

# In Vitro Antileishmanial Activity of *Falcaria vulgaris* Fractions on *Leishmania major*

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## Abstract

**Background:** In recent years, many reports have been published regarding the increasing frequency of drug resistance in *Leishmania* species to antimonial compounds. Accordingly, many researchers have tried to develop new medicines for the treatment of cutaneous leishmaniasis.

**Objectives:** The aim of this study was to evaluate the in vitro antileishmanial activity of aqueous, ethyl acetate, and butanol fractions of *Falcaria vulgaris* against *Leishmania major* promastigotes.

**Methods:** The total extract was obtained using the percolation method. Subsequently, chlorophyll and oil were eliminated from the total extract, using medium pressure liquid chromatography (MPLC), followed by liquid-liquid extraction (LLE) for fractionation of the total extract. The antileishmanial activity of each fraction was examined at 6 concentrations (40, 80, 160, 320, 640, and 1280  $\mu\text{g/mL}$ ) and 4 intervals (6, 24, 48, and 72 hours).

**Results:** The results of repeated measures ANOVA showed P values of 0.16, 0.22, and 0.04 for the aqueous, ethyl acetate, and butanol fractions, respectively. Furthermore, one-way ANOVA test revealed a significant difference between these fractions at 48 and 72 hours ( $P = 0.04$  for both). In addition, Tukey's HSD test showed a significant difference between the butanol fraction and other fractions at 48 and 72 hours ( $P < 0.05$ ). The greatest antileishmanial effect was observed in the butanol fraction with a half-maximal inhibitory concentration ( $\text{IC}_{50}$ ) of 343.8  $\mu\text{g/mL}$  at 48 hours.

**Conclusions:** As *Falcaria vulgaris* showed acceptable antileishmanial activity with no side effects in this study, it can be used as an effective complementary or alternative treatment for skin lesions of cutaneous leishmaniasis. However, further *in vivo* studies are required in the future.

**Keywords:** Extract, *Leishmania Major*, *Falcaria Vulgaris*, In Vitro

## 1. Background

Cutaneous leishmaniasis (CL) is an important zoonotic disease worldwide (1, 2). Currently, pentavalent antimonials, such as meglumine antimoniate (Glucantime®), are used as first-line treatment for CL (3). However, these medicines pose some health risks due to their inherent toxicity (4), especially when systemic therapy is necessary for CL (5).

In recent years, many reports have been published regarding the increasing frequency of drug resistance in *Leishmania* species to antimonial compounds (6). Accordingly, many researchers have tried to develop new agents for the treatment of CL (7). One of the major approaches to the treatment of various types of leishmaniasis is the use of medicinal plants (8). The present study aimed to examine

the effects of various fractions of *Falcaria vulgaris* on *Leishmania major*.

The genus *Falcaria*, which belongs to the family *Apiaceae*, is an herbaceous plant with 2 species endemic to Iran, i.e., *F. vulgaris* and *F. falcarides*. Spathulenol and carvacrol are 2 essential oils found at high concentrations in *F. vulgaris* extracts. Additionally, germacrene B is the most frequently found essential oil in *F. falcaroides* extracts (9). A previous study revealed that some essential oil compounds of this plant have antileishmanial activities (10).

The use of *F. vulgaris* in the treatment of skin wounds dates back to several decades ago. In a study conducted by Khazaei and Salehi, the effects of *F. vulgaris* extract on gastric ulcer were evaluated in animal models; the results

showed that the ethanolic extract could significantly reduce the ulcer index (11). Likewise, another study confirmed the valuable effect of this plant on gastric ulcers (12). The therapeutic effects of *F. vulgaris* on skin wounds have been also discussed in the literature (13). In this regard, a previous study confirmed the antibacterial effects of this plant (14). However, no study has yet examined the effects of *F. vulgaris* extract on *L. major*, as the causative agent of skin wounds.

## 2. Objectives

We aimed to evaluate the *in vitro* antileishmanial activities of aqueous, ethyl acetate, and butanol fractions of *F. vulgaris* on *L. major* promastigotes.

## 3. Methods

### 3.1. Parasites

The standard strain of *L. major* (MRHO/IR/75/ER) was provided by the department of parasitology, Isfahan University of Medical Sciences, Isfahan, Iran. The parasites were maintained under laboratory conditions, allowing the repeated passage of BALB/c mice. Amastigotes were isolated from the infected mice and transformed into promastigotes in Novy-MacNeal-Nicolle (NNN) medium. The promastigotes, obtained from the third passage in the NNN medium, were gradually added to RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 µg/mL of streptomycin, and 100 IU/mL of penicillin at 25°C.

### 3.2. Plants

The aerial parts of *F. vulgaris* were collected in the spring from Khomein, Markazi Province, Iran. Subsequently, the collected plants were authenticated at the department of pharmacognosy, Isfahan University of Medical Sciences, Isfahan, Iran and stored in the herbarium (No., 2955).

### 3.3. Total Extract

The collected plants were left in darkness at room temperature to dry and were then ground to pass through a sieve. The powder was extracted using the percolation method (15), and the total extract was concentrated by a rotary evaporator.

### 3.4. Chlorophyll and Oil Elimination

Chlorophyll and oil were eliminated from the total extract via medium pressure liquid chromatography (MPLC), using petroleum ether and acetone (16).

### 3.5. Total Extract Fractionation

Fractionation was performed using the liquid-liquid extraction (LLE) method (17). Equal volumes of ethyl acetate and distilled water were mixed. The total extract was added to a separatory funnel, stirred vigorously, and allowed to form 2 separate phases. Subsequently, ethyl acetate was separated from the aqueous phase. Afterwards, butanol was added to the remaining aqueous phase at the same volume as the aqueous phase, stirred vigorously, and allowed to form 2 phases; then, the butanol phase was separated from the aqueous phase; the remaining aqueous phase was also collected. The fractions were lyophilized and kept at -20°C until further use.

### 3.6. Experimentation

Initially, butanol and ethyl acetate fractions were dissolved in isotonic saline, containing dimethyl sulfoxide (DMSO); it should be noted that the final volume of DMSO in the testing medium was approximately 0.2%. DMSO was not added to the aqueous fraction. Each fraction was studied at 6 concentrations (40, 80, 160, 320, 640, and 1280 µg/mL) in the RPMI medium, supplemented with FBS (10%), 100 µg/mL of streptomycin, 100 IU/mL of penicillin, and  $1 \times 10^6$  exponentially growing promastigotes.

The single-blind experiments were performed in triplicate for each concentration at each interval (6, 24, 48, and 72 hours). It should be noted that a positive control containing amphotericin B (1 µg/mL) and the parasite, as well as 2 negative controls (1 with 0.2% DMSO and parasite and 1 with only the medium and parasite), was considered each time.

The negative control with DMSO was used for the ethyl acetate and butanol fractions, while the control without DMSO was applied for the aqueous fraction. At each interval (6, 24, 48, and 72 hours), 10 µL of each sample was gently mixed with 10 µL of 0.4% trypan blue on a glass slide. The mortality of parasites was determined by counting the number of alive (not stained) and dead (stained) promastigotes according to the following formula (1).

Accordingly, at least 50 microscopic fields were examined for each sample.

### 3.7. Data Analysis

The normal distribution of data was evaluated using nonparametric Kolmogorov-Smirnov test. Interactions between concentration and time were separately analyzed by repeated measures analysis of variance (ANOVA) for each fraction. The groups were compared using one-way ANOVA test, followed by Tukey's honest significant difference (HSD) test at P value below 0.05. The Microsoft Excel software (Microsoft Inc.) was used to determine the lethal

$$\text{Mortality rate} = \frac{\text{The number of killed promastigotes}}{\text{The number of killed promastigotes} + \text{The number of viable promastigotes}} \times 100 \quad (1)$$

dose 50 (LD<sub>50</sub>) by plotting the percentage of death versus different concentrations of each fraction at a given time.

Considering the nonlinearity of the effect of each fraction at different concentrations, type II regression analysis was performed; by putting  $Y = 50$  in the equation,  $X$  values were calculated. The half-maximal inhibitory concentration (IC<sub>50</sub>) was calculated for only the butanol fraction at a concentration of 1280  $\mu\text{g/mL}$  within 48- and 72-hour intervals, which produced the greatest effects, respectively. The statistical analyses were performed using SPSS version 16.

#### 4. Results

The results of repeated measures ANOVA showed no significant differences for the aqueous ( $P = 0.16$ ) and ethyl acetate ( $P = 0.22$ ) fractions, whereas a significant difference was observed for the butanol fraction ( $P = 0.04$ ). One-way ANOVA revealed a significant difference among fractions at 48 and 72 hours ( $P = 0.04$  for both). Moreover, Tukey's HSD test showed a significant difference between the butanol fraction and the other 2 fractions ( $P < 0.05$ ) at 48 and 72 hours.

In addition, the IC<sub>50</sub> values for the butanol fraction were 343.8 and 639.1  $\mu\text{g/mL}$  at 48 and 72 hours, respectively. The data regarding the antileishmanial effects of fractions are presented in Figures 1 - 3. Moreover, the effects of *F. vulgaris* fractions on *L. major* promastigotes at 48 hours are compared in Figure 4. The butanol extract showed maximum antileishmanial effects in comparison with other fractions.

#### 5. Discussion

Considering its efficacy in the treatment of wounds, *F. vulgaris* was examined in the present study (18). Previous studies have shown that beta-caryophyllene, alpha-pinene, and limonene are compounds with antileishmanial activities (19-21). In this regard, one previous study showed that *F. vulgaris* is rich in these compounds (10). In addition, saponin is another compound in this plant with antileishmanial effects (22, 23).

According to some research, a few plants show mild to strong activities against *Leishmania* species (24-27). Asadi et al. (28) showed that the hydroalcoholic extracts of *Stachys lavandulifolia* and *Mespilus germanica* have antileishmanial activities. Some studies have also shown that these extracts are suitable adjuvants in vaccination against *Leishmania*

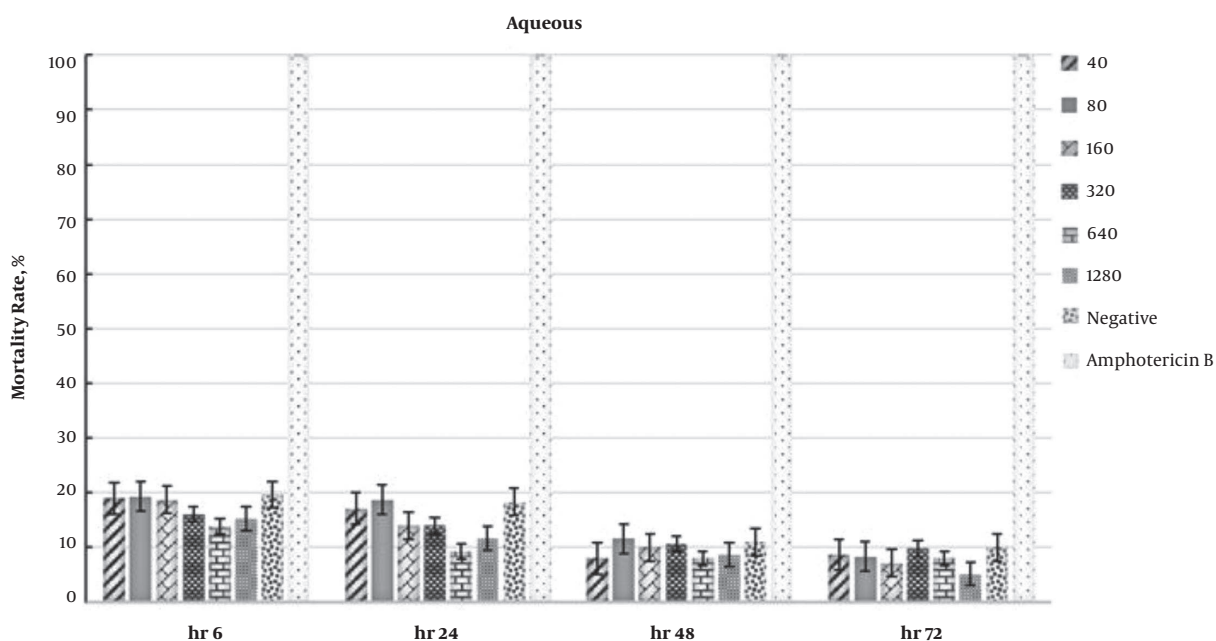
species (29, 30). Recently, *invivo* studies have revealed that alcoholic extract of *Hedera helix* and ethanol extract of *Peganum harmala* exert antileishmanial effects on *L. major* (31, 32). Furthermore, another study showed that the aqueous extract of *Artemisia sieberi* has more antileishmanial activities than artemisinin (33).

Moreover, the mucilage extract of *Cordia myxa* has shown antileishmanial activities against *L. major* and *L. infantum* (34). The results of a previous study on the effects of 6 plants showed that *Allium hirtifolium* and *Ziziphus spinachristi* had greater inhibitory effects on parasites (35). Another study showed that the extracts of *Stachys lavandulifolia* Vahl. and *Mespilus germanica* L. leaves could inhibit the proliferation of *L. major* promastigotes.

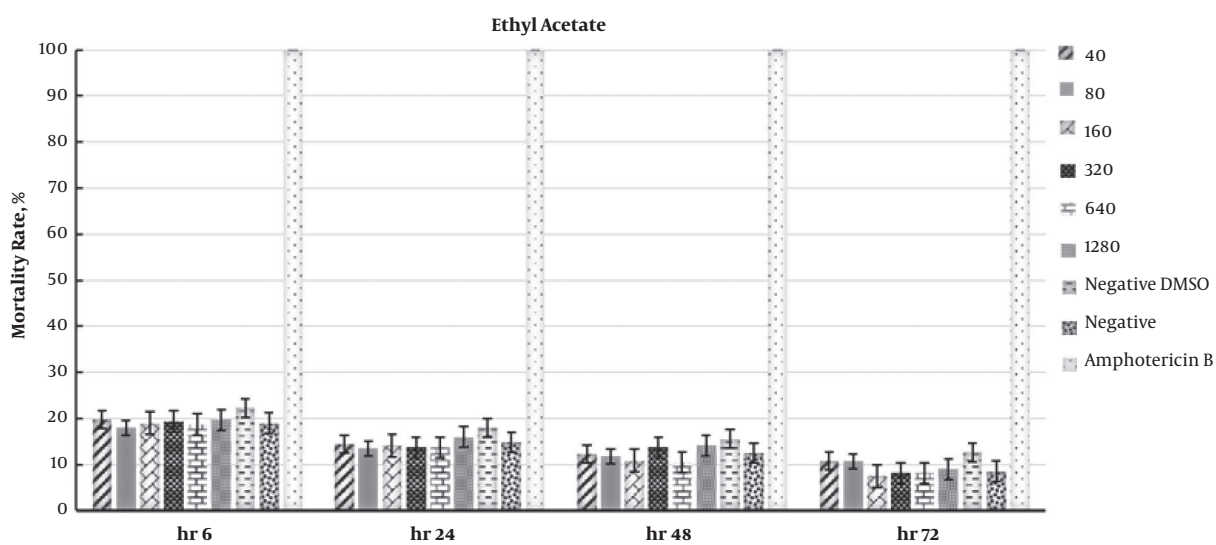
In the present study, the aqueous fraction of *F. vulgaris* showed the greatest effect on parasite death during the first 6 hours (Figure 1). After this interval, changes reduced at all concentrations, and the difference was almost insignificant among different concentrations within 48 and 72 hours. As most solute compounds in the aqueous fraction are proteins and carbohydrates, they may act as a source of energy for parasite survival and proliferation; this may be the reason for the reduced death of parasites in this interval.

Likewise, the effects of ethyl acetate fraction on parasite mortality were almost similar to the aqueous fraction. In addition, the greatest effect of ethyl acetate fraction was observed during the first 6 hours. The changes were almost similar for all the fractions during the first 24 hours. In this study, the highest antileishmanial effect was observed for the butanol fraction with an IC<sub>50</sub> of 343.8  $\mu\text{g/mL}$  at 48 hours. As presented in Figure 3, the antileishmanial effect of butanol fraction was less significant than amphotericin B; in fact, amphotericin B destroyed 100% of parasites within 6 hours. Nevertheless, the butanol fraction showed a reliable and dose-dependent antileishmanial effect at 48 hours (Figure 3).

So far, researchers have introduced various antileishmanial compounds (36). Accordingly, the world health organization (WHO) has recommended the use of medicinal plants in the treatment of leishmaniasis (34). Based on the present findings, *F. vulgaris* has acceptable antileishmanial activity and can act as an effective agent in the treatment of wounds without any side effects. Therefore, it may be used as a useful complementary or alternative treatment for skin lesions of cutaneous leishmaniasis. However, further *invivo* studies are necessary in the future.



**Figure 1.** The Effects of Different Concentrations of Aqueous Fraction of *F. Vulgaris* on *Leishmania Major* at Different Intervals



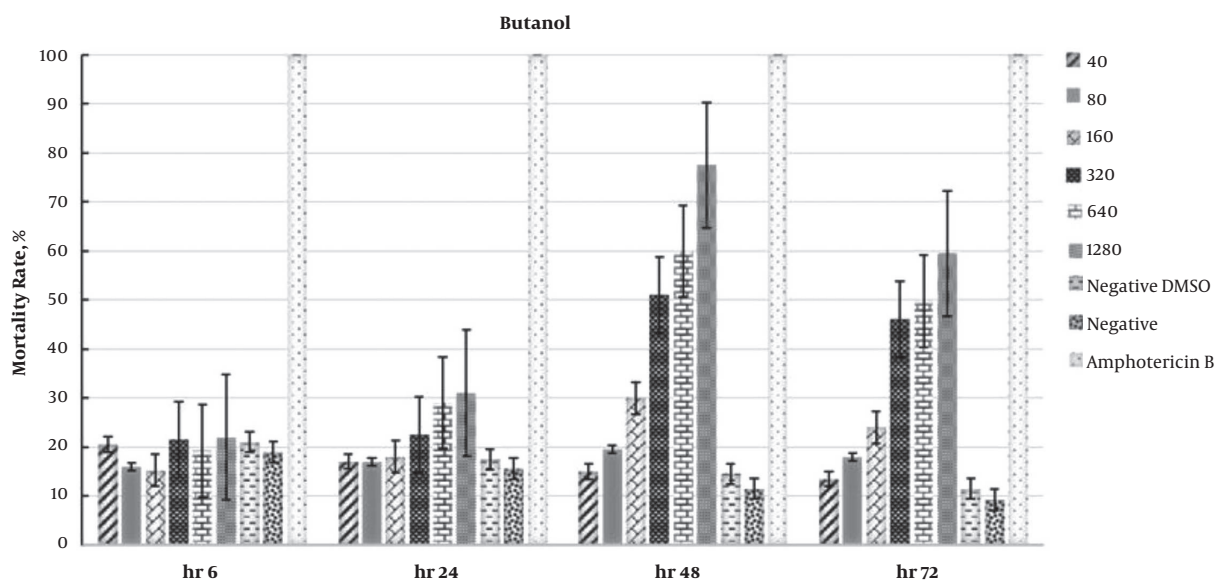
**Figure 2.** The Effects of Different Concentrations of Ethyl Acetate Fraction of *F. Vulgaris* on *Leishmania Major* at Different Intervals

### Footnotes

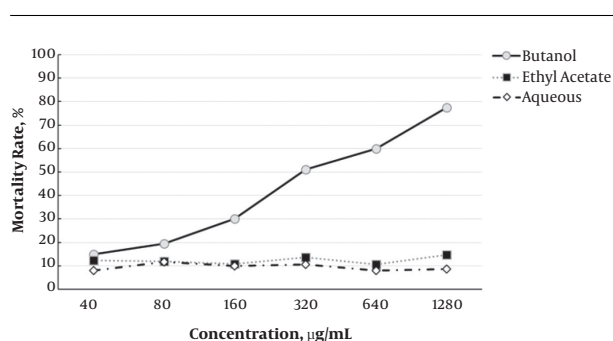
**Authors' Contribution:** Abbas Ali Eskandarian designed the study, prepared the samples, and performed the culture studies of parasites. Hamed Jafari performed the experiments. Gholamreza Asghari designed the study, analyzed and interpreted the data, and contributed to the writing of the manuscript. Mohammad Ali Mohaghegh

examined the samples. Hosein Ali Yousefi prepared the laboratory materials and performed the culture studies of parasites. Mustafa Ghanadian performed the extraction. Hamed Kalani prepared the samples and contributed to the writing of the manuscript.

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**Figure 3.** The Effects of Different Concentrations of Butanol Fraction of *F. Vulgaris* on *Leishmania Major* at Different Intervals



**Figure 4.** The Effects of Different Concentrations of *F. Vulgaris* Fractions on *Leishmania Major* at 48 Hours

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