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Antileishmanial, antioxidant, and cytotoxic activities of *Quercus infectoria* Olivier extract

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ABSTRACT

Currently, there is no effective vaccine available, and chemotherapy is the main approach for treatment of cutaneous leishmaniasis (CL). During recent decades, studies have demonstrated that a number of plant-derived compounds may act as new therapeutic tools against leishmaniasis. This study was evaluated the antileishmanial, antioxidant, and cytotoxic activities of *Quercus infectoria* Olivier (oak) extract. The total amount of phenolic and flavonoid compounds was measured in oak extract. High performance liquid chromatography (HPLC) analysis was also performed to determine the amount of quercetin and gallic acid in this plant. This extract (0–80 g/mL) was evaluated *in vitro* against promastigote and intracellular amastigote forms of *Leishmania major* (MRHO/IR/75/ER) using MTT assay and in a macro-phage model, respectively. Then oak extract was tested on CL in infected male BALB/c mice with *L. major* in order to evaluate the antileishmanial activity topically. Moreover, cytotoxicity effects of oak in murine macrophage cells were tested by MTT assay. Antioxidative activity of oak was also determined by the 2,2-diphenyl-1,1-picrylhydrazyl (DPPH) scavenging test. The amount of phenolic and flavonoid compounds in the oak extract was 57.50 and 1.86%, respectively. The amount of quercetin and gallic acid in the oak extract was 0.0064 and 0.22%, respectively. The findings revealed that oak significantly ($P < 0.05$) inhibited the growth rate of promastigote of (IC_{50} 12.65 μg/mL) and amastigotes (IC_{50} 10.31 μg/mL) as a dose-dependent response. In the *in vivo* assay, after 4 weeks of treatment, 91.6, 66.66, and 50% recovery was observed in the infected mice treated with 20, 10, and 5 mg/kg of oak extract, respectively. After treatment of the infected mice with the concentration of 10 and 20 mg/kg of oak, the mean diameter of lesions, parasite load and mean number of parasites was significantly ($P < 0.05$) reduced. Selectivity index of greater than 10 for oak revealed that oak extract had no cytotoxic effects on macrophage cells. Moreover, DPPH test demonstrated that radical inhibition occurred at greater power with increasing the concentration of oak. To conclude, the present study showed potent antileishmanial and antioxidant activity of oak extract; whereas this plant had no toxic effect on mammalian cells.

1. Introduction

Leishmaniasis is a prevalent parasitic disease found in 98 countries in 5 continents, which causes 20,000 to 40,000 deaths per year [58]. This disease has three main types of manifestations including visceral, cutaneous, and mucocutaneous leishmaniasis. Cutaneous leishmaniasis (CL) as the most common form of leishmaniasis has the occurrence rate of 0.7–1.2 million cases per year [10]. The most affected countries include Afghanistan, Algeria, Iran, and Brazil.

There are several reports of different parasitic infections including leishmaniasis in Iran [2,23,24,30]. CL is usually characterized by chronic skin lesions and permanent scars of leaves as the deformation of the infected area [27]. Currently, there is no effective vaccine available, and chemotherapy is the main approach for the treatment of CL [31]. Several researches on new therapeutic approaches such as molecular methods and the herbal medicine effect in the treatment of leishmaniasis have been conducted

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[22,26,29,35,49]. Pentavalent antimonial compounds such as meglumine antimoniate and sodium stibogluconate are the first-line antileishmanial agents that have been used clinically since last decades [13]. Treatment of CL by antimonial drugs has some limitations in use, due to the need for intramuscular administration and long treatment periods, side effects, and emergence of resistant cases [8,47]. Furthermore, second-line drugs such as amphotericin B and pentamidine can be toxic and expensive [48]. Therefore, new alternative treatments to provide safe, cheap, and effective antileishmanial agents are urgently needed.

During recent decades, studies have demonstrated that a number of plant-derived compounds may act as new therapeutic tools against parasites such as leishmaniasis [6,25,37,44]. Oak plant with the scientific name of *Quercus*, a shrub that grows in Asia Minor, Iran, and Greece, is one of the popular medicinal plants which has been traditionally used [9,18]. Genus of *Quercus* belongs to the Fagaceae family which has more than 45 species, among which *Quercus infectoria* Olivier is highly distributed in Zagros Mountains, West of Iran [18]. Different parts of oak are known to have multiple therapeutic properties and are used widely in several folk medicine as an analgesic CNS depressant, antiparkinsonian, antidiabetic, and anti-inflammatory drug [17]. Reviews have also related various antimicrobial properties including antibacterial [56], antiviral [16], and antifungal [3] to *Q. infectoria*. Oak consists of a large number of polyphenol compounds and tannins Sakar et al., 2005. However, some factors such as plant species and parts, cultivar sex, geographical origin, harvesting time, and climatic conditions could affect the constituents and functional activity of plants [45]. To the best knowledge of the present authors, there is no study on the antileishmanial effects of oak. Therefore, this study is aimed to evaluate *in vitro* and *in vivo* antileishmanial activities of oak fruit hull (Jaft) extract and compare its adequacy with Glucantime as the reference drug. In addition, as the second objective of this study, antioxidant and cytotoxic effects of this plant are assessed.

2. Materials and methods

2.1. Ethical statement

This study was carried out in strict accordance with the recommendations in Guide for the Care and Use of Laboratory Animals of National Institute of Health (publication no. 85-23, revised in 1985). The protocol was approved by Committee on the Ethics of Animal Experiments, Lorestan University of Medical Sciences (permit number: 89/6). Moreover, all the efforts were made to minimize suffering.

2.2. Chemicals

Meglumine antimoniate (Glucantime®, MA) as a control drug was purchased from Aventis, France. Penicillin and streptomycin were obtained from Alborz Pharmacy, Karaj, Iran, and stored at room temperature (25 °C) until testing. MTT powder [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide], and Schneider's medium were prepared from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI-1640 medium with L-glutamine were also purchased from Gibco- BRL, Gaithersburg, MD. All other chemicals and solvents had the highest commercially available purity.

2.3. Collection of plant materials

Oak fruit hulls were collected from the rural regions of Khorramabad district, Lorestan Province, west of Iran, in September 2013. They were identified by a botanist from Razi Herbal Medicine Research Center, Lorestan University of Medical Sciences (Khorramabad, Iran). A voucher specimen (RH 1165) of the plant materials was deposited at the herbarium of Razi Herbal Medicine Research Center, Khorramabad, Iran.

2.3.1. Preparation of hydroalcoholic extract

The dried materials of the plants were milled and extracted using methanol solvent and soxhlet extractor at 50 °C [32]. The obtained extract was condensed by vacuum rotary, completely dried under shadow, and stored at 4 °C for later use. The extraction efficiency was reported as 45%.

2.3.2. Determination of total phenolic compounds

The total amount of phenolic compounds was measured using Folin-Ciocalteu [51]. In this method, 500 μL of the extract with 2.5 mL Folin (Folin 0.2 N in 50% methanol) was mixed and incubated at room temperature for 5 min. Then, 2 mL of sodium carbonate (75 g/L) in water was added. After 1 h incubation, the absorption of the samples against the blank (water) was measured at 765 nm.

2.3.3. Determination of total flavonoid compounds

The total amount of flavonoid compounds was measured by Dowd method [1]. In this method, 4 mL of oak extract was mixed with 4 mL of AlCl3 2% in methanol and incubated for 15 min at room temperature. Then, the absorption of the samples was measured at 415 nm. The blank was 4 mL of methanol in 4 mL of oak extract without AlCl3.

2.3.4. High performance liquid chromatography (HPLC) analysis

HPLC device, model Shimadzu (SCL-10AVP), with C-18 column, model Wakosil II 5C18R, with the length of 24 cm, diameter of 6.4 mm, filler particle size of 5 μm, and a protective column with the length of 1 cm was used to analyze the actual sample. This device was equipped with a reciprocating pump, an oven, a continuous degassing device, a sample loop with the size of 20 μm, and a UV/Visible detector, model SPD-10 AVP. Class-VP V.R 6.1 was used to control the HPLC device and process the data. A 100 μL micro-syringe, made by Hamilton Company, was used to collect the sample from the container and inject it into the device. Quercetin was detected using HPLC device by isocratic elution program with methanol, isopropanol solvent, and 20 mM phosphate buffer with pH equal to 2 with the ration of (v:v:v) 70:10:20. The UV detector was set at 380 nm. The chromatograms were run at the flow rate of 0.7 mL/min. Also, gallic acid was detected using HPLC device by isocratic elution program with methanol solvent, water, and phosphoric acid with the ration of (v:v:v) 20:79.9:0.1. The UV detector was set at 210 nm. The chromatograms were run at the flow rate of 1 mL/min [41].

2.4. Antioxidant activity

In order to perform the experiment, 0.3 mL with different concentrations of solutions containing the extract and butyl hydroxy tuloene standard (BHT) anti-oxidant was separately poured into the tubes and 2.7 mL of 2,2-diphenyl-1,1-picrylhydrazyl (DPPH) methanol solution (6×10^{-5} M) was added to each tube. The resulting solution was stirred in a continuous shaker device for 60 min under darkness. Then, using a spectrophotometer, the absorptivity of the solution was measured at 517 nm. The free radical scavenging activity of DPPH was calculated according to the following equation [52]:

$$\mathrm{RSA}(\%) = 100 \times \left(\frac{A_{\mathrm{blank}} - A_{\mathrm{sample}}}{A_{\mathrm{blank}}}\right)$$

Then, a concentration that inhibited 50% of the free radicals (IC_{50}) was calculated. It is clear that the smaller the resulting number, the higher the inhibiting property of the anti-oxidative capacity or free-radicals would be.

2.5. In vitro *antileishmanial effects of oak extract*

2.5.1. Parasite

L. major (MRHO/IR/75/ER) was kindly obtained from Center for Research and Training in Skin Diseases and Leprosy (Tehran, Iran). The parasites were cultured at 26 ± 1 °C in NNN medium and sub-cultured in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycine.

2.5.2. Isolation of murine macrophage cells

Murine macrophages were collected from male BALB/c mice (6–8 weeks old) by injecting 2–5 mL of cold RPMI-1640 medium into mouse peritoneal cavity. Then, the aspirated macrophages were washed twice and resuspended in the RPMI 1640 medium [33].

2.5.3. Activity of antipromastigotes

Antipromastigote effects of oak extract on the promastigote stage of *L. major* by colorimetric cell viability MTT assay were performed according to the method described previously with some modification [45]. MTT stock solution was prepared with the concentration of 5 mg/mL in phosphate-saline buffer and kept in the dark at 4 °C for 2 weeks. Briefly, 100 μL of the promastigotes of both species (2×10^6 cell/mL) harvested from logarithmic growth phase was added to a 96-well tissue culture plate. Then, 100 μL of various concentrations of oak extract (0–80 μg/mL) and MA (0–125 μg/mL) as control drug was added to each well and incubated at 25 ± 1 °C for 72 h. After the incubation, the supernatants were discarded by a centrifuging plate in 2000 rpm for 5 min. Also, 100 μL of $RPMI_{1640}$ culture medium lacking phenol red ($RPMI\text{-}PR^-$) and FBS which included 10 μL MTT (5 mg/mL) was added to each well. The plates were incubated for 4 h at 22 °C in the dark. Then, the plates were centrifuged again for 5 min at 2000 rpm and their supernatants were discarded. Also, 100 μL of dimethyl sulfoxide (DMSO) was added to each well as the solvent of formazan. The promastigotes were cultured in the complete medium with no drug as a positive control and the complete medium with no promastigotes and drugs as blank. Finally, absorbance was measured by an ELISA reader at 492 nm. Antipromastigote activity was determined as the 50% inhibitory concentrations (IC_{50}) using linear regression.

2.5.4. Effect on intramacrophage amastigotes

To determine the antiamastigote effects of oak extract, peritoneal macrophages were recovered, washed, and transferred to glass coverslips inserted in 6-well plates at 5×10^4 cell/well and allowed to adhere for 5 h at 37 °C in 5% CO_2. Nonadherent cells were removed and the plates were incubated overnight at $RPMI_{1640}$ supplemented with 10% FBS. After 24 h, adherent macrophages were infected with stationary phase promastigotes of *L. major* at the parasite/macrophage ratio of 10:1 and incubated in 5% CO_2 at 37 °C for 4 h. After 4 h of incubation, the free promastigotes were removed by washing with phosphate-buffered saline (PBS) (0.01 M). Then, $RPMI\text{-}_{1640}$ medium containing FBS 10%, 100 IU/mL penicillin, and 100 μg/mL streptomycin was added to each well and incubated overnight. No parasite was added to the group of plates, which was considered for the viability assessment of macrophage during the study. Treatment of the infected macrophages with oak extract (0–80 μg/mL) was done one day after the infection. MA (0–125 μg/mL) was also tested for its antileishmanial efficacy as the reference drug. The macrophages containing amastigotes without extract and those with no parasite and extract were considered positive and negative controls, respectively. All the tests were performed in three series: 24, 48, and 72 h after the treatment, the cover slips were fixed in methanol and stained with Giemsa [34]. The number of infected macrophages and number of amastigotes per infected macrophage were determined by counting at least 200 macrophages in triplicate cultures. IC_{50} values of the extract and MA were calculated.

2.5.5. Inhibiting infection in peritoneal macrophages

To assess the inhibitory effect of oak extract on the invasion of *L. major* promastigotes into macrophages, the promastigotes were pre-incubated in the oak extract (2.5 μg/mL) for 2 h at room temperature. The oak extract dilution was selected based on the initial experiments. Then, the promastigotes were washed with RPMI 1640 medium and incubated with peritoneal macrophages in 5% CO_2 at 37 °C for 4 h. The cells were washed and incubated for 48 h again. Then, they were stained with Giemsa and studied using a light microscope to assess the percentages of the infected macrophages by counting 100 cells [36].

2.6. Cytotoxic effects

In order to assess cytotoxic activities on peritoneal macrophage, CC_{50} (cytotoxicity concentration for 50% of cells) of various concentrations of the oak extract was determined. In this method, 1×10^5 macrophages were incubated in 96-well flat-bottomed culture plates in 5% CO_2 at 37 °C for 24 h. Cytotoxicity on the macrophages was evaluated in the presence of increasing concentrations of oak extract (0–320 μg/mL) and (0–250 μg/mL). The plates were re-incubated for 72 h. In parallel, the control groups containing cultured macrophage without extract and drug were considered. MTT assay was performed as mentioned above. Absorption rate in the wells was measured by an ELISA reader at the wavelength of 570 nm [11]. CC_{50} was assessed using linear regression. Also, selectivity index (SI) was calculated based on the ratio of CC_{50} for peritoneal macrophage/IC_{50} for intramacrophage amastigotes, as described by Jamzed et al. [18].

2.7. In vivo *antileishmanial activity*

Eighty four male BALB/c mice (6–8 weeks old) were purchased from Pasteur Institute of Iran (Tehran, Iran). The animals were housed in a colony room with a 12: 12 h light/dark cycle at 21 ± 2 °C and handled according to the standard protocols for the use of laboratory animals [40]. The mice were infected subcutaneously with 0.1 mL (2×10^6 cells/mL) stationary phase promastigotes of *L. major* at the base of the tails [11]. After 6 weeks, when the lesions were developed at the site of parasite inoculation, the mice were randomly divided into 7 groups; so, every group had 12 mice.

Group 1: Non-infected and non-treated
Group 2: Infected, but non-treated (control group)
Group 3: Non-infected and treated with 20 mg/kg oak extract
Group 4: Treated with 20 mg/kg oak extract
Group 5: Treated with 10 mg/kg oak extract
Group 6: Treated with 5 mg/kg oak extract
Group 7: Treated with 60 mg/kg/day MA, intraperitoneally

The treatment with oak extract was topically applied to the lesions twice a day for 30 days.

2.7.1. Measurement of lesion size

The diameter of the lesions was measured by a metric caliper before the treatment and at weekly intervals for 4 weeks after the challenge. Measurement was performed at two diameters (D and d) at right angles to each other and the size (mm) was determined based on the formula: S = (D + d)/2 [39].

2.7.2. Microscopical examinations of lesions

Laboratory demonstration of the parasite load was done in the lesions by making stained smears at the end of the experimental period to confirm the clinical diagnosis. The lesions were cleaned with ethanol and punctured at the margins by a sterile lancet and the exudation material was smeared. The smears were dried in air, fixed by methanol, and stained with Giemsa in order to detect amastigotes by light microscopy. Grading of *Leishmania* parasites was obtained by average amastigote density using ×10 eyepiece and ×100 oil immersion lens as follows:

4+: 1–10 parasites/1 field,
3+: 1–10 parasites/10 fields,
2+: 1–10 parasites/100 fields,
1+: 1–10 parasite/1000 fields [28,42].

2.7.3. Assessment of parasite burden

In week 4 after the challenge, the number of live parasites at draining lymph nodes was quantified by limiting dilution assay (LDA) [55]. Briefly, the lymph nodes were aseptically removed, weighed, and then homogenized in 2 mL Schneider\s medium (Sigma, USA) supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Different serial dilutions of the lymph nodes were prepared in the same media, cultured in quadruplicate in sterile flat-bottom-96 well plates, and incubated at 25 ± 1 °C for 10 days. The motile and non-motile parasites (positive and negative wells, respectively) were determined by an inverted microscope. The number of viable parasite per mg of tissue was determined in the highest dilution which promastigotes grow using ELIDA software [53].

2.8. Statistical analysis

Data analysis was done using SPSS statistical package, version 16.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test was used to analyze the data. To assess the interaction of time and experimental group, repeated measures analysis test was used. Differences were significant when the *p*-value was lower than 0.05.

3. Results

3.1. Total phenolic and flavonoid compounds

The total amount of phenolic and flavonoid compounds were measured by Folin-Ciocalteu Dowd methods, respectively. The amount of phenolic and flavonoid compounds in the oak extract was 57.50 and 1.86%, respectively.

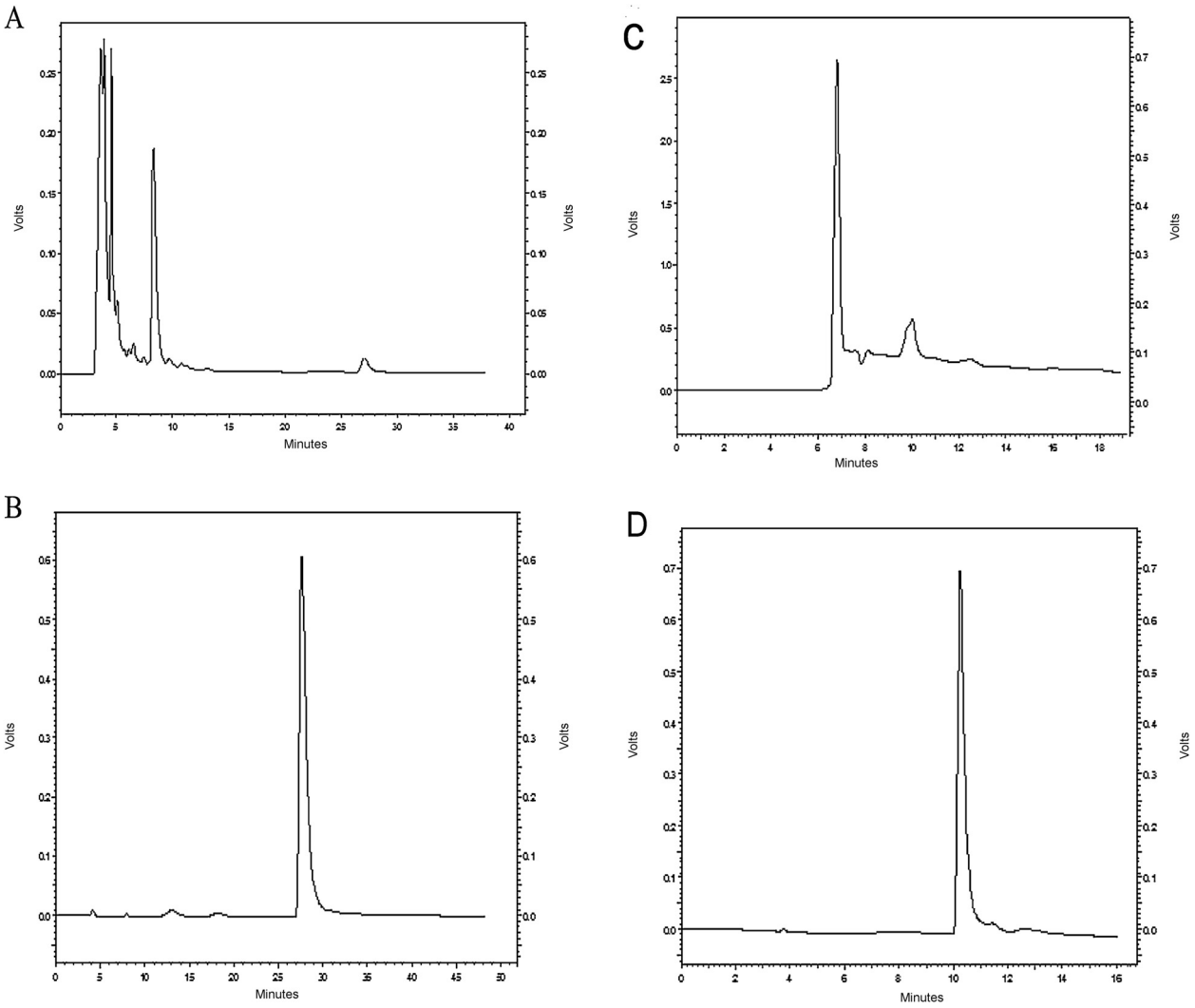

Fig. 1. HPLC analysis to determine the amount of quercetin and gallic acid in oak extract. Chromatograms of (A) oak extract and (B) quersitin standard solution; (C) oak extract and (D) gallic acid standard solution.

3.2. HPLC analysis

HPLC analysis was perfomed to determine the total amount of quercetin and gallic acid in the oak extract. The amount of quercetin and gallic acid in the oak extract was 0.0064 and 0.22%, respectively. Chromatograms related to the standard quercetin and gallic acid are shown in Fig. 1.

3.3. Antioxidant activity

The capability of the extract to inhibit the free radicals was assessed by DPPH test. In this test, radical inhibition occurred at greater power with increasing the concentration of the extract. Comparison of radical scavenging activity of extract with BHT is reported in Table 1. The IC_{50} values for extract and BHT were 30.78 μg/mL and 31.50 μg/mL, respectively.

3.4. Antipromastigotes activity

Results obtained in this study demonstrated that the oak extract had remarkable anti-promastigote activity based on a dose-dependent response ($P < 0.05$). The findings of MTT assay indicated significant difference between the intervention and control groups ($P < 0.001$). IC_{50} values for extract and MA were 12.65 μg/mL and 14.36 μg/mL, respectively. Overall, the results showed the effectiveness of oak extract for the *L. major* promastigotes.

3.5. Effect on infected macrophages and amastigotes

The findings revealed that the number of infected macrophages and the mean number of amastigotes per infected macrophage were significantly reduced in the groups receiving the extract ($P < 0.001$). The results also exhibited that, similar to promastigote stage, oak extract inhibited the growth rate of the infected macrophages and intramacrophage amastigotes as a dose dependent response ($P < 0.001$). IC_{50} values for extract and MA were 10.31 μg/mL and 11.48 μg/mL, respectively (Table 2).

3.6. Cytotoxic effect

Cytotoxic effect of oak extract on peritoneal macrophages was evaluated using MTT assay. Table 2 shows the CC_{50} value for oak extract and MA against peritoneal macrophages and, subsequently, their SIs for amastigote forms of *L. major*. CC_{50} values for extract and MA were 210.75 μg/mL and 106.81 μg/mL, respectively (Table 2). SI of greater than 10 for oak extract indicated safety to the macrophages and specificity to the parasite according to Weninger et al. [57].

Table 1
Antioxidant activity of oak extract using the free radical scavenging activity of DPPH.

Sample	Concentration (μg/mL)	RSA[a] (%)	IC_{50}(μg/ml)
Oak extract	10	33.87 ± 0.01	30.78
	20	46.55 ± 0.19	
	40	52.89 ± 0.17	
	80	62.21 ± 0.16	
	80	73.59 ± 0.1	
	320	76.91 ± 0.04	
	640	83.41 ± 0.01	
BHT[b]	10	32.21 ± 0.13	31.50
	20	38.76 ± 0.32	
	40	54.51 ± 0.19	
	80	66.94 ± 0.14	
	160	79.46 ± 0.01	
	320	82.30 ± 0.09	
	640	91.08 ± 0.00	

[a] Radical scavenging activity.
[b] Butyl hydroxy tuloene.

Table 2
IC_{50} values of oak leaf extract (OLE) and MA (meglumine antimoniate) against the growth rate of promastigotes and intramacrophage amastigote forms of *L. major*, and their cytotoxicity concentration for 50% of cells (CC_{50}) values on peritoneal macrophages and selectivity index (SI) against intramacrophage amastigote form of *L. major*. Data are expressed as the mean ± SD.

Sample	CC_{50} (μg/mL) Macrophage	IC_{50} (μg/mL)		SI[b]
		Promastigote	Amastigote	
Oak extract	210.75 ± 5.18	12.65 ± 0.73	10.31 ± 0.21	20.44
MA[a]	106.81 ± 6.17	14.36 ± 0.87	11.48 ± 1.34	9.30

[a] Meglumine antimoniate.
[b] Selectivity index (CC_{50} for macrophage/IC_{50} for amastigote form).

3.7. Inhibiting infection in peritoneal macrophages

Since infectivity is one of the most important pathogenic and biological criteria of Leishmania parasites. The results showed that, in the absence of drugs, 76.5% of the peritoneal macrophages was infected by *L. major* promastigotes. *L. major* promastigotes treated with oak extracts were able to infect only 33.2% of the peritoneal macrophages and its infectiveness reduction was 51.3%.

3.8. In vivo *antileishmanial effects*

The results of *in vivo* assay demonstrated that the infected mice treated with the oak extract at the concentrations of 10 and 20 mg/kg, number of parasites, and parasite load of pooled draining lymph nodes significantly ($P < 0.05$) decreased, while 5 mg/kg of oak extract decreased the number of parasites and parasite load intermediately. Furthermore, the results indicated that oak extract at the concentration of 20 mg/kg significantly ($P < 0.05$) decreased the mean number of parasites and parasite load compared with other groups and MA. Control group also had no decrease in the number of parasites. After the treatment of the groups with the concentrations of 5, 10, and 20 mg/kg of oak extract, the mean diameter of the lesions was reduced by 2.77, 4.08, and 7.75 cm, respectively. In contrast, in the group treated with MA, the mean diameter of the lesions was decreased to about 3.35 cm. In the control group, the mean diameter of the lesions was increased to about 5.9 cm (Table 3). After 4 weeks of treatment, 91.6, 66.6, 50, and 66.6% recovery were observed in the infected mice treated with oak extract at the concentrations of 20, 10, 5, and MA, respectively (Fig. 2). Table 4 shows the mean diameter of the lesions.

4. Discussion

According to the report by World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Plant-derived components and plant extracts are valuable sources in traditional medicine due to having less side-effects, low cost, and high availability and are mainly used to treat a wide range of disease conditions, including microbial infections [20,21,44]. Since the introduction of industrial and synthetic antimicrobial drugs in the last decades, lack of interest in plants as a natural and valuable source has been observed [7]. However, with the emergence of some limitations in the use of these agents, the situation has changed and field of ethnobotanical research has been considered.

Table 3
Effect of oak extract and MA (meglumine antimoniate) on the mean size of lesions (mm) in BALB/c mice infected by *L. major*.

Concentrations	Mean size of lesions (mm) before treatment	Mean size of lesions (mm) after 8 weeks of treatment	*P* value*
Control	8.24 ± 0.35	14.14 ± 0.22	<0.05
20 mg/kg	8.61 ± 0.15	0.86 ± 0.63	<0.05
10 mg/kg	8.28 ± 0.29	4.20 ± 0.27	<0.05
5 mg/kg	7.88 ± 0.39	5.11 ± 0.34	<0.05
MA	8.33 ± 0.22	4.98 ± 0.16	<0.05

[a]Meglumine antimoniate.
* The difference between beginning and end of the study was statically significant.

The obtained results of optical density (OD) and, consequently, IC_{50} values revealed that oak extract significantly inhibited the growth rate of promastigote forms of *L. major*. Oak extract also significantly reduced mean infection rate and, subsequently, the viability of amastigote forms in the macrophages compared with the control group. These findings indicated that amastigote forms were more susceptible to oak extract than promastigote forms. This difference in the susceptibility of promastigote and amastigote forms could be related to their structural, biochemical, and morphological features [35,37]. In the *in vivo* assay, we found that oak extract, especially at the concentration of 20 mg/kg, had potent suppression effects on CL in male BALB/c mice infected with *L. major* with 91.6% recovery; the extract at the concentration of 10 mg/kg also displayed the suppression effects (66.6%) similar to MA. Moreover, oak extract significantly reduced the number of parasites and parasite load of pooled draining lymph nodes in BALB/c mice infected with *L. major*.

Since infectivity is one of the most important pathogenic and biological criteria of *Leishmania* parasites, the effects of oak extract on the infectivity of the promastigotes of *L. major* to murine macrophages were evaluated. We found that, in the absence of any drugs, 76.5% of the peritoneal macrophages were infected by *L. major* promastigotes; however, *L. major* promastigotes treated with oak extracts were able to infect only 33.2% of the peritoneal macrophages and its infectiveness reduction was 51.3%.

So far, in various studies, antibacterial, antiviral, and antifungal properties of *Q. infectoria* have been reported. For example, Fredalina Basri et al. [4] showed potent antibacterial effect of *Q. infectoria* extracts on two Gram-positive bacteria (*Streptococcus mutans* and *Streptococcus salivarius*) and two Gram-negative bacteria (*Porphyromonas gingivalis* and *Fusobacterium nucleatum*) and concluded that *Q. infectoria* extracts can be considered effective phytotherapeutic agents for the prevention of oral pathogens.

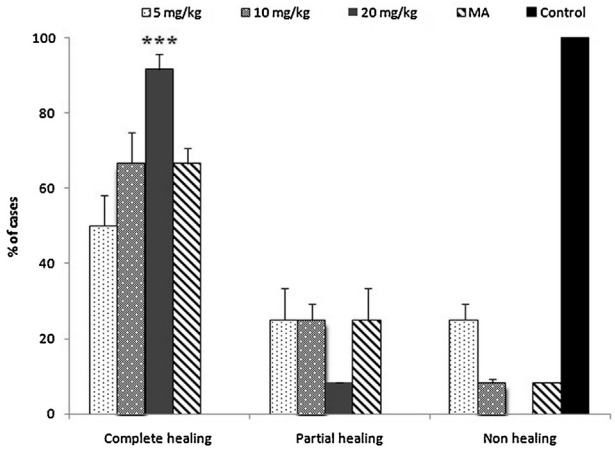

Fig. 2. Comparison of healing rate of lesions in infected male BALB/c mice with *L. major* after treatment with various concentrations of oak extract and MA (meglumine antimoniate). *** $P < 0.001$, the difference was statically significant.

Regarding the antiparasitic effects of *Q. infectoria*, there is no previous study on the antiparasitic activity of oak extract; therefore, the present work for the first time showed the antileishmanial effects of oak extract *in vivo* and *in vitro*. Reviews have reported that the main compounds of oak are polyphenolic compounds including tannins and gallic acid and flavonoids compounds such as quercetin [14]; individual activities of these compounds have been also proven [7]. However, some factors such as plant species and part, sex of cultivars, geographical origin, harvesting time, and climatic conditions could affect the concentrations of active components and functional activity of plants [59]. Consistent with previous studies, we found that the amount of phenolic compounds in the oak extract was 57.5%. Therefore, high amounts of phenolic compounds present in the extract of *Q. infectoria* implied that they might be the active compound responsible for the antileishmanial activity in this study. Various investigations have demonstrated that polyphenols potentially inhibit amastigote and promastigote forms of different species of *Leishmania* spp. [15,49]. Flavonoids are also broad classes of plant phenolics that have been shown to possess noticeable antiplasmodial, trypanocidal, and antileishmanial effects on *L. mexicana, L. donovani, and L. amazonensis* [15,38]. Sifaoui et al. [50] demonstrated that the relative leishmanicidal activity of 5 different Tunisian olive tree varieties against *L. tropica* and *L. major* was correlated with the content of phenols and flavonoids. In the present study, our results revealed that the amount of quercetin and gallic acid in the oak extract was 0.0064 and 0.22%, respectively. Regarding the antileishmanial activity of these components, Fonseca-Silva et al. [12] demonstrated that quercetin inhibited *L. amazonensis* promastigote growth in a dose- and time-dependent manner beginning at 48 h of treatment and with maximum growth inhibition observed at 96 h. Ribeiro et al. [43] showed that gallic acid had potent antileishmanial effects on the promastigotes of *L. amazonensis* with the IC_{50} value of 1.7 μg/mL. Therefore, phytoconstituents in this plant could be responsible for the antileishmanial activity of oak extract, whereas their exact mechanism of action is unclear. However, phenolic structures seem to produce its antimicrobial effect by damaging the membrane and/or disrupting cell peptidoglycans. Some researchers have also proposed it to be due to the presence of the orthodiphenolic system (catechol) [5]. Effective interference with the production procedures of certain amino acids necessary for the growth of specific microorganisms has been also suggested. Meanwhile, one of the most mechanisms is the direct stimulation of phagocytosis as a response of the immune system to the microbes of all types [46].

Here, we found that oak at the used concentrations had no toxic effect on macrophage cells. The SI of greater than 10 for oak represented its safety to the macrophages and specificity to the parasite [50]. So, it can be proposed that oak is safe for mammalian cells. Therefore, reducing the number of infected macrophages and amastigotes related to oak affects parasites. Moreover, the results

showed considerable antioxidative activity of the oak extract, which was directly related to the increase in the concentration of the applied material. It is well-known that, in plants, antioxidant activity is directly related to the level of phenol and flavonoid compounds. Previously it has been proven that phenolic compounds generally act as antioxidants by trapping free radicals; whereas flavonoids can scavenge free radicals and chelate metals as well Engeseth and Geldof, 2001. The key role of phenol compounds as the eliminator of free radicals has been reported by several studies [19,54]. Therefore, it can be concluded that the antioxidative activity of oak extract is resulted from the presence of phenol and flavonoid compounds in this plant.

5. Conclusion

In conclusion, the findings of this research provided the scientific data that herbal medicines could be used for the prevention and treatment of cutaneous leishmaniasis. Therefore, it can be concluded that, in addition to antiviral, antibacterial, and anti-fungal properties, oak extract has an anti-parasitic effect as well.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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