

# The therapeutic effects of olive leaf extract on *Leishmania major* infection in BALB/c mice

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## ABSTRACT

At present, no effective vaccine has been found for CL; thus, the upgrading and improvement of the present drugs and development of the novel agents have been demonstrated to be the merely control choice for this disease. The present study aims to evaluate the effects of olive leaf extract (*Olea europaea* L. OLE) on *Leishmania major* infection in BALB/c mice. OLE tested on cutaneous leishmaniasis (CL) in male BALB/c mice with *L. major* promastigotes (MRHO/IR/75/ER) at the doses of 10, 20, and 30 mg/kg. Results demonstrated that after treatment

of the groups with the concentrations of 10, 20, and 40 mg/kg of OLE, mean diameter of lesions was reduced by 2.16, 5.87, and 7.61 cm, respectively. Moreover, after 4 weeks of treatment, 83.3%, 66.64%, and 33.32% recovery was observed in the infected mice treated with 40, 20, and 10 mg/kg of OLE, respectively. In conclusion, the present study showed OLE has potent therapeutic effects on cutaneous leishmaniasis.

**Keywords:** Olive; *Leishmania*; mice; cutaneous leishmaniasis; *in vivo*

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## 1. Introduction

Leishmaniasis as a protozoan parasitic disease is widespread in more than 90 countries around the world; whereas approximately 350 million people are at risk and more than one million new cases were reported annually [1]. Most commonly form of leishmaniasis is cutaneous leishmaniasis (CL) which is described by prolonged cutaneous wounds and continual scars in the infected zone [2]. Based on the World Health Organization (WHO), the majority of the CL cases are reported from Iran, Afghanistan, Algeria, Iraq, Saudi Arabia, and Syria in the Old World; and Bolivia, Brazil, Colombia, and Peru in the New World [3- 5]. At present, no effective vaccine has been found for CL; thus, the upgrading and improvement of the present drugs and development of the novel agents have been demonstrated to be the merely control choice for this disease [6]. Nowadays, the use of antimonial agents such as meglumine antimoniate and sodium stibogluconate have been faced with some problems because of possessing a number of restrictions including the emergence of drug resistance and serious complications [7]. These issues highlight the crucial requirement for the development of novel effective agent replacements for CL.

Throughout the history of humanity, natural products, such as plants extracts have been used as an infinite chance for development of a new drug to treat a broad spectrum of disease because of having few side effects, and high accessibility [8]. *Olea europaea* L. (olive), the most well-known species of the genus *Olea* is one of the most famous fruit crops all over the Mediterranean Basin such as Iran [8, 9]. According to the previous studies, different parts of *O. europaea* including bark, wood, leave, fruit, and aerial parts are applied in folk medicine to remedy some illness such as asthma, gallstones, hypertension, as well diarrhea, respiratory, and urinary tract infections [10]. Moreover, in modern medicine various pharmacological features such as anticancer, antidiabetic, antihypertensive, antiinflammatory, and antimicrobial effects have been linked to this plant. Although previous investigations have demonstrated the main chemical components of different parts of *O. europaea* [10]; however some factors such as plant species and used part, geographical source, the time of plant harvesting, and weather conditions might impress the amount of chemical compositions and consequently the biological properties of plants [11]. The present study aims to evaluate the effects of olive leaf extract on *Leishmania major* infection in BALB/c mice.

## 2. Materials and methods

### Plant materials

Leaves of *O. europaea* were obtained from rural regions of Khorramabad district, Lorestan province, West of Iran, in September 2013. They were identified by a botanist of Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences (Khorramabad, Iran). A voucher specimen of the plant materials was deposited at the herbarium of Razi Herbal Medicines Research Center, Khorramabad, Iran (RH 1165).

### Preparation of plant extract

Sample (20 g air-dried and pulverized *O. europaea* leaves) was put into a cellulose cartridge and extracted in a 250 mL Schott Duran Soxhlet extractor (Germany) with 200 mL ethanol: H<sub>2</sub>O (70:30) for 16 h. The solvent was evaporated on a rotatory evaporator at 40°C, and the remains were stored at 4°C until analysis.

### HPLC analysis

The high-performance liquid chromatography (HPLC) device of model Shimadzu (SCL-10AVP) with the C-18

column, model Wakosil II 5C18R, with the length of 24 cm, the diameter of 6.4 mm, filler particles with a size of 5 µm, and a protective column with a 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 of 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 of Hamilton Company, was used to collect the sample from the container and inject it into the device. Oleuropein was detected using HPLC device by isocratic elution program with acetonitrile solvent and 50 µM phosphate buffer with pH equal to 9.2 with a ration of (v:v) 70:30. The UV detector was set at 254 nm. The chromatograms were run for 10 min at a flow rate of 1.2 ml/min at ambient temperature [12]. A stock standard solution (1000 µg/mL) was prepared by dissolving oleuropein in methanol. Working standard solutions at the concentrations of 0.5–100 µg/mL were prepared by diluting suitable volumes of the stock standard with ethyl acetate.

### Parasite strains

*L. major* (MRHO/IR/75/ER) was compassionately acquired from the Center for Research and Training in Skin Diseases and Leprosy (Tehran, Iran). Parasites were cultured at 26 ± 1°C in the complete tissue culture medium [RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin].

### Animals

To evaluate the antileishmanial activity of OLE on CL in mice, 70 male BALB/c mice (6–8 weeks old) were obtained from Pasteur Institute (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 standard protocols for the use of laboratory animals. The Committee on the Ethics of Animal Experiments of the Lorestan University of Medical Science (Permit Number: 89/6) approved the protocol. Furthermore, all efforts were made to minimize suffering.

### Establishment of cutaneous leishmaniasis

To establish CL, mice were infected subcutaneously at the base of the tails with 0.1 ml (2×10<sup>6</sup> cells/ml) of promastigotes of *L. major* in the stationary phase [13]. After 5 weeks, when the lesions created at the location of parasites inoculation,

the mice were randomly divided into 6 groups (10 mice per each);

Group 1: Non-infected and non-treated

Group 2: Infected but non-treated (control group)

Group 3: Infected and treated with OLE 40 mg/ml

Group 4: Infected and treated with OLE 20 mg/ml

Group 5: Infected and treated with OLE 10 mg/ml

Group 6: Infected and treated with MA 60 mg/kg/day; intraperitoneally.

The treatment with OLE was applied to the lesions twice a day for 30 days topically by a sterile medical cotton swap.

### Measurement of lesion size

The diameter of lesions was calculated by means of a metric caliper before treatment and at weekly intervals for 4 weeks after challenge. The measurement was carried out in two diameters (D and d) at right angles to each other, and the size (mm) was determined based on the formula  $S = (D + d)/2$  [14].

### Parasite load in smear

In order to confirm the clinical diagnosis laboratory exhibition of the parasite load was carried out in the lesions by staining smears at the end of the test time.

Lesions were cleaned by ethanol and punctured at the margins with a sterile lancet and exudation substance was smeared. The smears were dried in air, fixed by methanol and stained with Giemsa with the aim of amastigotes detection by light microscopy. Grading of *Leishmania* parasites was obtained by average amastigote density using x10 eyepiece and x100 oil immersion lens consistent with previous studies:

4+: 1-10 parasites/1 field, 3+: 1-10 parasites/10 fields, 2+: 1-10 parasites/100 fields, 1+: 1-10 parasite/1000 fields as it was reported previously [15].

### Parasite burden assay

A number of live parasites in draining lymph nodes were assessed by limiting dilution assay (LDA), at week 4 after experiment as it was reported previously [16]. The lymph nodes were aseptically removed, weighed and then homogenized in 2 ml Schneider's medium (Sigma, USA) supplemented with 10% FBS, 2mM glutamine, 100 U/mL

penicillin and 100 µg/mL streptomycin. Different serial dilutions of lymph nodes were prepared in the same media and cultured in quadruplicate in sterile flat-bottom-96 well plates and incubated at  $25 \pm 1$  °C for 10 days. The motile and non-motile parasites (positive and negative wells, respectively) were determined with an inverted microscope. The number of viable parasite per mg of tissue was determined by the highest dilution at which growing promastigotes using ELIDA software [17].

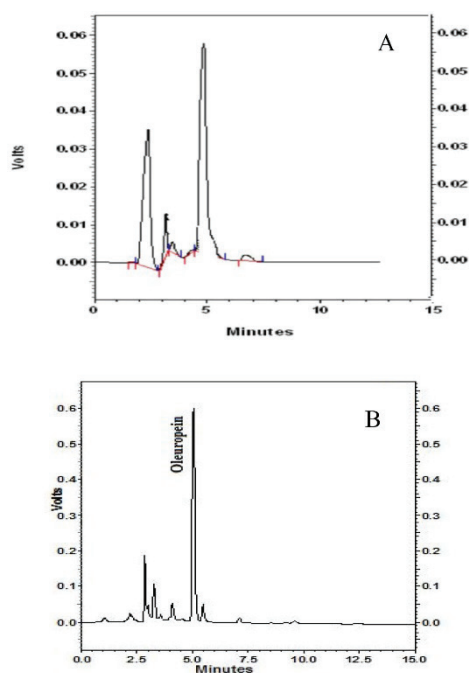
### Statistical analysis

Data analysis was conducted using SPSS statistical package, version 16.0 (SPSS Inc., Chicago, IL, USA). One-way Analysis of Variance (ANOVA) followed by Tukey Post hoc test was used to analyze the data. To evaluate the interaction of time and the experimental group, repeated measures analysis test was used. Statistical significance was taken at  $P \leq 0.05$ .

## 3. Results

### Analysis of Oleuropein level

The level of oleuropein available in the extract was reported as 18.45%. Chromatogram related to the standard oleuropein is shown in Figure 1.



**Figure 1.** Chromatograms of (A) OLE and (B) oleuropein standard solution (1000 µg/mL).

### Therapeutic effects on CL in mice

*In vivo* assay, in the infected mice treated with the extract concentration of 20 and 40 mg/kg, showed that the number of parasites and parasite load of pooled draining lymph nodes significantly ( $P < 0.05$ ) reduced, whereas 10 mg/kg of OLE decreased the number of parasites and parasite load weekly ( $P > 0.05$ ). Moreover, the findings revealed that OLE at the concentration of 40 mg/kg significantly ( $P < 0.05$ ) decreased the mean number of parasites and parasite load in comparison with other groups and MA. The control group also had no decrease in the number of parasites. After treatment of the groups with the concentrations of 10, 20, and 40 mg/kg of OLE, mean diameter of lesions was reduced by 2.16, 5.87, and 7.61 cm, respectively. In contrast, in the group treated with MA, mean diameter of lesions was decreased about 4.13 cm. In the control group, the mean diameter of the lesions was increased about 6.4 cm (Table 1). After 4 weeks of treatment, 83.3, 66.64, 33.32 and 58.31% recovery were observed in the

infected mice treated with OLE at the concentrations of 40, 20, 10 mg/kg and MA (meglumine antimoniate), respectively (Figure 2). Table 2 shows the mean diameter of lesions.

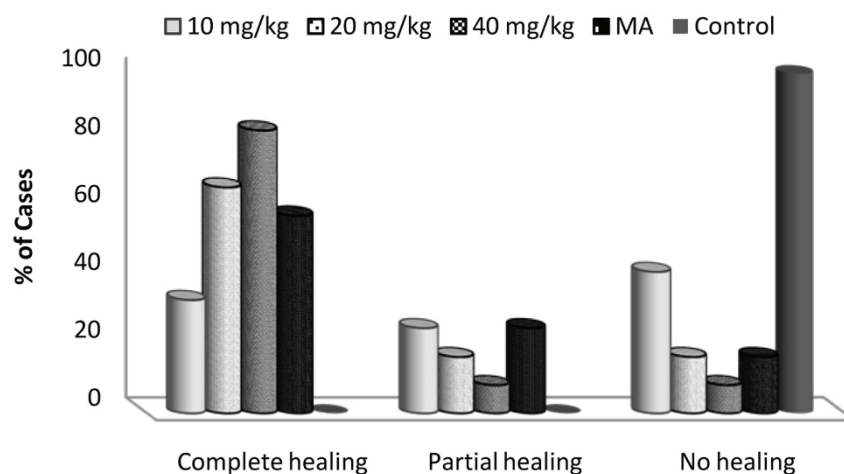
### 4. Discussion

Leishmaniasis is considered as a health-medical problem in many countries of the world [1]. Despite the existence of different methods for the treatment of this disease, there is still no definite and effective cure for it [18]. The side effects caused by the available drugs increased the demand for use of herbal compounds with plan origin and high efficacy for the treatment of diseases. Recently, many developments have been achieved in the field of using herbal drugs for the treatment of leishmaniasis [8]. Based on the reviews, more than the two-thirds of world's population believe folk medicine for their early healthcare requirements. Since, herbal medications usually have high availability and high

**Table 1.** Effect of olive leaf extract (OLE) 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 treatment	P value*
Control	7.44 ± 0.21	13.84 ± 0.16	<0.01
40 mg/kg	8.42 ± 0.19	0.81 ± 0.13	<0.001
20 mg/kg	8.39 ± 0.27	2.52 ± 0.17	<0.001
10 mg/kg	7.37 ± 0.33	5.21 ± 0.21	<0.05
MA	8.33 ± 0.31	4.18 ± 0.19	<0.01

\* The difference was statistically significant ( $P < 0.05$ )



**Figure 2.** Comparison of healing rate of lesions in tested groups after treatment with various concentrations of olive leaf extract and MA.

efficacy considered a proper alternative for the chemical drugs [19].

The findings obtained in the *in vivo* assay revealed that OLE at the concentration of 40 and 20 mg/ml had potent suppression effects on CL in male BALB/c mice infected with *L. major* with 83.3% and 66.64% recovery, respectively; whereas OLE extract at the concentration of 10 mg/ml indicated the lower suppression effects. After treatment of the infected mice with OLE at the concentrations of 20 and 40 mg/kg, the mean diameter of lesions, parasite load and mean a number of parasites were significantly ( $P < 0.05$ ) reduced in comparison with the other groups.

Previously, Sifaoui *et al* (2014) reported that the inhibitory effect of some Tunisian olive tree varieties against the promastigote stage of *L. donovani*, *L. tropica*, *L. major* and *L. amazonensis* with  $IC_{50}$  ranging from 2.1 to 71.5  $\mu\text{g/ml}$  [20]. Despite the number of papers published on olive leaf and its antileishmanial effects, none of them has focused on *in vivo* efficacy OLE on cutaneous leishmaniasis in a mouse model of infection.

Formerly, the principle compounds of OLE are found to be polyphenolic compounds like hydroxytyrosol, tyrosol, and benzoic acids, secoiridoids such as the oleuropein, flavonoids, and triterpenoid including oleanolic acid, maslinic acid, and ursolic acid [10]; which, individual activities of these compounds have been proven [19]. 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 the plants [21-23]. There are several studies which indicate polyphenols potentially inhibit amastigote and promastigote forms of different species of *Leishmania species* [24, 25]. In addition, Sifaoui *et al.* (2014) showed that the relative leishmanicidal activity of five varieties against *L. tropica* and *L. major* was moderately correlated to phenols and flavonoids content [20]. Flavonoids are also broad classes of plant phenolics that have been under study for antiparasitic activity [26]. Regarding antileishmanial activity of triterpenic acid several studies have been done [20, 27]. For example study of Torres-Santos *et al.*, (2014) demonstrated that oleanolic acid, ursolic acid and triterpenic acids isolated from the methanolic fraction of *Pourouma guianensis* exhibit a strong activity against *L. amazonensis* [27].

In the present study, we found that level of oleuropein available in the OLE was 18.45%. Olive leaf is rich in a bitter material called oleuropein which is most frequent phenol compounds in the olive leaves and fruits and various pharmacological properties such as antioxidant, anticancer, anti-inflammatory,

anti-aging, and neuroprotective activity have been related to this component [28]. Kyriazis *et al* (2013) have demonstrated that Oleuropein had the potent inhibitory effect in both stationary and middle logarithmic phase promastigotes of *L. infantum*, *L. donovani*, and *L. major*. In addition, they have reported that oleuropein reduced the spleen parasitic burden >80% in an experimental *L. donovani*-infected [29].

Moreover, in several investigations antimicrobial and antiviral effects of oleuropein against some pathogenic bacterial and viral strains have been proven [31]. Therefore, phytoconstituents in this plant particularly oleuropein could be answerable for the antileishmanial activity of OLE but their accurate mechanism of action is vague. However, phenolic structures such as oleuropein appear to create its antimicrobial effect through destructive the bacterial wall and/or interrupting cell peptidoglycans. Some researchers also have proposed that it is due to the presence of the ortho-diphenolic system (catechol) [30]. In the study conducted by Saija and Uccella (2001), it has been shown that the glycoside group changes the capability to penetrate the cell membrane and get to the target site [31]. Effective meddling with the making procedures of certain amino acids essential for the growth of specific microorganisms has also been recommended. Meanwhile, one of the most mechanisms proposed is the direct stimulation of phagocytosis as a response of the immune system to microbes of all types.

## 6. Conclusion

According to the results of present study, OLE seems to possess remarkable therapeutic effects on *L. major* infection in BALB/c mice. Furthermore, this activity could be the result of the presence of several molecules especially phenolic compounds.

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## Conflict of interest

The authors declare that there is no conflict of interest in this study.



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