



Anti-leishmanial Effect and Cytotoxicity of Hydroalcoholic Extract of *Laser trilobum*: An in vitro Evaluation

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Abstract

Background: With the hope of finding a less expensive, safe, and common drug for leishmaniasis, this study aimed to evaluate the leishmanicidal activity and cytotoxicity of *Laser trilobum* hydroalcoholic extract in vitro conditions.

Methods: The fruit extract of *L. trilobum* was prepared and examined for cytotoxic activity against RAW264.7 macrophages using the colorimetric MTT assay. The hydroalcoholic extract was evaluated against *Leishmania major* promastigote and amastigote stages. The infectivity rate and the number of amastigotes inside macrophages were investigated and compared with no treatment (control) and glucantime (reference drug) groups.

Results: *Laser trilobum* exhibited significant leishmanicidal activity against both promastigote and amastigote forms compared to the control groups. The inhibitory concentration (IC_{50}) values of the plant on uninfected RAW264.7 cells were 1727 μ g/mL. Herbal lethality against promastigotes was observed from $15 \pm 7.4\%$ for 37 μ g/mL up to at least $79 \pm 7.8\%$ for 5000 μ g/mL. The percentage of infected macrophages and the number of amastigotes in macrophages significantly declined at concentrations of 1000, 1250, and 1500 μ g/mL ($P < 0.05$, $P = 0.001$, and $P = 0.000$, respectively) over 24 to 72 h compared to control groups. The infected macrophage rates between 1500 μ g/mL extract and 5 μ g/mL glucantime were not statistically significant at each time point. The crude extract at 1000, 1250, and 1500 μ g/mL was significantly more effective on the number of amastigote forms in treated macrophages than the 5 μ g/mL glucantime ($P < 0.05$, $P = 0.001$, and $P = 0.000$, respectively), whereas its 1250 μ g/mL was not statistically significant with 10 μ g/mL glucantime at 48 - 72 h post-incubation. Additionally, the percentage of amastigote viability in treated macrophages was equal between 1500 μ g/mL crude extract and 15 μ g/mL glucantime at each time point, so the mean number of amastigotes/100 macrophages was 2.67 ± 1.58 for this concentration versus 2.67 ± 0.58 for 15 μ g/mL glucantime, and also 7.33 ± 1.53 for 10 μ g/mL glucantime after 48 h of incubation.

Conclusions: Considering the remarkable leishmanicidal effects and absence or very low cytotoxicity, we can introduce the plant as a valuable natural source for further studies on the treatment and control of leishmaniasis.

Keywords: *Laser trilobum*, Herbal Medicine, in vitro, Leishmania, Leishmaniasis, Hydroalcoholic Extract

1. Background

Leishmaniasis is a zoonotic disease caused by obligate intracellular protozoan parasites of the genus *Leishmania* (L), which pose a public health problem in many tropical and subtropical regions of the world (1, 2). This parasitic infection is present on five continents,

with 350 million people at risk worldwide (3, 4). The disease has a broad clinical spectrum, and cutaneous leishmaniasis (CL) is the most prevalent form, commonly caused by *Leishmania tropica* and *L. major* (1, 4). *Leishmania* has extracellular promastigotes and intracellular amastigotes in the female sand fly vectors and mammalian host (human) macrophages,

respectively (2, 5). Chemotherapy is essential for leishmaniasis control and treatment, with the first line of chemotherapy being the antimonial compounds meglumine antimoniate (glucantime) and sodium stibogluconate. Second-choice drugs include amphotericin B, pentamidine, and miltefosine (6-8). These therapeutic drugs have adverse effects, including toxicity to the liver, kidneys, and spleen of patients, high cost, lengthy treatment, and drug resistance (6, 7). Hence, new drug discovery is essential (7, 9, 10), and the use of natural products can be valuable in the treatment of leishmaniasis (6, 11). *Laser trilobum* (L.) Borkh. (Apiaceae family) is a perennial, herbaceous, aromatic plant known as Kefe cumin, horse caraway, or three-lobed sermountain, and it grows in Iran, Southeast Asia, the Balkan Peninsula, and Central and Eastern Europe (12). One species, namely *L. trilobum* (L.) Borkh, has been reported in Iran. Its fruits are used as a spice and condiment.

The chemical compositions of the essential oils obtained from *L. trilobum* have been published, revealing ten monoterpenes hydrocarbons, nine sesquiterpenes, and three diterpenes (12-15). The main compounds of the fruit of the plant include limonene, perillaldehyde, α -pinene, cis-limonene oxide, diethyl phthalate, carvone, and myrcene (12, 15). *Laser trilobum* exhibits antimicrobial activity against gram-positive and gram-negative bacteria and *Candida albicans* (12, 15), and demonstrates immunobiological (16), antioxidant, and antihaemolytic properties (17). The amastigote forms can be a very useful tool for studying the leishmanicidal activity of medicinal compounds in vitro research because this form is located in human macrophages and can cause pathogenesis and a broad spectrum of clinical manifestations. On the other hand, there is no investigation into the effects of *L. trilobum* extracts on *Leishmania* amastigote and promastigote forms. Therefore, the present study was designed and performed to find a new drug with more effectiveness and fewer or absent adverse effects, which can be an excellent approach for treating and controlling leishmaniasis.

2. Objectives

This experimental study was performed to evaluate the effect of *L. trilobum* hydroalcoholic extracts against promastigotes and intracellular amastigotes of *L. major* (MRHO/IR/75/ER) and its cytotoxic activities on

macrophages RAW264.7 in vitro conditions, with the hope of finding a less expensive and common drug for this neglected disease.

3. Methods

3.1. Cultivation of Parasite and Macrophages

An Iranian strain of *L. major* (MRHO/IR/75/ER) was provided by the Tehran University of Medical Sciences (Tehran, Iran) and cultured in NNN and RPMI-1640 medium as previously described (8). The RAW264 macrophage cell line was obtained from the Iranian Biological Resource Center (IBRC; Iran). The RAW264 macrophages are widely used due to their phagocytosis and pinocytosis activity, ease of maintenance, and phenotypic stability (8, 18). The RAW 264.7 was cultured according to a previous report (8), and the passage range used was up to 12 passages during the experiments.

3.2. Plant Materials and Extraction

Laser trilobum (L.) Borkh was identified and collected from the Namin road to Fandoghloo forest in Ardebil province, northwestern Iran. The plant was deposited in the herbarium of Ferdowsi University of Mashhad (Mashhad, Iran) under the voucher number 35702. *Laser trilobum* fruits were washed, shade-dried, and ground into powder. The ground material (100 g) was dissolved in 100 mL of aqueous ethanol (70%) solvent, and maceration was performed three times, each time for 24 h in the dark with constant agitation at room temperature. The extracts were sterilized by filtration using a 0.22 μ m syringe filter (Sartorius, Germany). Finally, the extracts were concentrated under a vacuum at 40°C using a rotary evaporator apparatus (Heidolph, Germany), and the residue was stored at -20°C until required for testing (8).

3.3. Cytotoxic Effects of Extract on Macrophage Cells

The cytotoxicity effects of *L. trilobum* against RAW264.7 macrophage cells were assessed by cultivating macrophages (5×10^5) with 100 μ L of various concentrations of hydroalcoholic extract (0 to 3000 μ g/mL) in 96-well tissue culture plates. These plates were incubated at 37°C in 5% CO₂ for 24, 48, and 72 h, and each part was done in triplicate. Culture medium RAW264.7 cells without the plant extract and complete medium

with no macrophages and drugs/extracts were respectively considered as untreated (negative) control and blank. The effects of *L. trilobum* extract on RAW264.7 macrophages were determined using the colorimetric MTT assay. The cytotoxicity was calculated as the inhibitory concentration (IC_{50}) that inhibited 50% of cell growth using GraphPad Prism Version 8.0 software.

3.4. Effect of Extract on Promastigotes

Initially, 100 μ L of RPMI-1640 medium containing logarithmic growth phase promastigotes (1×10^5 parasites/mL) were added into 96-well plates. Afterward, 100 μ L of different concentrations of *L. trilobum* extracts (final concentrations of extract were obtained at 37 - 5000 μ g/mL) were put into each well and incubated at $25 \pm 1^\circ\text{C}$ for 24, 48, and 72 h. The Parasite Viability Index was determined by trypan blue solution (1:1, trypan blue/sample volume), and the percentage of viable promastigotes (unstained) compared to untreated cultures (control cultures) was measured using a Neubauer hemocytometer under a light microscope in triplicate. Promastigotes cultured in a complete medium with no extract were used as control. The inhibition rate (%) was calculated in triplicate as reported previously (8).

3.5. Effect of Extract on Infectivity Rate and the Number of Amastigotes in Macrophages

Assessment of the infection rate and parasite load in macrophages was determined with a brief modification according to previously described methods (8). Plate culture was incubated at 37°C in 5% CO_2 for 4 - 5 h, then the medium, together with non-adherent RAW264.7 cells, was removed. *Leishmania major* promastigotes in the stationary phase (at a ratio of 10 parasites per 1 macrophage) were added to the wells and then incubated at 37°C in 5% CO_2 for 24 h. The medium was aspirated, and free promastigotes were removed by washing with RPMI 1640 medium. The infected macrophages were treated with various concentrations of *L. trilobum* hydroalcoholic extracts (500, 750, 1000, 1250, 1500 μ g/mL) under the same conditions for 24, 48, and 72 h. The macrophages containing the parasite without extract and those with glucantime (Meglumine antimoniate; Sanofi Aventis, France) were considered as a control group and drug (reference) control, respectively, for comparison of parasite inhibition.

Finally, slides were fixed in absolute methanol, stained with Giemsa dye, and the percentage of infected macrophages (% infection rate of macrophages), as well as the mean number of amastigotes in at least 100 macrophages (% amastigotes viability), were studied under light microscopy compared to those obtained with the control group and reference drug (8).

3.6. Statistical Analysis

All experiments were carried out in triplicate, and the findings were determined as mean \pm standard error of the mean (SEM). The GraphPad Prism 8 statistical software was used to determine the IC_{50} values for the plant extracts. The data and statistical analysis were determined using the Wilcoxon signed ranks test and Student's *t*-test. The mean infection rate of macrophages and the number of amastigotes in macrophages between test and control groups were compared by the Wilcoxon signed ranks test. A *P*-value < 0.05 was considered remarkably significant.

4. Results

4.1. Cell Cytotoxicity Activity

The cytotoxicity of *L. trilobum* hydroalcoholic extracts on uninfected RAW264.7 macrophages was determined by colorimetric MTT assay at 24, 48, and 72 h post-incubation and compared with the control groups. The IC_{50} for cytotoxicity of the plant extracts on uninfected RAW264.7 macrophages was 1727 μ g/mL at 24 and 48 h, and R^2 values for the same time were 0.9654 and 0.9843, respectively (Figure 1).

4.2. Antileishmanial Effects of Laser *trilobum* Extracts on Promastigotes

As shown in Figure 2, the fatality rate of *L. trilobum* hydro-ethanolic extracts on *L. major* promastigotes displayed a dose-dependent manner (*P* < 0.05), such that the inhibition rate led to the death of promastigotes of *L. major* from $15 \pm 7.4\%$ for 37 μ g/mL up to $79 \pm 7.8\%$ for 5000 μ g/mL. The inhibition rate of the herbal extract was not time-dependent.

4.3. Antileishmanial Effects of the Herbal Extract on Infected Macrophages and Intracellular Amastigote Numbers

The effect of different concentrations (500 - 1500 μ g/mL) of *L. trilobum* hydroalcoholic extract on infected

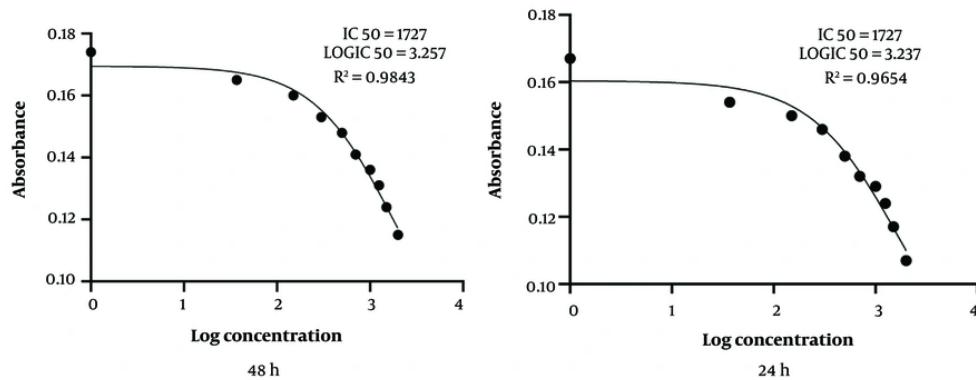


Figure 1. Inhibitory concentration (IC_{50}) for cytotoxicity of *Laser trilobum* hydro-ethanolic extracts (increasing concentrations: 500 - 3000 μ g/mL) on the macrophage cell line (RAW264.7) after 24 and 48 h by using MTT assay and GraphPad Prism Version 8.0 software. All data reported as a mean of three repeated experiments.

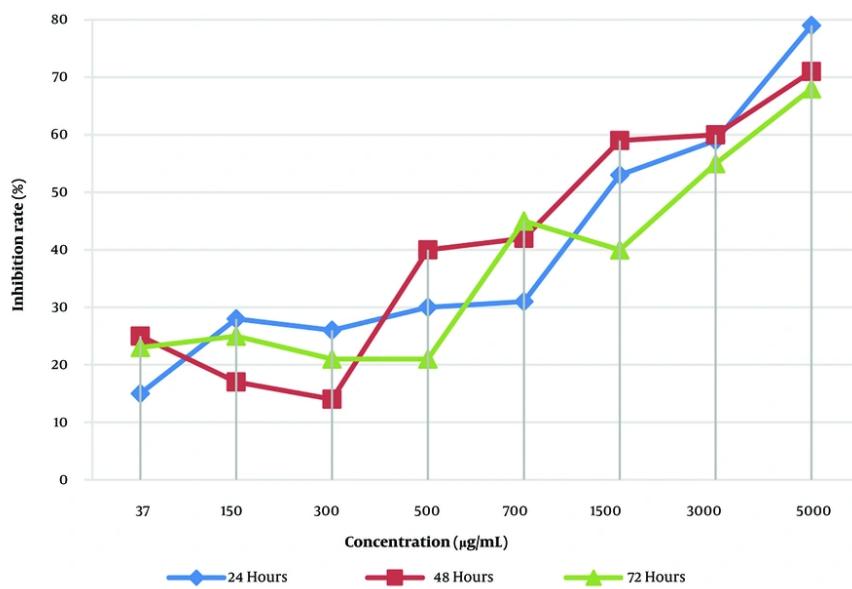


Figure 2. Inhibition rates of promastigotes treated with different concentrations (37 - 5000 μ g/mL) of *Laser trilobum* extract at 24, 48, and 72 h incubation; data represent experiments done in triplicate.

macrophage cells and intracellular amastigote numbers was dose-dependent over 24 - 72 h (Table 1). Hence, the percentage of infectivity in the infected RAW264.7 cells decreased from $66.67 \pm 1.53\%$ for 500 μ g/mL to $19.67 \pm 1.53\%$ for 1500 μ g/mL of *L. trilobum* at 48 h.

According to Table 1, statistically significant reductions in the percentage of infected macrophages and the average number of amastigotes in macrophages were observed at concentrations of 1000, 1250, and 1500 μ g/mL ($P < 0.05$, $P = 0.001$, and $P = 0.000$, respectively)

Table 1. Leishmanicidal Effects of Different Concentrations of *Laser trilobum* Hydroalcoholic Extracts on Infected Macrophages with *Leishmania major* and the Number of Amastigotes in Macrophages at 24, 48 and 72 h After Incubation ^a

Conc. (µg/mL); Time (h)	Percentage of Infected Macrophages				No. of Amastigotes per 100 Macrophages			
	Treated	Control	P-Value	Reduction	Treated	Control	P-Value	Reduction
500								
24	56.33 ± 1.00	58.00 ± 1.00	0.95	2.88	23.33 ± 2.08	25.33 ± 1.53	0.257	7.9
48	66.67 ± 1.53	69.67 ± 2.08	1.00	4.31	25.67 ± 1.53	28.33 ± 1.15	0.22	9.39
72	71.00 ± 2.00	75.33 ± 1.53	1.00	5.75	30.67 ± 1.53	32.67 ± 1.53	0.32	6.12
750								
24	52.33 ± 0.58	58.0 ± 1.0	0.086	9.78	21.00 ± 2.00	25.33 ± 1.53	0.17	17.09
48	62.33 ± 1.53	69.67 ± 1.53	0.095	10.53	23.33 ± 1.53	28.33 ± 1.15	0.096	17.65
72	66.0 ± 2.00	75.33 ± 1.53	0.079	12.38	28.33 ± 1.53	32.67 ± 1.53	0.10	13.28
1000								
24	40.67 ± 1.53	58.0 ± 1.0	0.023	29.88	11.0 ± 1.0	25.33 ± 1.53	0.044	56.57
48	45.33 ± 1.53	69.67 ± 1.53	0.03	34.94	9.67 ± 1.15	28.33 ± 1.15	0.01	68.87
72	54.00 ± 2.65	75.33 ± 1.53	0.05	28.32	13.33 ± 0.58	32.67 ± 1.53	0.04	59.20
1250								
24	31.67 ± 1.53	58.0 ± 1.0	0.001	45.40	8.33 ± 1.53	25.33 ± 1.53	0.001	67.11
48	34.33 ± 1.53	69.67 ± 1.53	0.001	50.72	6.00 ± 1.00	28.33 ± 1.15	0.001	78.82
72	43.33 ± 1.53	75.33 ± 1.53	0.001	42.48	8.00 ± 1.00	32.67 ± 1.53	0.001	75.51
1500								
24	23.33 ± 1.53	58.0 ± 1.0	0.000	59.78	4.67 ± 1.53	25.33 ± 1.53	0.000	81.56
48	19.67 ± 1.53	69.67 ± 1.53	0.000	71.77	2.67 ± 0.58	28.33 ± 1.15	0.000	90.58
72	32.00 ± 2.65	75.33 ± 1.53	0.000	57.52	3.67 ± 0.58	32.67 ± 1.53	0.000	88.77

^a Values are expressed as mean ± SEM of experiments done in triplicate.

over 24 to 72 h in comparison with infected macrophages with no treatment as control (Table 1).

4.4. Comparative Effects of Extract and Glucantime on Infected Macrophages and the Number of Intra-macrophage Amastigotes

The infection rates of treated macrophages with effective concentrations of the plant extract compared with glucantime (as a reference drug) are shown in Figure 3. A significant difference in infected macrophage rates between 5 µg/mL glucantime (standard drug) and 1500 µg/mL of the plant extract in treated macrophages was not observed at each time point, but for 10 µg/mL drug at 48 h only ($P > 0.05$) (Figure 3). The inhibitory effect of the herbal extract was not time-dependent; however, a concentration-dependent inhibition was observed.

The percentage of intracellular amastigote viability in treated macrophages with *L. trilobum* extracts compared with glucantime is presented in Figure 4. Concentrations of 1000 and 1250 µg/mL and higher of the plant extract were significantly more effective on the number of amastigote forms in treated macrophages

than the 5 µg/mL glucantime ($P < 0.05$, $P < 0.01$, respectively) at the three tested times, whereas the extract values of 1250 µg/mL were not statistically significant compared to 10 µg/mL glucantime as the control drug.

According to the results shown in Figure 4, the percentage of amastigote viability in treated macrophages was equal between 1500 µg/mL plant extract and 15 µg/mL glucantime at each time point, so the mean number of amastigotes/100 macrophages was 2.67 ± 1.58 for this concentration versus 2.67 ± 0.58 for 15 µg/mL glucantime, and also 6.0 ± 1 for 1250 µg/mL plant extract versus 7.33 ± 1.53 for 10 µg/mL glucantime after 48 h of incubation (Figure 4).

5. Discussion

The emergence of some restrictions reported about conventional therapy for the disease, such as excessive side effects and parasitic resistance to these drugs, justifies the search for new effective and safe antileishmanial drugs (6, 19). Therefore, the *L. trilobum* herbal plant can be used as a novel natural resource. In

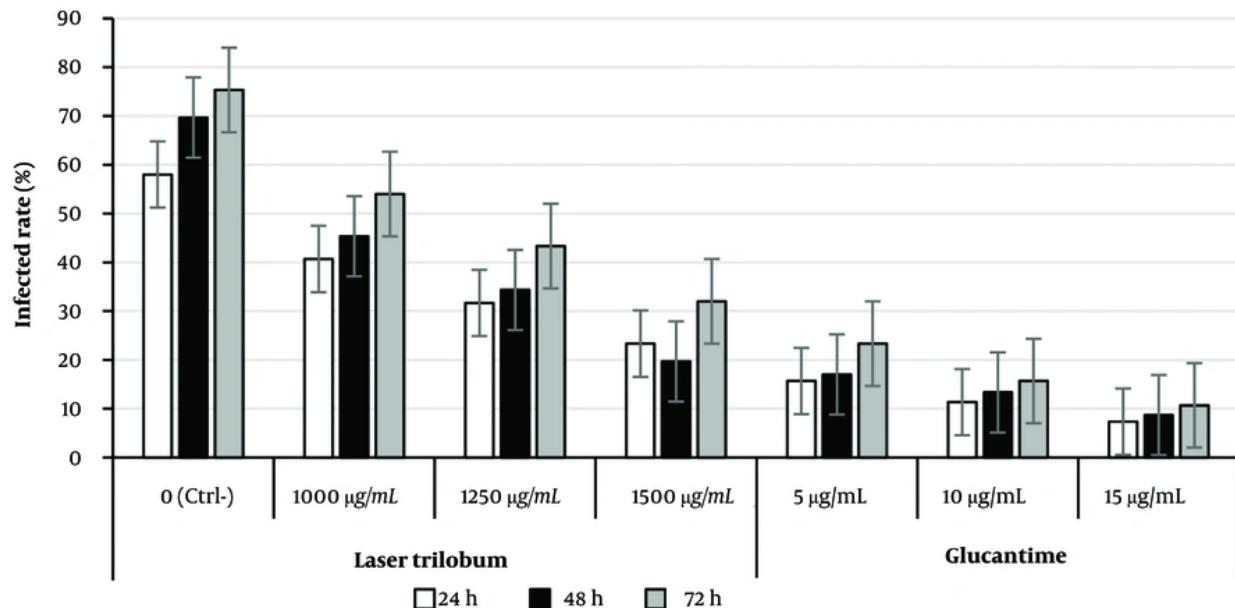


Figure 3. Percentage of infected macrophages when treated with effective concentrations of *Laser trilobum* extract compared to glucantime (as reference drug) and untreated (negative control) groups at 24, 48, and 72 h after incubation; results are shown as mean \pm standard error of the mean (SEM; $n=3$).

the present study, the IC_{50} for cytotoxicity of the plant extracts on uninfected RAW264.7 macrophages was 1727 $\mu\text{g}/\text{mL}$ at 24 and 48 h, and R^2 values for the same time were 0.9843 and 0.9654, respectively (Figure 1). The results showed that *L. trilobum* had very low cytotoxicity on RAW264.7 macrophages and can be of great significance for its traditional usefulness in potentially treating leishmaniasis. These findings are in concurrence with the Nematollahi et al. study (8). In this study, *L. trilobum* acted as a leishmanicidal agent against both promastigotes and intracellular amastigote forms. Different concentrations of the herbal extract caused the death of *L. major* promastigotes (Figure 2), and inhibitory rates demonstrated a dose-dependent response. The results presented in Figure 2 showed that by increasing the concentration of *L. trilobum* extracts, the proliferation of *L. major* promastigotes decreased remarkably ($P < 0.05$), and the leishmanicidal activity was in line with the findings of another study (8, 20).

In the present study, the percentage of infected RAW264.7 cells with *L. major* significantly declined at 1000 and 1250 $\mu\text{g}/\text{mL}$ and higher concentrations of the fruit extract over 24 to 72 h compared with infected

macrophages with no treatment as control. The findings reported by Najm et al. and Nematollahi et al. support our results (8, 20). In our results, the lethal activity of the plant extract against the parasite was dose-dependent, with the reduction of the percentage of infected macrophages increasing from 4.31% for 500 $\mu\text{g}/\text{mL}$ to 71.77% for 1500 $\mu\text{g}/\text{mL}$ ($P < 0.01$). These findings suggest that *L. trilobum* compounds such as α -pinene could prevent the growth and proliferation of *L. major* amastigotes and promastigotes (21). Recent studies have reported that the fruit of the herbal extracts had significant antimicrobial effects on pathogenic bacteria such as *Staphylococcus aureus*, *Proteus vulgaris*, *Proteus mirabilis*, *Bacillus cereus*, *Aeromonas hydrophila*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Enterobacter aerogenes*, and *Escherichia coli* (15). In this study, the anti-amastigote effects of the herbal extracts on the average number of amastigotes in macrophages significantly inhibited parasite growth at 24, 48, and 72 h after incubation for 1000 - 1500 $\mu\text{g}/\text{mL}$ concentrations compared with the control group. Our results observed that the reduction in the number of amastigotes per 100 macrophages increased from 9.39 for 500 $\mu\text{g}/\text{mL}$ to 90.58 for 1500 $\mu\text{g}/\text{mL}$ ($P = 0.000$) at 48

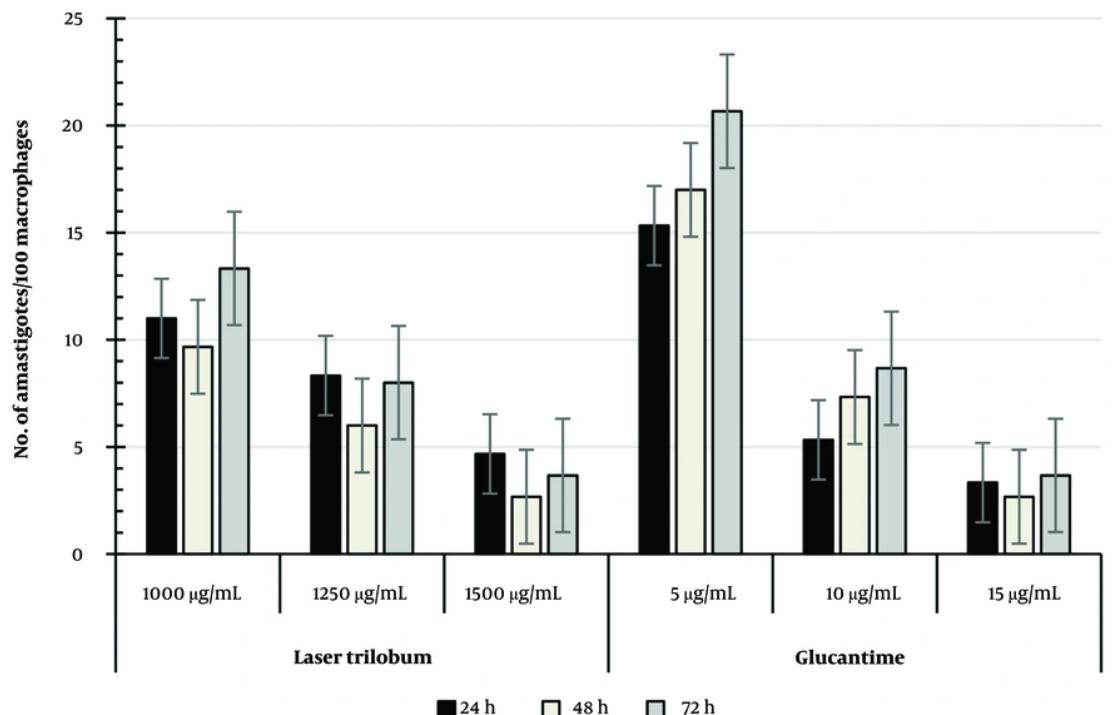


Figure 4. Percentage of intracellular amastigote viability in treated macrophages with *Laser trilobum* extracts compared with glucantime at 24, 48 and 72 h after incubation. The results refer to the mean \pm standard error of the mean (SEM) of experiments done in triplicate.

h. The anti-amastigote effects of these herbal concentrations were significantly effective against intramacrophage amastigotes. This study is consistent with previous studies that demonstrate *Thymus kotschyanus* (8), *Berberis vulgaris*, and berberine (22) significantly decreased the mean number of intra-macrophage amastigotes compared with the control.

Our findings revealed that the difference in infected macrophage rates between 5 µg/mL glucantime (standard drug) and 1500 µg/mL of the plant extract in treated macrophages was not statistically significant at each time point (Figure 3), except for the 10 µg/mL drug at 48 h only ($P > 0.05$). Similar to our findings, Nematollahi et al. (8) reported that the difference in infected macrophage rates between the 350 µg/mL of *T. kotschyanus* extract and the 5 µg/mL standard drugs (glucantime) was not statistically significant ($P > 0.05$). Additionally, Wabwoba et al. (23) revealed that there was no significant difference in infection rates between the

100 µg/mL of *Allium sativum* extract and the standard drugs.

This study showed that the concentrations of 1000 and 1250 µg/mL and higher of the plant crude extract were significantly more effective on the number of amastigote forms in treated macrophages than the 5 µg/mL glucantime ($P < 0.05$, $P < 0.01$, respectively) at the three times tested. The extract values of 1250 and 1500 µg/mL were not statistically significant compared to 10 and 15 µg/mL glucantime as the control drug, respectively (Figure 4). The study of *T. kotschyanus* hydroalcoholic extract values at 300 and 350 µg/mL on the intracellular amastigotes by Nematollahi et al. agreed with this study (8). According to published reports, *L. trilobum* hydroalcoholic crude extract contains effective compounds such as alpha-pinene and beta-caryophyllene (21, 24). The alpha-pinene of the plant is a biologically active compound against the viability and proliferation of both amastigote and promastigote forms of *L. amazonensis* in vitro (21).

The results showed that the percentage of amastigote viability in treated macrophages with 1250 and 1500 $\mu\text{g}/\text{mL}$ plant extract was equal to 10 and 15 $\mu\text{g}/\text{mL}$ glucantime at each time point, respectively. Hence, the mean number of amastigotes per 100 macrophages was 6.0 ± 1 and 2.67 ± 1.58 for 1250 and 1500 $\mu\text{g}/\text{mL}$ crude extracts versus 7.33 ± 1.53 and 2.67 ± 0.58 for 10 and 15 $\mu\text{g}/\text{mL}$ glucantime (standard drug), respectively. The leishmanicidal activity of *L. trilobum* fruits may be due to effective compounds such as alpha-pinene and β -caryophyllene. For example, β -caryophyllene induces apoptosis via ROS-mediated MAPKs and suppression of AKT/PI3K/mTOR/S6K1 pathways (24). Therefore, this study revealed that inhibition dose ranges of 1000 - 1500 $\mu\text{g}/\text{mL}$ from *L. trilobum* hydroalcoholic crude extract were as effective on the tested intra-macrophage *L. major* as glucantime. This suggests the plant extract could be a potential choice when considering the adverse effects of glucantime. However, even if the studies are very promising, these are only in vitro, and further insights and clinical trials are required for future human application. The hydroalcoholic extraction method is a safe and effective process for crude herbal extraction; however, the major limitation of this work remains due to the lack of phytochemical characterization of the extract.

5.1. Conclusions

The percentage of infected macrophages and the number of amastigotes in macrophages significantly declined at concentrations of 1000 - 1500 $\mu\text{g}/\text{mL}$ over 24 to 72 h compared to control (no treatment) and 5 $\mu\text{g}/\text{mL}$ glucantime (reference drug) groups. However, the effect of 1250 and 1500 $\mu\text{g}/\text{mL}$ of crude extract on the amastigote forms in treated macrophages had equal leishmanicidal effects as 10 and 15 $\mu\text{g}/\text{mL}$ glucantime, respectively. Considering the absence or very low cytotoxicity on mammalian cells and remarkable leishmanicidal effects, it seems that *L. trilobum* can be proposed as a natural potential compound for leishmanicidal activity. Nevertheless, supplementary investigations will be necessary to achieve these findings in laboratory animal and human subjects.

Footnotes

Authors' Contribution: All authors contributed equally the same in this article.

Conflict of Interests Statement: The authors declare no conflict of interest.

Data Availability: The dataset presented in the study is available on request from the corresponding author.

Ethical Approval: This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.RETECH.REC.1404.076).

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