In Vitro and In Vivo Antileishmanial Activities of Pistacia vera Essential Oil

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Abstract
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This study aims to evaluate the *in vitro* and *in vivo* antileishmanial activities of *Pistacia vera* essential oil and compare their efficacy with a reference drug, meglumine antimoniate (Glucantime®). This essential oil (0–100 µg/mL) was evaluated *in vitro* against the intracellular amastigote forms of *Leishmania tropica* (MHOM/IR/2002/Mash2) and then tested on cutaneous leishmaniasis of male BALB/c mice by *Leishmania major* (MRHO/IR/75/ER). In the *in vitro* assay, it could be observed that *P. vera* essential oil significantly ($p < 0.05$) inhibited the growth rate of amastigote forms (IC_{50} of 21.3 ± 2.1 µg/mL) in a dose-dependent response compared with the control drug. Meglumine antimoniate also demonstrated antileishmanial effects with an IC_{50} value of 44.6 ± 2.5 µg/mL for this clinical stage. In the *in vivo* assay, the results indicated that 30 mg/mL of the essential oil had potent suppression effects on cutaneous leishmaniasis in BALB/c mice (87.5% recovery), while 10 and 20 mg/mL of the essential oil represented the suppression effects as weak to intermediate. The mean diameter of the lesions decreased about 0.11 and 0.27 cm after the treatment of the subgroups with the essential oil concentrations of 10 and 20 mg/mL, respectively. In contrast, in the subgroup treated with the essential oil concentration of 30 mg/mL, the mean diameter of the lesions decreased about 0.56 cm. In the control subgroups, the mean diameter of the lesions increased to 1.01 cm. The main components of *P. vera* essential oil were limonene (26.21%), α-pinene (18.07%), and α-thujene (9.31%). It was also found that *P. vera* essential oil had no significant cytotoxic effect on J774 cells. The present study found that *P. vera* essential oil showed considerable *in vitro* and *in vivo* effectiveness against *L. tropica* and *L. major* compared to the reference drug. These findings also provided the scientific evidence that natural plants could be used in traditional medicine for the prevention and treatment of cutaneous leishmaniasis.

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Introduction
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Cutaneous leishmaniasis (CL) is a protozoan infection caused by protozoa of the genus *Leishmania*. This disease is characterized by chronic skin lesions and leaves permanent scars as the deformation of the infected area [1]. CL is a public health problem at a global level affecting 1.5 million people annually worldwide [1]. In Iran, the principal pathogenic species of CL are *Leishmania tropica* and *Leishmania major* [2]. The current first-line chemotherapy of leishmaniasis with antimonial drugs such as meglumine antimoniate and sodium stibogluconate is a challenge due to having the problems of emerging resistance, severe side effects, or high costs [3]. Moreover, the second-line drugs have some limitations for use because of the prolonged length of therapy as well as adverse reactions [4]. Since vaccines are not yet available for CL, the maintenance and improvement of the existing treatment regimens and discovery initiatives have been found to be the only ways of controlling this important tropical disease [5]. For decades plant extracts and plant-derived compounds, due to having few side effects, low cost, and high availability, have been valuable sources that are commonly used to treat a wide range of disease conditions including infectious diseases [6]. The genus *Pistacia* belongs to the family Anacardiaceae. Among the 15 known species of pistachios, only three species grow in Iran, which include *Pistacia vera* L., *Pistacia khinjuk* Stocks ex Stocks, and *Pistacia atlantica* Desf. [7]. *P. vera* is the only species of the genus cultivated commercially and the rest of the species are mostly used as rootstocks for *P. vera* [8]. In folk

Table 1 Essential oil composition of *P. vera* identified by GC/MS.

No	Components	RI[a]	% Composition
1.	Tricyclene	956	1.24
2.	α-Thujene	973	9.3
3.	α-Pinene	987	18.1
4.	Camphene	993	4.4
5.	Sabinene	1017	0.6
6.	β-Pinene	1026	3.1
7.	β-Myrcene	1031	1.9
8.	α-Terpinene	1035	1.6
9.	Δ-2-Carene	1060	2.8
10.	p-Cymene	1072	0.7
11.	Limonene	1097	26.2
12.	γ-Terpinene	1101	2.1
13.	α-Terpinolene	1179	9.3
14.	1,8-Menthadien-5-ol	1184	1.1
15.	3-Cyclohexen	1196	1.3
16.	α-Terpineol	1199	0.6
17.	Borneol	1234	7.5
18.	Thujopsene	1288	0.4
19.	β-Himachalene	1297	0.7
20.	Cembrene	1354	0.9
21.	E-Caryophyllene	1428	2.1
22.	Bis(2-ethylhexyl) phthalate	1448	0.6
23.	1,13-Teridecadiene	1498	0.6
24.	delta-Cadinene	1581	0.6
25.	Cyclohexadecane	1587	0.25
	Total		97.93

[a] Retention indices on nonpolar DB-5 ms column in reference to *n*-alkanes

Table 2 The IC50 and CC50 values and SI determined for *P. vera* essential oil and for meglumine antimoniate. The antileishmanial activity was determined against intracellular amastigotes of *L. tropica*; the cytotoxicity was determined on J7774 cells.

SI[a]	CC_{50} (µg/mL) on J774 cells (± SD)	IC_{50} (µg/mL) on amastigotes (± SD)	Tested material
10.1	216.3 ± 8.15	21.3 ± 2.1	Essential oil
27.5	1225.6 ± 11.1	44.6 ± 2.5	Meglumine antimoniate

[a] CC_{50} for J774 cells/IC_{50} for amastigote forms of *L. tropica*

Table 3 Inhibition of the infection in macrophage cells after treatment of *L. tropica* promastigotes with *P. vera* essential oil. Data are expressed as the mean ± SD (n = 3).

Infectiveness reduction	Percentage of infected macrophages	Promastigotes
–	84.1 ± 3.5	Non-treated
77 ± 3.1	19.3 ± 1.2	Treated with essential oil (5 µg/mL)

Table 4 Comparison of NO production in macrophage cells after treatment with various concentrations of *P. vera* essential oil.

Production of nitric oxide (nM)	Concentration
16	3.125
9.3	6.25
6	12.5
11	Non-treated

medicine, different parts of *P. vera* including resin, leave, fruit, and aerial parts are traditionally used for analgesic, carminative, astringent, stomachic, aphrodisiac, antitussive, diuretic, and expectorant purposes [7]. Reviews have also reported that *P. vera* has various biological activities such as anti-inflammatory, antinociceptive, antiatherogenic, and hypoglycemic ones [9–11]. Moreover, in several studies, antibacterial, antiviral, antifungal, and antiparasitic activities of this plant against several pathogenic strains have been proven [12, 13]. The major chemical constituents of *P. vera* essential oil are hydrocarbon and oxygenated monoterpens such as α-pinene [14]. However, some factors such as plant species and part, cultivar sex, geographical origin, harvesting time, and climatic conditions could affect the chemical composition and functional activity of this plant [15]. The present study aimed to evaluate the chemical composition of *P. vera* essential oil and investigate its antileishmanial activities against *L. tropica* and *L. major* in *in vitro* and *in vitro* models, respectively.

Results
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⊙**Table 1** shows the results obtained by gas chromatography/mass spectrometry (GC/MS) analysis of *P. vera* essential oil. Twenty-five compounds were identified, which represented 97.9% of the total oil. The main components were limonene (26.2%), α-pinene (18.1%), α-thujene (9.3%), and α-terpinolene (9.3%).
The findings demonstrated that *P. vera* essential oil inhibited the growth rate of intramacrophage amastigotes of *L. tropica* as a dose-dependent response. The obtained IC_{50} values were 21.3 ±

2.05 and 44.6 ± 2.5 µg/mL for the *P. vera* essential oil and meglumine antimoniate, respectively (⊙**Table 2**). The findings demonstrated that the promastigote forms of *L. tropica* with no drugs were able to infect 84.1% of the macrophage cells, while promastigotes treated with *P. vera* essential oil had a potency to infect only 19.3% of the macrophages cells (⊙**Table 3**). These results indicate that the infectivity of promastigotes of *L. tropica* is significantly ($p < 0.05$) reduced after incubation with *P. vera* essential oil. The findings also exhibited that the essential oil of *P. vera* triggered the production of 16 µM of nitric oxide (NO) at the low concentration of 3.125 µg/mL compared to 11 µM for the untreated macrophages. In contrast, the essential oil at a higher concentration (≥ 6.25 µg/mL) reduced the production of NO to 6 µM compared to the untreated macrophages (⊙**Table 4**). Moreover, the *in vitro* assay demonstrated that *P. vera* essential oil had no significant cytotoxicity in J774 cells. The cytotoxic concentration for 50% of the cells (CC_{50}) value of the essential oil was 216.3 ± 8.15 µg/mL. In addition, the selectivity index (SI) of greater than 10 for *P. vera* essential oil represented its safety to the macrophages and specificity to the parasite (⊙**Table 2**).
The findings demonstrated that in the infected mice treated with 30 mg/mL of *P. vera* essential oil, the number of parasites was significantly ($p < 0.05$) decreased compared to the untreated mice, while 10 and 20 mg/mL of the essential oil reduced the number of parasites in a dose-dependent manner (⊙**Fig. 1**). The untreated group did not show any decrease in the number of parasites. After 30 days of treatment, 87.5% recovery (seven cases) was observed in the cases treated with 30 mg/mL essential oil,
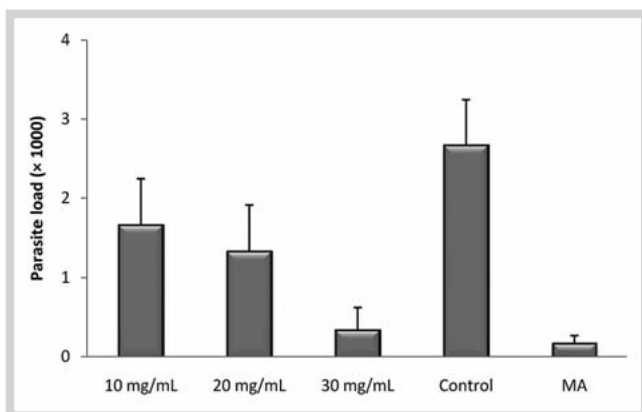
Fig. 1 Comparison of mean number of parasites (parasite load) in infected mice after treatment with various concentrations of *P. vera* essential oil.
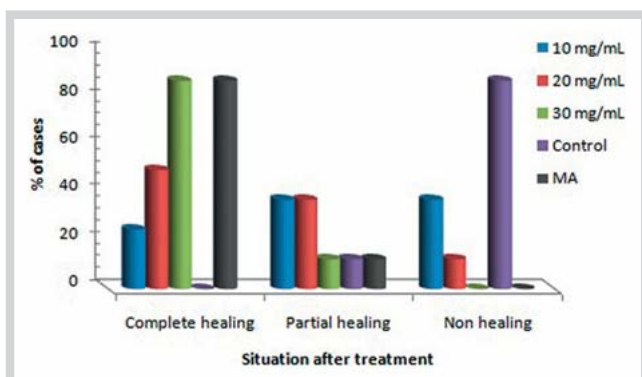

Fig. 2 Comparison of the healing rate of lesions in infected mice after treatment with various concentrations of *P. vera* essential oil. (Color figure available online only.)

whereas with 10 and 20 mg/mL of *P. vera* essential oil, 25 and 50% recovery was observed, respectively (○ **Fig. 2**). The mean diameter of the lesions decreased by 0.11 and 0.27 cm after the treatment of the groups with concentrations of 10 and 20 mg/mL of *P. vera* essential oil, respectively. In the group treated with *P. vera* essential oil at the concentration of 30 mg/mL, the mean diameter of the lesions decreased by 0.56 cm, while in the untreated group, the mean diameter of the lesions increased by 1.01 cm (○ **Table 5**).

Discussion
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Natural products, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity [16]. According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary health care needs. In the past decades, the advent of synthetic antimicrobial drugs has caused reluctance in plants as a rich resource of antimicrobial agents [17]. However, in recent years, the emergence of some limitations in the use of these drugs has caused changes in the situation of and interest

in the field of ethnobotanical research [18]. In this study, the *in vitro* and *in vivo* antileishmanial effects of *P. vera* essential oil against *L. tropica* and *L. major* were investigated. In the *in vitro* assay, *P. vera* essential oil significantly reduced the mean infection rate and subsequently the viability of amastigote forms of *L. tropica* in the macrophages. In line with the present work, Orhan et al. [12] reported that *P. vera* branch extract with a 4.8 µg/mL concentration significantly inhibited (77.3%) the growth of *L. donovani*, whereas the dry leaf extract (PV-DL) was active against *Plasmodium falciparum* (60.6% inhibition). They also indicated that the IC_{50} values of these extracts were 2.3 µg/mL for the amastigotes of *L. donovani* grown in axenic culture and 3.65 µg/mL for *P. falciparum* [12]. Of course, it should be noted that this difference in susceptibility of amastigote stages between the present study and the study conducted by Orhan et al. [12] might be due to the use of extracellular amastigotes of *L. donovani* grown in axenic culture, while in this investigation we used intramacrophage amastigotes.

In the *in vivo* assay, the results demonstrated that 30 mg/mL of the essential oil had potent suppression effects on the CL in male BALB/c mice infected with *L. major* (87.5% recovery), while 10 and 20 mg/mL resulted in a weak and moderate suppression, respectively. Since NO produced in macrophages plays a pivotal role as a leishmanicidal agent, in this survey we evaluated NO production in macrophages exposed to various concentrations of *P. vera* essential oil. The results obtained showed that the essential oil of *P. vera* triggered the production of NO at the low concentration, but in higher concentrations, it reduced the production of NO compared to the untreated macrophages.

The phytochemical screening of *P. vera* essential oil showed the presence of terpenoids, phenols, and flavonoids in this plant [7]. Furthermore, the chemical composition of *P. vera* essential oil using GC/MS revealed that the main components were limonene (26.21%), α-pinene (18.07%), α-thujene (9.31%), and α-terpinolene (9.28%). Therefore, the plant analyzed in this research was a new chemotype of *P. vera.* It has been previously proven that the chemical composition of essential oils depends on species, climate, collection time, and growth stage, altering the studied biological activities [15].

So far, individual activities of these compounds have been demonstrated [17]. Furthermore, in various studies, potent antileishmanial activities of these compounds, such as terpenic derivates, carvacrol, *p*-cymene, thymol, carvone, limonene, and terpinene, have been demonstrated [19–22]. Therefore, phytoconstituents in these plants could be responsible for their antileishmanial activity although their exact mode of action is poorly understood. However, in the case of the antimicrobial mechanism of some terpenoid compounds such as monoterpens, the main component of *P. vera*, Sikkema et al. [23] revealed that they are diffused into pathogens and damage cell membrane structures. On the other hand, other reports have suggested that the antimicrobial activity is related to the ability of terpenes in affecting not only permeability but also other functions of cell membranes; these compounds might cross the cell membranes, penetrate into the interior of the cell, and interact with critical intracellular sites [24, 25].

In the case of cytotoxic effects, it was found that *P. vera* essential oil had no cytotoxic effects on J774 cells. The SI value ≥ 10 of the essential oil showed its safety to the macrophages and specificity to parasites [26]. Similar to the present findings, Orhan et al. [12] showed that *P. vera* extract did not exert any cytotoxic effect on mammalian L6 cells, even at very high concentrations. Therefore,

p value	Size of lesions (cm) after treatment (30 days) (± SD)	Size of lesions (cm) before treatment (± SD)	Concentrations (mg/mL)	
< 0.05	1.16 ± 0.3	1.27 ± 0.3	10	
< 0.05	1.04 ± 0.2	1.31 ± 0.3	20	Essential oil
< 0.05	0.77 ± 0.2	1.33 ± 0.3	30	
< 0.05	0.42 ± 0.1	1.37 ± 0.2	30	Meglumine antimoniate
–	2.40 ± 0.2	1.39 ± 0.25	–	Control

Table 5 Effects of *P. vera* essential oil on the size of lesions in BALB/c mice infected by *L. major*.

it can be suggested that *P. vera* essential oil is safe for mammalian cells.

To conclude, the present study found that *P. vera* essential oil showed *in vitro* and *in vivo* effectiveness against *L. tropica* and *L. major*, respectively. The findings of this study also provide scientific evidence that natural plants could be used in traditional medicine for the prevention and treatment of CL.

Materials and Methods
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Chemicals
Meglumine antimoniate (Glucantime®, 99% purity), used as the control drug (1.5 g/5 mL) of antileishmanial activity, was purchased from Aventis. Penicillin and streptomycin were obtained from Alborz Pharmacy and were stored at room temperature (25 °C) until testing. MTT powder [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide)], fetal calf serum (FCS), RPMI-1640 medium with L-glutamine, and Griess reagent (A and B) were purchased from Sigma-Aldrich. All of the other chemicals and solvents used were of the highest purity commercially available.

Collection of plant materials
The plant materials (branch) were collected from rural regions of Kerman province, south east of Iran, from May to September 2013. They were identified by a botanist (Dr. Sharififar) of the Botany Department of Shahid Bahonar University, Kerman, Iran. A voucher specimen of the plant materials was deposited at the Herbarium of Department of Pharmacognosy of School of Pharmacy, Kerman University of Medical Science, Iran (KF 1136). The collection of plant materials in this study was carried out on private land, and no specific permissions were required for this location. In addition, we confirmed that the present field study did not involve endangered or protected species.

Isolation of essential oil
One hundred g of powdered plant materials (branch) were subjected to hydrodistillation for 3 h using an all-glass clevenger-type apparatus. The essential oil obtained was dried over anhydrous sodium sulfate and stored in darkness at 4 °C in airtight glass vials closed under nitrogen gas until testing [27].

Drug dilutions
To prepare the dilutions of the *P. vera* essential oil, 0.1 mL of the essential oil was dissolved in 0.97 mL of normal saline. In addition, to enhance the dispersal of the essential oil in normal saline, 0.03 mL of Tween 20 was added to the test tube. The resulting solution was mixed adequately by a magnetic stirrer. Serial dilutions were carried out to obtain the essential oil at concentrations of 3.125 to 100 µg/mL. The selection of dilutions of *P. vera*

essential oil were based on initial experiments, which also showed that normal saline plus Tween 20 did not cause an effect on the growth of parasites.

Gas chromatography/mass spectrometry analysis of essential oil
In this study, GC analysis was carried out by a Hewlett-Packard 6890 with an HP-5MS column (30 mm × 0.25 mm, film thickness 0.25 mm). The column temperature was maintained at 55 °C for 3 min and programmed to 180 °C at a rate of 5 °C per min, and kept constant at 220 °C for 5 min. Injector and interface temperatures were 220 °C and 290 °C, respectively. The flow rate of helium as a carrier gas was 1 mL/min C. F. The percentages were calculated by electronic integration of FID peak areas without the use of response factors correction. Linear retention indices for all components were determined by coinjection of the samples with a solution containing a homologous series of C8-C22 *n*-alkanes. GC/MS analysis was performed using a Thermoquest-Finnigan gas chromatograph equipped with fused silica capillary DB-5 column (30 mm × 0.25 mm, film thickness 0.25 mm) coupled with a TRACE mass. Helium was used as a carrier gas with an ionization voltage of 70 eV. Ion source and interface temperatures were 220 °C and 290 °C, respectively. The mass range was from 40 to 400 u. The oven temperature program was the same as above for the GC.

Identification of the essential oil components
The components of the essential oil were identified by comparison of their retention indices and mass spectra with those of the standards of the Wiley 2001 library data of the GC/MS system or with those reported in the literature [28].

Parasite and cell culture
Reference strains of *L. tropica* (MHOM/IR/2002/Mash2) and *L. major* (MRHO/IR/75/ER) were kindly provided by the Center for Research and Training in Skin Diseases and Leprosy (Tehran, Iran). The parasites were cultured in NNN medium and subcultured in RPMI 1640 supplemented with penicillin (200 IU/mL), streptomycin (100 µg/mL), and 15% heat-inactivated FCS. The murine macrophage cell line (J774-A1) was obtained from the Pasteur Institute of Iran (Tehran, Iran). The cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 37 °C in 5% CO_2.

In vitro antileishmanial activity
Anti-amastigote assay: The intracellular amastigote assay with the *P. vera* essential oil was carried out according to the method described elsewhere [29]. Briefly, before adding the macrophages to the plates, 1 cm² cover slips were placed in the wells of 6-chamber slides (Lab-Tek, Nalge Nunc International). In the next step, 200 µL of macrophage cells (10^5/mL) were incubated at 37 °

C in 5% CO_2 for 2 h in DMEM. Then, 200 uL of *L. tropica* promastigotes (10^6/mL) in the stationary phase were added to the murine macrophages so that proportion of *Leishmania*/macrophage was 10 : 1 and were incubated again in a similar condition for 24 h. Free parasites were removed by washing with RPMI 1640 medium and the infected macrophages were treated with 50 μL of various concentrations of essential oil (0–100 μg/mL) at 37 °C in 5% CO_2 for 72 h. At the end, the dried slides were fixed with methanol, stained by Giemsa, and observed under a light microscope. Also, the macrophages containing amastigotes without extract and those with no parasite and extract were considered positive and negative controls, respectively. Activity of the essential oil on intracellular amastigotes was evaluated by counting the number of amastigotes in each macrophage by examining 100 macrophages in comparison with those obtained with the positive control. The 50% inhibitory concentrations (IC_{50} values) were measured for all of the tested drugs by the Probit test in SPSS software. All of the tests were performed in triplicate.

Inhibition of infection in macrophage cells: To determine the inhibitory effect of *P. vera* essential oil against the promastigote invasion of macrophages, promastigotes of *L. tropica* (10^6/mL) were preincubated in essential oil (5 μg/mL) for 2 h at room temperature (the selection of 5 μg/mL was based on the initial experiments which showed no significant effect on viability of promastigote cells). Then, promastigotes were washed with RPMI-1640 medium and incubated with murine macrophages (J774 cells) for 4 h. After washing the cells again, the macrophages were stained by Giemsa and studied by a light microscope to evaluate the frequency of infection by counting 100 macrophages [30]. All of the tests were performed in triplicate.

Nitric oxide production determination: NO release in the supernatants of the macrophage culture was measured by the Griess reaction for nitrites. The supernatants (100 μL) were collected 72 h after introducing the *P. vera* essential oil into the culture medium. The assay was done in triplicate wells in a 96-well tissue culture plate. To this, 60 μL of Griess reagent A and then 60 μL of Griess reagent B were added. The plates were read at 540 nm in an ELISA plate reader (BioTek-ELX800).

Cytotoxic effects: Cytotoxicity effects of *P. vera* essential oil against J774-A1 cells were evaluated by cultivating macrophages (5 × 10^5) with various concentrations of essential oil (0 to 500 μg/mL) in 96-well tissue culture plates at 37 °C in 5% CO_2 for 48 h. Cell viability was determined by the colorimetric MTT assay and the results are displayed as the percentage of dead cells compared to non-treated macrophages (100% of viability). Moreover, CC_{50} was calculated by the Probit test in SPSS software [31].

Selectivity index: SI, calculated based on the equation of CC_{50} for murine macrophage cells/IC_{50} for amastigote forms of *L. tropica*, was used to compare toxicity and activity of *P. vera* essential oil as described by Weniger et al. [26].

In vivo antileishmanial activity

Ethical statement: This study was carried out in strict accordance with the recommendations of the Guide for Care and Use of Laboratory Animals of National Institutes of Health. The protocol was approved by the Committee on Ethics of Animal Experiments, Lorestan University of Medical Sciences (Permit number: 91/27, 2013).

Animals: Forty male BALB/c mice (6–8 weeks old) were obtained from the Animal Breeding Stock Facility, Razi Institute of Iran (Karaj, Iran). They were housed in a colony room with a 12-h/ 12-h light/dark cycle at 21 ± 2 °C and handled according to the standard protocols for the use of laboratory animals [32].

Induction of cutaneous leishmaniasis in BALB/c mice: In this study, male BALB/c mice were infected subcutaneously with 0.1 mL of promastigotes of *L. major* (2×10^6 cells/mL) harvested from the stationary phase at the base of the tail [33]. The mice were randomly divided into five groups and each group contained eight mice.

Treatment of infected mice: The infected mice were treated after 5 weeks when leishmanial lesions appeared. At first, the diameter of the lesions was measured before the treatment. Then, the lotion of essential oil with concentrations of 10, 20, and 30 mg/mL was applied by rubbing to each tested group on a daily basis for 30 days. The positive control group received meglumine antimoniate (30 mg/kg) as an intralesional injection. Before and after the treatment, the diameter of the lesions was measured (lesion borders) using a vernier caliperand to determine the number of parasites. Impression smears were prepared from the lesions, dried in air, fixed by methanol, and stained with Giemsa to specify the load of parasites by light microscopy [34].

Statistical analysis

Data analysis was carried out using SPSS statistical package version 17.0 (SPSS, Inc.). Differences between the test and control groups were analyzed by a t-test. In addition, $p < 0.05$ was considered statistically significant.

Acknowledgments
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Conflict of Interest
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The authors declare no conflict of interest

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