72 hours. Then, the parasites were cleaned with cold PBS to remove extra extracts and collected by centrifugation at 1400 g for 10 minutes. Following the addition of 500 mL of buffer, the samples were incubated for an additional 15 minutes on ice before receiving 5 mL of annexin V and 5 mL of PI (annexin-V kit, IQ Products BV, Groningen, Netherlands). The specimens were then evaluated by a flow cytometer (BD FACSCanto II, USA). The device's output is presented as graphs and percentage charts. FlowJo software (version 10) was employed to analyze the data. This kit could distinguish between apoptotic (just annexin-V positive as primary apoptosis/lower right-both annexin-V and PI positive as secondary apoptosis/upper right), normal, living (both annexin-V and PI

negative/lower left), and necrotic (PI positive/upper left) cells.[35,38,41-44]

Promastigote Assay

The promastigotes of *L. tropica* (MHOM/IR/02/Mash10) and *L. infantum* (MHOM/TN/80/IPT1) were cultured at 25 ± 1°C in RPMI 1640 (Gibco, Germany) supplemented with 10% fetal bovine serum (FBS) (Gibco, Germany) and antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin). Every day, promastigotes in the culture were monitored to collect cells in the logarithmic phase.[38]

Then, 100 µL of promastigotes (2×10^6 cell/mL) were seeded in a 96-well plate containing 100 µL of fortified RPMI1640 with 10% FBS in the presence of 100 µL of various doses (i.e., 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100, and 200 µg/mL) of the *A. aucheri* solution and maintained at 24 ± 2°C for 24, 48, and 72 hours, and during these times, the parasite number was assessed by the hemocytometer chamber to reveal the anti-*Leishmania* effects of *A. aucheri* on parasites. Glucantime (Sanofi-Aventis France, 50 µg/mL) was also used as a positive control group. Finally, the outcomes were compared to control groups, and each experiment was repeated three times.[35,42,45]

Statistical Analysis

IBM SPSS (version 21) was applied for statistical analyses. To ensure data normalization, the Kolmogorov-Smirnov test was utilized, and mean differences were compared using one-way ANOVA and LSD. Eventually, GraphPad Prism (version 8.0.1.) was used to create graphs.[35]

Results

MTT for Assessment of Artemisia aucheri Cytotoxicity on Macrophage Cells

The results of MTT for determining *A. aucheri* cytotoxicity on macrophages demonstrated that at high doses of *A. aucheri* (e.g., 200 and 100 µg/mL) it revealed more toxic effects on macrophage cells than low doses in comparison to the control group. Figure 1 contains additional information in this regard.

MTT for the Artemisia aucheri Cytotoxicity Effects on Promastigotes

The *A. aucheri* cytotoxicity effects on *L. tropica* and *L. infantum* promastigotes were assessed by optical density after the MTT test. The viability of parasites depends on a dose-response relationship (Figures 2 and 3), and parasite viability decreases by increasing the dose of *A. aucheri* because the greatest toxicity is found at concentrations of 200 and 100 µg/mL. After 24 hours, at low concentrations of *A. aucheri,* the results were somewhat similar to those of the control drug (Glucantime).

Flow Cytometry Assay

Flow cytometry tests following staining with Annexin-V and PI were employed to determine the apoptotic, necrotic, and alive cell percentages in *L. tropica* and *L. infantum* promastigote populations. It was observed that the percentages of apoptotic and necrotic promastigote cells in contact with 50 µg/mL concentrations of *A. aucheri* after 72 hours of incubation were 22.28 and 3.99, as well as 22.28 and 3.99 for *L. tropica* and *L. infantum*, respectively. Furthermore, the percentage of alive cells in the control group (no treatment) was 98.8 and 98.8 for *L. tropica* and *L. infantum*, respectively. More information is provided in Figures 4 and 5.

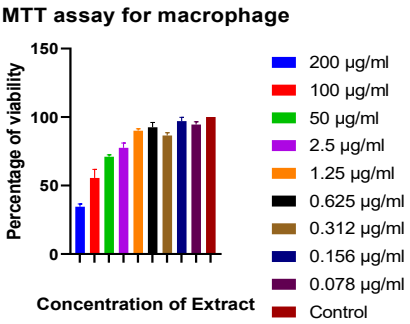

Figure 1. MTT Assay for Macrophage. *Note.* MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; *A. aucheri: Artemisia aucheri*. MTT assay was performed to assess the viability percentage (live percentage) of macrophages in exposure to six concentrations of *A. aucheri*. This figure shows that higher *A. aucheri* concentrations (e.g., 200 and 100 µg/mL) have the most toxic effects on macrophages, while its lower concentrations have the lowest toxic effects on macrophage cells, and these effects are ignorable compared to the control group. Thus, with an increase of *A. aucheri* doses, their toxicity effects on macrophages increase as well.

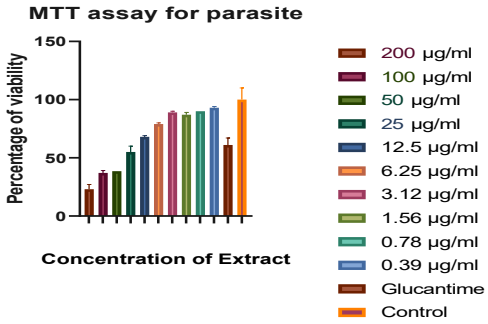

Figure 2. MTT Assay for *Leishmania infantum*. *Note.* MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; *A. aucheri: Artemisia aucheri*. MTT test was conducted to evaluate the viability percentage (live percentage) of *Leishmania infantum*. This figure displays that the viability percentage (live percentage) of *Leishmania infantum* is high in groups treated with low concentrations of *A. aucheri,* while the viability percentage (live percentage) of *Leishmania infantum* is low in those treated with high concentrations of *A. aucheri*. As a result, as the concentration of *A. aucheri* increased, the viability percentage (live percentage) of *Leishmania infantum* decreased further than in the control group. Further, at high concentrations of *A. aucheri* (e.g., 200 and 100 µg/mL), the viability percentage (live percentage) of *Leishmania infantum* is lower than in conventional therapies such as Glucantime.

The influence of Artemisia aucheri on the Growth Inhibition of Promastigotes

Figures 6 and 7 illustrate the influence of various doses of *A. aucheri* on, *L. tropica*, and *L. infantum* promastigotes

assessed after incubation for 24, 48, and 72 hours. Based on the results, proliferation significantly reduces by rising doses of *A. aucheri*, *L. tropica*, and *L. infantum* promastigotes. Doses of 200 and 100 µg/mL of *A. aucheri* after 24, 48, and 72 hours of incubation had the most performance, while the doses of 0.39 and 0.78 µg/mL after 24 and 48 hours of incubation, had the least performance in hindering the proliferation and activity of *L. tropica* and *L. infantum* promastigotes.

Determination of the Half-maximal Inhibitory Concentration (IC_{50})

After 72 hours, the IC_{50} value of *A. aucheri* was 15.17 µg/mL and 9.13 for *L. tropica* and *L. infantum,* respectively, as determined by the promastigote assay. Additionally, the anti-leishmanial property of *A. aucheri* was positively correlated with exposure time and dose (Figures 8 and 9).

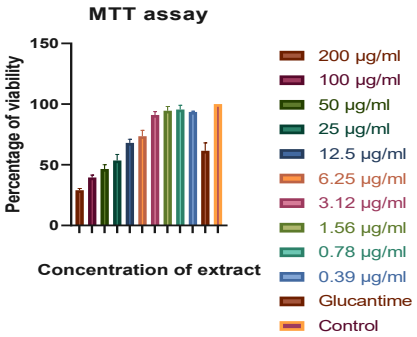

Figure 3. MTT Assay for *Leishmania tropica*. *Note.* MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; *A. aucheri: Artemisia aucheri.* This test was performed to assess the viability percentage (live percentage) of *Leishmania tropica*. This figure illustrates that in groups treated with low concentrations of *A. aucheri*, the viability percentage (live percentage) of *Leishmania tropica* is high, while in groups treated with high concentrations of *A. aucheri*, the viability percentage (live percentage) of *Leishmania tropica* is low. Accordingly, as the concentration *A. aucheri* increased, the viability percentage (live percentage) of *Leishmania tropica* further decreased than in the control group. Moreover, at high concentrations of *A. aucheri* (e.g., 200 and 100 µg/mL), the viability percentage (live percentage) of *Leishmania tropica* is lower than in conventional therapies such as Glucantime.

Discussion

Leishmaniasis is already broadly renowned as the world's key health concern. The present leishmaniasis treatment is ineffective, and the applied drugs (Glucantime and Pentostam) are not comprehensive choices due to numerous adverse effects, high costs, high toxicity, painful injections, and the emergence of drug resistance in certain endemic regions.[46-49] As a result, investigations are now focused on finding lower-cost and more efficient drugs with minimal or even no side effects.[20] *A. aucheri* is one of these drugs because the therapeutic benefits of this drug, including antiparasitic, antioxidant, anti-inflammatory, antifungal, and antimicrobial activities, have been more clearly identified in recent decades.[24,38,50,51] The present study evaluated the anti-parasitic effects of the *A. aucheri* extract on *L. tropica* and *L. infantum in-vitro*. Asghari et al used gas chromatography–mass spectrometry to identify mixtures in *A. aucheri* extracts, including bornyl acetate, decane, caryophyllene oxide, lavandulol, and dehydro aromadenderene.[52] Several researchers assumed that the existence of phenol, terpene, lactone, flavonoids, coumarin, and sterol in the *A. aucheri* extract was responsible for its antimicrobial effects.[53] Rezaei et al asserted that the presence of terpene, sesquiterpene, and anti-inflammatory compounds in the *A. aucheri* extract resulted in a great therapeutic effect on the hepatoxic problem.[54]

The MTT results of Karimipour-Saryazdi et al showed that as the concentration of the *A. aucheri* extract was raised, macrophage cell survival decreased after 24 hours

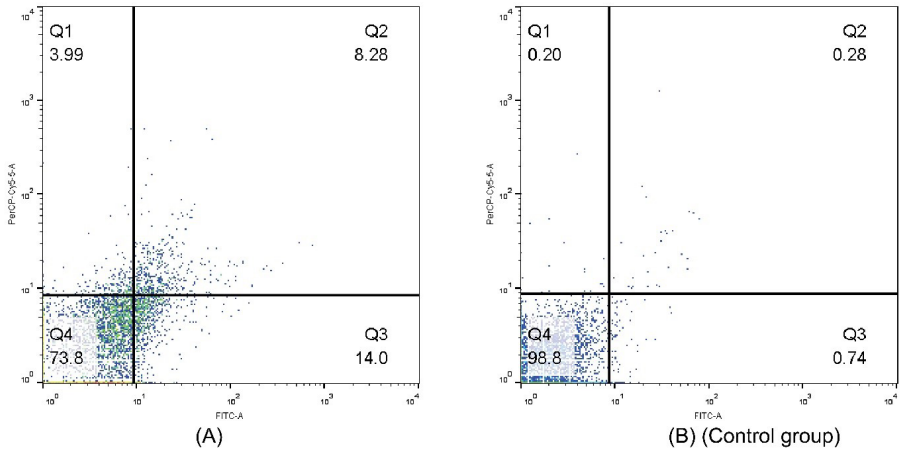

Figure 4. Flow Cytometry Analysis for *Leishmania infantum*. *Note. A. aucheri: Artemisia aucheri.* This test was conducted to determine the viability percentage (live percentage) and apoptotic percentage of macrophages in exposure to a 50 µg/mL concentration of *A. aucheri*. Panel (A) shows that at a 50 µg/mL concentration of *A. aucheri*, 22.28 % of *Leishmania infantum* parasites underwent apoptosis, and 73.8% of *Leishmania infantum* parasites are alive in exposure to this concentration of *A. aucheri*. Furthermore, Panel (B) depicts that in the untreated control group, 98.8% of *Leishmania infantum* parasites are alive. In this figure, the upper and lower right sections indicate secondary and primary apoptosis, respectively. Additionally, the upper and lower left sections represent necrosis and alive cells, respectively.

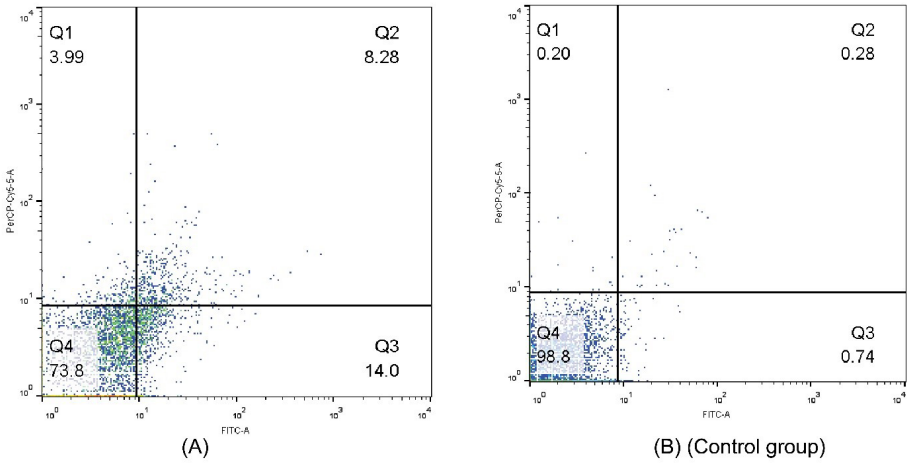

Figure 5. Flow Cytometry Analysis for *Leishmania tropica*. *Note. A. aucheri: Artemisia aucheri.* This test was conducted to determine the viability percentage (live percentage) and apoptotic percentage of macrophages in exposure to a 50 µg/mL concentration of *A. aucheri*. Panel (A) illustrates a 50 µg/mL concentration of A. aucheri. Overall, 22.28% of *Leishmania tropica* parasites underwent apoptosis, as well as 73.8% of *Leishmania tropica* parasites are alive in exposure to this concentration of *A. aucheri*. In addition, Panel (B) shows that in the untreated control group, 98.8% of *Leishmania tropica* parasites are alive. In this figure, the upper and lower right sections demonstrate secondary and primary apoptosis, respectively. Further, the upper and lower left sections indicate necrosis and alive cells, respectively.

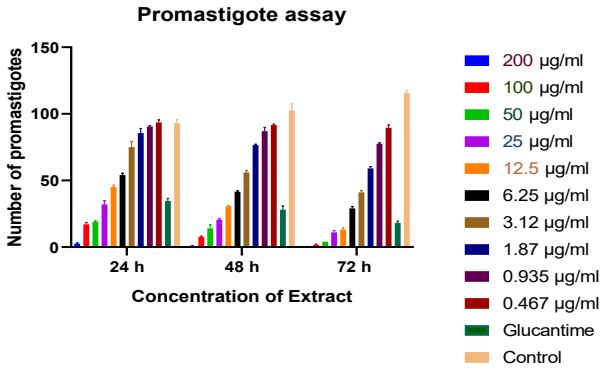

Figure 6. Promastigote Assay for *Leishmania infantum*. *Note. A. aucheri: Artemisia aucheri.* This figure depicts that the effect on the decreasing number of *Leishmania infantum promastigotes* increases with an increased concentration of *A. aucheri*. Thus, at higher doses (e.g., 200 and 100 µg/mL concentrations), *A. aucheri* has the most effect on the decreasing number of *Leishmania infantum promastigotes*. Even at these doses, their effects are higher than those of conventional therapies such as Glucantime. These effects are minimal at lower *A. aucheri* concentrations. Furthermore, this figure displays that the effects of *A. aucheri* on the decreasing number of *Leishmania infantum promastigotes* increase with increasing time.

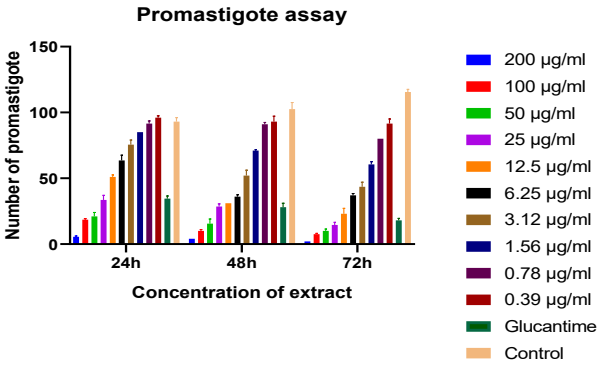

Figure 7. Promastigote Assay for *Leishmania tropica*. *Note. A. aucheri: Artemisia aucheri.* This figure shows that increasing the concentration of *A. aucheri* increases the decreasing number of *Leishmania tropica promastigotes*. Accordingly, at higher doses (e.g., 200 and 100 µg/mL concentrations), *A. aucheri* has the highest effect on the decreasing number of *Leishmania tropica promastigotes*. Even at these doses, their effects are higher than those of conventional therapies such as Glucantime. These effects are minimal at lower *A. aucheri* concentrations. In addition, this figure displays that the effects of *A. aucheri* on the decreasing number of *Leishmania tropica promastigotes* increase with increasing time.

of exposure to various doses of this extract,[37] which is similar to our results.

According to their flow cytometry results, *A. aucheri* extracts from the spring harvest (100 µg/mL) induced approximately 32% *L. major* promastigote apoptosis.[35] Ghaffarifar et al also found 68.16% apoptosis in *L. major* promastigotes that were exposed to 100 µg/mL *artemisinin*.[38] In our study, this value was 22.28 and 22.28 for *L. tropica* and *L. infantum* promastigotes, respectively. Ahmadi et al highlighted the apoptotic influence of the *A. aucheri* extract on human cancer cell lines (e.g., SKNMC cells) via mitochondrial caspase activation.[50] Mojarrab et al employed the beta-hematin formation method to examine the *in-vitro* effect of the *A. aucheri* extract on malaria parasites and found that the IC_{50} value was 1.83 ± 0.03 mg/mL.[55] Moreover, in the study by Karimipour-Saryazdi et al, the IC_{50} values of *A. aucheri* spring extracts against *L. major* amastigotes were 90 µg/mL,[35] whereas in our study, the IC_{50} values of *A. aucheri* spring extracts against promastigote of *L. tropica* and *L. infantum* were 15.17 and 9.13, respectively.

Similarly, Emami et al revealed that all 11 *Artemisia* plant species studied in Iran had anti-leishmanial effects, while *A. ciniformis* (IC_{50}: 25 µg/mL) and *A. santolina* (IC_{50}: 80 µg/mL) had the greatest toxicity levels.[56]

Overall, the efficiency of the *A. aucheri* extract as an appropriate candidate drug against leishmaniasis was confirmed by MTT and flow cytometry analyses.

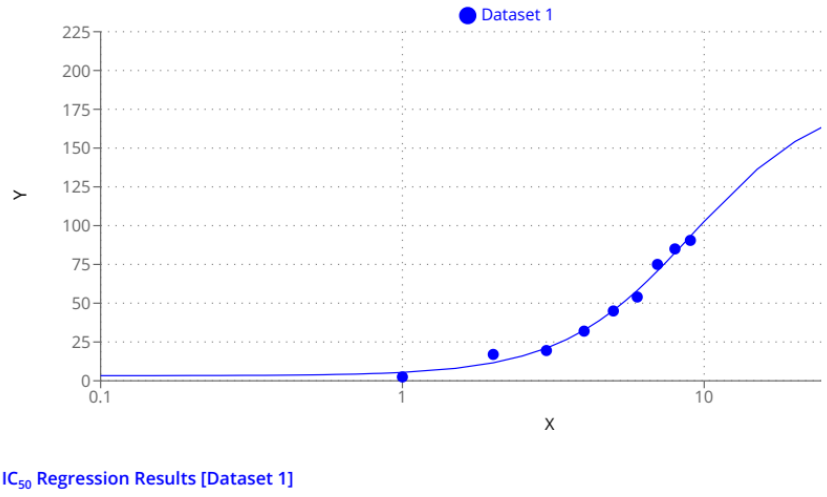

Figure 8. Determination of IC_{50} for *Leishmania infantum*. *Note*. IC_{50}: Half-maximal inhibitory concentration. This chart has been drawn to determine the IC_{50} for *Leishmania infantum*. According to this chart, the IC_{50} for *Leishmania infantum* was 9.13. This concentration was determined according to the promastigote assay after 72 hours of exposure to parasites with *A. aucheri* at various dilutions.

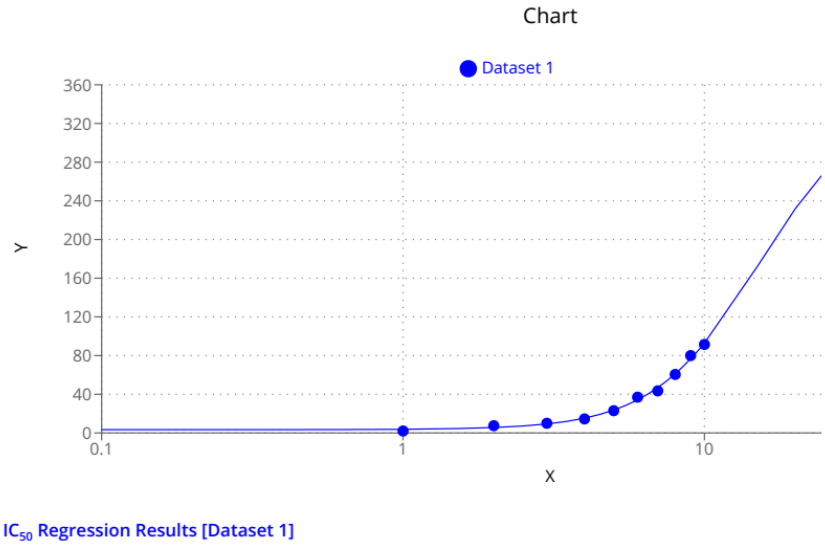

Figure 9. Determination of IC50 for *Leishmania tropica*. *Note*. IC_{50}: Half-maximal inhibitory concentration. This chart was drawn to estimate the IC_{50} for *Leishmania tropica*. According to this chart, the corresponding IC_{50} for *Leishmania tropica* was 15.17. This concentration was determined according to the promastigote assay after 72 hours of exposure to parasites with *A. aucheri* at various dilutions.

Conclusion

The *in vitro* efficacy of an *A. aucheri* extract against promastigotes of *L. tropica* and *Leishmania infantum* was found to be promising in this study. Furthermore, the findings of this study suggest that more research should be conducted on the plant's leishmanicidal activities *in vivo*. It should be noted that the leishmanicidal activity of the *A. aucheri* extract is probably attributed to the existence of bioactive substances, which appears to be highly efficient as an herbal drug candidate that is suitable for the cure of *Leishmania* spp. infections. Further research will focus on isolating various substances from extracts and testing these substances for anti-Leishmanial efficacy.

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Supervision: Abdolhossein Dalimi, Zeinab Moghadamizad.
Validation: Majid Pirestani.
Writing – original draft: All Authors.
Writing – review & editing: Majid. Pirestani.

Conflict of Interest Disclosures

The authors declare that they have no competing interests.

Ethical Approval
Not applicable.

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