



# Anti-leishmanial Activity of *Zataria multiflora* and *Thymus vulgaris* Essential Oils Against *Leishmania major*: Phytochemical Analysis and Therapeutic Efficacy

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## Abstract

**Background:** Leishmaniasis, a neglected tropical disease, impacts millions, especially in resource-limited areas.

**Objectives:** This study examines the chemical composition and anti-leishmanial potential of *Zataria multiflora* and *Thymus vulgaris* essential oils against *Leishmania major*, a key cause of cutaneous leishmaniasis (CL).

**Methods:** The chemical composition of *Z. multiflora* and *T. vulgaris* essential oils was analyzed by gas chromatography-mass spectrometry (GC-MS). Their anti-leishmanial effects against *L. major* promastigotes (31.25 - 1000 µg/mL) were evaluated via flow cytometry, while anti-amastigote activity was determined by counting infected macrophages using light microscopy. Cytotoxicity against J774 cells was assessed with the MTT assay after 48 hours.

**Results:** The main compounds in *T. vulgaris* and *Z. multiflora* essential oils were thymol (49.83% and 46.46%, respectively), along with γ-terpinene (14.93%) and cymene (10.99%) in the former, and carvacrol (32.47%) in the latter. Both oils showed activity against *L. major* promastigotes ( $IC_{50} = 92.75$  and  $59.69$  µg/mL, respectively) and amastigotes (48 hours) ( $IC_{50} = 100.68$  and  $87.28$  µg/mL, respectively). Cytotoxicity on J774 cells ( $CC_{50} = 820.4$  and  $799.6$  µg/mL) resulted in Selectivity Indices (SI) of 8.84 (promastigote) and 8.14 (amastigote) for *T. vulgaris*, and 13.39 (promastigote) and 9.16 (amastigote) for *Z. multiflora*. In the promastigote assay, amphotericin B [20 µg/mL, positive control (PC)] caused 89.71% mortality, confirming assay validity.

**Conclusions:** This study demonstrates the anti-leishmanial potential of *Z. multiflora* and *T. vulgaris* essential oils against *L. major*, but further *in vivo* validation is required.

**Keywords:** Essential Oil, Cutaneous Leishmaniasis, Phytochemical Composition, *Zataria multiflora*, *Thymus vulgaris*

## 1. Introduction

Leishmaniasis, a neglected tropical disease, represents a major global public health challenge affecting nearly 100 countries worldwide, with an estimated annual incidence of 700,000 to 1 million new cases (1, 2). This vector-borne disease exhibits disproportionate prevalence in resource-limited regions with inadequate healthcare infrastructure, particularly in parts of Asia, Africa, Latin America, and the Eastern

Mediterranean basin (3, 4). Leishmaniasis is caused by protozoan parasites of the genus *Leishmania*, which are transmitted to humans via the bites of infected female phlebotomine sand flies (5). The disease manifests in various clinical forms, including cutaneous leishmaniasis (CL), visceral leishmaniasis, and mucocutaneous leishmaniasis. Cutaneous leishmaniasis represents the most prevalent clinical form of the disease, characterized by debilitating and disfiguring skin lesions (6).

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Conventional pharmacotherapeutic agents employed for leishmaniasis management, including antimonials, amphotericin B, miltefosine, and paromomycin, have exhibited diverse efficacy profiles (7). Nevertheless, concerns surrounding drug resistance, extended treatment protocols, and adverse events have undermined the clinical viability of these conventional agents (8, 9). Furthermore, the elevated costs and restricted accessibility of these drugs in endemic regions have amplified the healthcare disparities. Consequently, the suboptimal efficacy, high expenditure, and toxicity associated with current leishmaniasis pharmacotherapies have motivated the exploration of alternative treatment modalities (10). In recent years, there has been a surge of interest in the utilization of natural products, particularly essential oils derived from medicinal plants, as potential sources of novel anti-leishmanial agents (11).

*Zataria multiflora* and *Thymus vulgaris* are two aromatic plant species that have been extensively recognized for their diverse medicinal attributes and have been traditionally employed in folk medicine for the treatment of various ailments (12-14). *Zataria multiflora* and *T. vulgaris* have been widely used not only in traditional medicine but also as culinary herbs, underscoring their safety profile and low toxicity in human consumption. This dietary use supports their potential as biocompatible alternatives to synthetic drugs, which often exhibit severe side effects (15).

Preliminary studies have provided evidence for the anti-leishmanial potential of these plants. For instance, a hydroalcoholic extract of *T. vulgaris* has been shown to be significantly more effective than systemic glucantime in reducing ulcer size in *Leishmania major*-infected BALB/c mice (16). Furthermore, thymol and carvacrol, the principal bioactive components of these plants, have demonstrated direct activity against Leishmania parasites, with synthesized derivatives showing high potency and promising Selectivity Indices (SIs) (17). More recently, green-synthesized silver nanoparticles using *T. vulgaris* extract exhibited significant anti-leishmanial activity against both promastigote and amastigote forms of *L. major* (18).

*Zataria multiflora*, commonly referred to as Avishan-e-Shirazi or Shirazi thyme, is indigenous to Iran and other regions of the Middle East. This plant is known to be rich in essential oils and has been extensively documented to exhibit antimicrobial, antioxidant, and anti-inflammatory properties (19, 20). *Thymus vulgaris*,

commonly referred to as common thyme, is widely distributed throughout Europe and the Mediterranean region, and has been utilized in traditional medicinal practices for centuries (21).

The chemical composition of essential oils extracted from *Z. multiflora* and *T. vulgaris* has been extensively investigated, unveiling the presence of a diverse array of bioactive compounds, including monoterpenes, sesquiterpenes, and phenolic compounds (22, 23). These compounds are renowned for their wide-ranging biological activities, encompassing antimicrobial and antiparasitic properties (24). While previous studies have primarily focused on crude extracts of these plants or their use in synthesizing nanoparticles, our study targets their essential oils directly, which are more concentrated in bioactive compounds due to their hydrophobic nature and distillation-based extraction. Essential oils typically exhibit higher potency against pathogens compared to extracts, as they contain volatile terpenoids and phenolics that directly interfere with microbial membranes (25, 26).

## 2. Objectives

Given the limited and indirect data on the anti-leishmanial activity of the essential oils themselves (particularly from *Z. multiflora* and *T. vulgaris*) and their advantage of being natural, low-toxicity agents, this study aims to: Analyze the chemical composition of their essential oils, evaluate their efficacy against *L. major*, highlight their potential as safer therapeutic candidates for CL, addressing the unmet need for non-toxic treatments.

## 3. Methods

### 3.1. Plant Materials

The aerial parts of *Z. multiflora* and *T. vulgaris* were collected in May 2023 from the mountainous regions surrounding Darab city in Fars Province, Iran. Plant identification was conducted by Dr. Moein, a specialist in pharmacognosy at the Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran. Voucher specimens of *Z. multiflora* (PM-1431) and *T. vulgaris* (PM-1430) were deposited in the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Iran.

### 3.2. Preparation and Extraction of Essential Oils

The essential oils of *Z. multiflora* and *T. vulgaris* were extracted by hydrodistillation of the leaves using an all-glass Clevenger-type apparatus for a duration of 4 hours (27). The resulting essential oils were dehydrated using sodium sulfate and stored in a dark environment at 4°C until further analysis.

### 3.3. Gas Chromatography and Mass Spectrometry Analysis

Gas chromatography/mass spectrometry (GC/MS) analysis was performed using a Hewlett-Packard 6890/5973 instrument operating at an ionization energy of 70.1 eV. The system was equipped with an HP-5 capillary column (phenyl methyl siloxane, 25 m × 0.25 mm i.d.), and helium (He) was employed as the carrier gas with a split ratio of 1:20. The oven temperature program included a three-minute hold at 60 °C, followed by a ramp to 260 °C for the detector. The carrier gas flow rate was set to 0.9 mL/min. To calculate the retention indices (RIs), the retention times of n-alkanes injected under the same chromatographic conditions were utilized. The identification of oil components was achieved by comparing the mass spectra and RIs with those reported in the literature, as well as by comparing the mass spectra with the Wiley library or published mass spectral data (28).

### 3.4. Identifying the Constituents of Essential Oils

The identification of the essential oil components was accomplished by comparing their respective retention times and mass spectra with those of known standards, the data from the Wiley 2001 library of the GC/MS system, as well as the information provided in the literature (29).

### 3.5. The Dilutions Preparation

Various dilutions of the essential oils were prepared following the methodology described by Saedi Dezaki *et al* (12). Specifically, 2 µL of the essential oil was dissolved in 988 µL of normal saline. To facilitate the dispersion of the essential oil within the normal saline, an additional 10 µL of Tween 20 was added to the experimental tube. The final solution was thoroughly mixed using a magnetic stirrer. The essential oils underwent a series of dilutions to achieve concentrations of 31.25, 62.5, 125, 250, 500 and 1000 µg/mL. The dilutions of the essential oils were chosen based on preliminary tests, which also confirmed that the combination of normal saline and Tween 20 did not affect parasite growth (12).

### 3.6. Parasite Preparation and Cell Culture

The promastigote form of the *L. major* parasite from the standard strain (MRHO/IR/75/ER) was utilized in this study. The parasite strain was obtained from the Department of Parasitology and Medical Mycology at Shiraz University of Medical Sciences. For the cultivation of parasite promastigotes, RPMI 1640 medium was supplemented with 15% fetal calf serum (FCS), 100 IU/mL of penicillin, and 100 µg/mL of streptomycin. The incubation temperature was maintained at 25°C. Mouse macrophage cells (J774) were obtained from the Central Laboratory at Isfahan University of Medical Sciences, Isfahan, Iran, and employed for cell culture. The cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 IU/mL of penicillin, and 100 µg/mL of streptomycin. The incubation conditions for the cell culture were set at 37°C and 5% CO<sub>2</sub>.

### 3.7. Anti-leishmanial Effects Were Assessed Using Flow Cytometry

To evaluate the effects of *Z. multiflora* and *T. vulgaris* essential oils on *L. major* promastigotes, a flow cytometry method was employed. Initially, 2 µL of the essential oils under investigation were dissolved in 10 µL of Tween 20 and subsequently diluted to a final volume of 1 ml with normal saline to create a stock solution. Serial dilutions were then prepared in normal saline to achieve the final testing concentrations. Subsequently, promastigotes in the logarithmic phase were assessed for evaluation. Initially, 2 × 10<sup>5</sup> promastigotes were added to 12 Eppendorf tubes. Subsequently, six tubes received concentrations of *Z. multiflora* essential oil ranging from 31.25 to 1000 µg/mL, while the other six tubes were supplemented with concentrations of *T. vulgaris* essential oil in the same range.

The positive control (PC) tube contained 20 µg/mL of amphotericin B, while the negative control (NC) tube contained 1% Tween 20. After 2 hours of incubation at 25°C, all tubes were stained with 10 µL of propidium iodide (PI) at a final concentration of 50 µg/mL for 5 minutes in the dark. Flow cytometry was then performed using a BD Calibur flow cytometer. For each sample, 10,000 to 30,000 events were acquired. Cell populations were first gated based on forward and side scatter characteristics to exclude debris. Viability was assessed based on PI fluorescence intensity; the population of PI-negative events (viable cells) was quantified against PI-positive events (non-viable cells).

The half-maximal inhibitory concentration (IC50) was calculated using statistical analysis (12, 30).

### 3.8. Investigation of Anti-amastigote Effects

To measure the anti-amastigote effects of *Z. multiflora* and *T. vulgaris* essential oils, experiments were performed in triplicate. For each replicate, 1 cm<sup>2</sup> cover slips were placed inside each of the wells of a 6-well microplate. Mouse macrophage cells were then seeded at a density of  $3 \times 10^5$  cells/ml and incubated at 37°C and 5% CO<sub>2</sub> for 24 hours. Stationary phase promastigotes were subsequently added to the wells at a parasite to macrophage ratio of 6:1 ( $18 \times 10^5$ /ml) and incubated for an additional 6 hours under the same conditions. Free promastigotes were removed by washing with RPMI-1640 medium, and the parasite-infected macrophages were treated with different concentrations of *Z. multiflora* and *T. vulgaris* essential oils (31.25 - 1000 µg/mL) for 24 and 48 hours at 37°C and 5% CO<sub>2</sub>. Amphotericin B at a concentration of 20 µg/mL was used as a PC. After the treatment, the slides were washed, dried, fixed with methanol, and stained with Giemsa. The stained slides were then examined under a light microscope at 1000X magnification. For each experimental condition, at least 100 macrophages were randomly counted, and the percentage of infected macrophages as well as the mean number of amastigotes per macrophage were determined.

### 3.9. Cytotoxic Effects on Cellular Viability

The effects of essential oils from *Z. multiflora* and *T. vulgaris* on J774 macrophages were investigated following exposure to various concentrations of the essential oils (31.25 - 1000 µg/mL). These experiments were conducted in triplicate. For each replicate, cell culture plates were incubated at 37°C with 5% CO<sub>2</sub> for 48 hours. Subsequently, cell viability was assessed using the MTT colorimetric assay, and the number of viable cells treated with various concentrations of the essential oils was compared to both the positive and NC groups.

### 3.10. Statistical Analysis

Data analysis was performed using IBM SPSS Statistics (Version 27) and GraphPad Prism (Version 10). The half-maximal IC50 values were determined by nonlinear regression analysis of dose-response curves using GraphPad Prism. For the comparison of multiple groups, one-way ANOVA followed by Tukey's post-hoc

test was used. A P-value threshold of less than 0.05 was established to indicate statistical significance.

## 4. Results

### 4.1. Gas Chromatography-Mass Spectrometry Analysis of the Essential Oils from *Thymus vulgaris* and *Zataria multiflora*

The extraction yielded 33 grams of essential oil (1.22%) from 2.7 kilograms of *T. vulgaris* and 43 grams of essential oil (2.86%) from 1.5 kilograms of *Z. multiflora*. The results of the gas chromatography-mass spectrometry (GC-MS) analysis of the essential oils from *T. vulgaris* and *Z. multiflora* are presented in Tables 1 and 2, respectively. A total of 16 compounds were identified in *T. vulgaris*, constituting 99.98% of the essential oils, while 14 compounds were identified in *Z. multiflora*, accounting for 100% of the analyzed essential oils. The main compounds identified in *T. vulgaris* included thymol (49.83%), γ-terpinene (14.93%), and cymene (10.99%), whereas the primary constituents of *Z. multiflora* were thymol (46.46%) and carvacrol (32.47%).

### 4.2. The Effect of *Zataria multiflora* and *Thymus vulgaris* Essential Oils on the Growth of *Leishmania* Major Promastigotes

The flow cytometry analysis demonstrated significant variations in the mortality rates of *L. major* promastigotes when subjected to different concentrations of essential oils derived from *T. vulgaris* and *Z. multiflora*. The NC, consisting of Tween 20 and normal saline, exhibited a mortality rate of 18.18%, suggesting that promastigote mortality remained relatively low when no leishmanicidal agents were present. In contrast, the PC exhibited a significant mortality rate of 89.71%, which confirms the efficacy and sensitivity of *L. major* promastigotes to amphotericin B (Figure 1). The IC50 values for *T. vulgaris* and *Z. multiflora* were determined to be 92.75 µg/mL and 59.69 µg/mL, respectively.

The lethality rates of *L. major* promastigotes exposed to concentrations of *Z. multiflora* essential oil (31.25, 62.5, 125, 250, 500, and 1000 µg/mL) were found to be 26.72%, 52.3%, 57.66%, 62.44%, 67.38%, and 75.82%, respectively (Figure 2). Additionally, the lethality rates corresponding to the specified concentrations of *T. vulgaris* were 24.23%, 41.97%, 56.07%, 58.18%, 62.98%, and 73.83%, respectively (Figure 3). Statistical analysis (one-way ANOVA followed by Tukey's post hoc test) confirmed both essential oils significantly increased promastigote

**Table 1.** The Compounds Isolated from *Thymus vulgaris* Essential Oil Were Analyzed Using Gas Chromatography-Mass Spectrometry

No.	Type of Compound	KI	Percentage
1	$\alpha$ -thujene	928	0.90
2	$\alpha$ -pinene	936	1.02
3	Octen-3-ol	980	1.12
4	Myrcene	993	1.33
5	$\alpha$ -terpinene	1021	2.81
6	Cymene	1033	10.99
7	1,8-Cineole	1037	0.65
8	$\gamma$ -terpinene	1069	14.93
9	Linalool	1103	2.98
10	Terpinene-4-ol	1181	2.87
11	Thymol methyl ether	1238	1.96
12	Carvacrol methyl ether	1247	1.12
13	Thymol	1292	49.83
14	Carvacrol	1304	2.42
15	Caryophyllene $\langle E \rangle$	1425	4.31
16	$\delta$ -cadinene	1530	0.74
17	Total		99.98

Abbreviation: KI, Kovats Index.

**Table 2.** The Compounds Isolated from *Zataria multiflora* Essential Oil Were Analyzed Using Gas Chromatography-Mass Spectrometry

No.	Type of Compound	KI	Percentage
1	$\alpha$ -pinene	937	1.77
2	Myrcene	993	0.85
3	$\alpha$ -terpinene	1020	1
4	$\alpha$ -cymene	1031	5.62
5	$\gamma$ -terpinene	1064	3.58
6	Linalool oxide	1075	0.48
7	Terpinene-4-ol	1185	0.72
8	Carvacrol methyl ether	1249	1.51
9	Thymol	1289	46.46
10	Carvacrol	1295	32.47
11	Caryophyllene $\langle E \rangle$	1423	3.54
12	Aromadendrene	1447	0.51
13	Bicyclogermacrene	1505	0.85
14	Caryophyllene oxide	1588	0.64
15	Total		100

Abbreviation: KI, Kovats Index.

mortality compared to the NC ( $P < 0.05$ ). The anti-promastigote activity of *Z. multiflora* essential oil was statistically superior to that of *T. vulgaris*. However, the efficacy of both essential oils was significantly lower than that of amphotericin B (PC) ( $P < 0.05$ ).

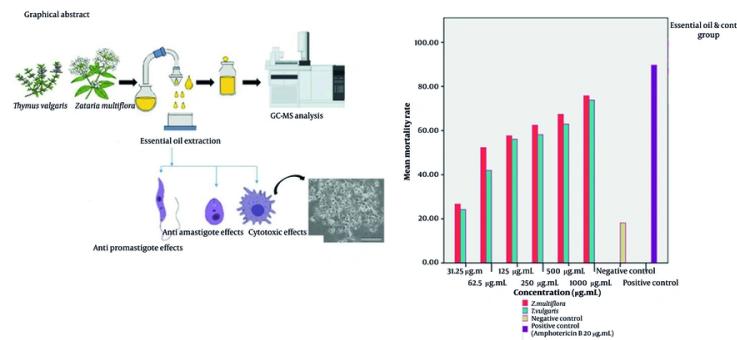
#### 4.3. The Cytotoxicity of *Zataria multiflora* and *Thymus vulgaris* Essential Oils on J774 Macrophage Cells Was Evaluated Using the MTT Assay

The cytotoxic effects of the essential oils of *Z. multiflora* and *T. vulgaris* on J774 macrophages were assessed after treatment with varying concentrations of the essential oils (31.25 - 1000  $\mu$ g/mL) in 96-well microplates under conditions of 37°C, 5% CO<sub>2</sub>, and 48 hours of incubation. Cell viability was evaluated using the MTT assay, and the results were expressed as the percentage of dead cells relative to those treated with amphotericin B and untreated macrophages (Figure 4).

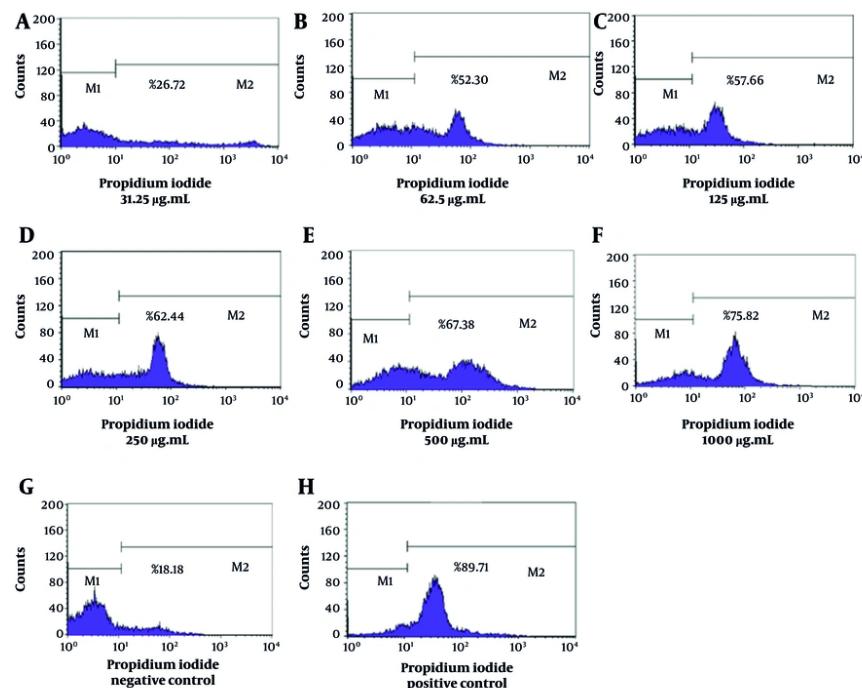
The CC<sub>50</sub> values for *T. vulgaris* and *Z. multiflora* were determined to be 820.4  $\mu$ g/mL and 799.6  $\mu$ g/mL, respectively. Cell viability after exposure to amphotericin B was found to be 47.30%.

#### 4.4. The Effect of *Zataria multiflora* and *Thymus vulgaris* Essential Oils on *Leishmania major* Amastigotes

Our findings indicate that the essential oils of *Z. multiflora* and *T. vulgaris* exert a significant effect on intracellular amastigotes. Statistical analysis (one-way ANOVA followed by Tukey's post-hoc test) confirmed that the essential oils of *Z. multiflora* and *T. vulgaris* exhibited a significant dose-dependent reduction in the intracellular amastigote load compared to the NC ( $P < 0.05$ ). As illustrated in Figures 5 and 6, the anti-amastigote activity of *Z. multiflora* essential oil was statistically superior to that of *T. vulgaris*. However, both essential oils demonstrated significantly lower efficacy



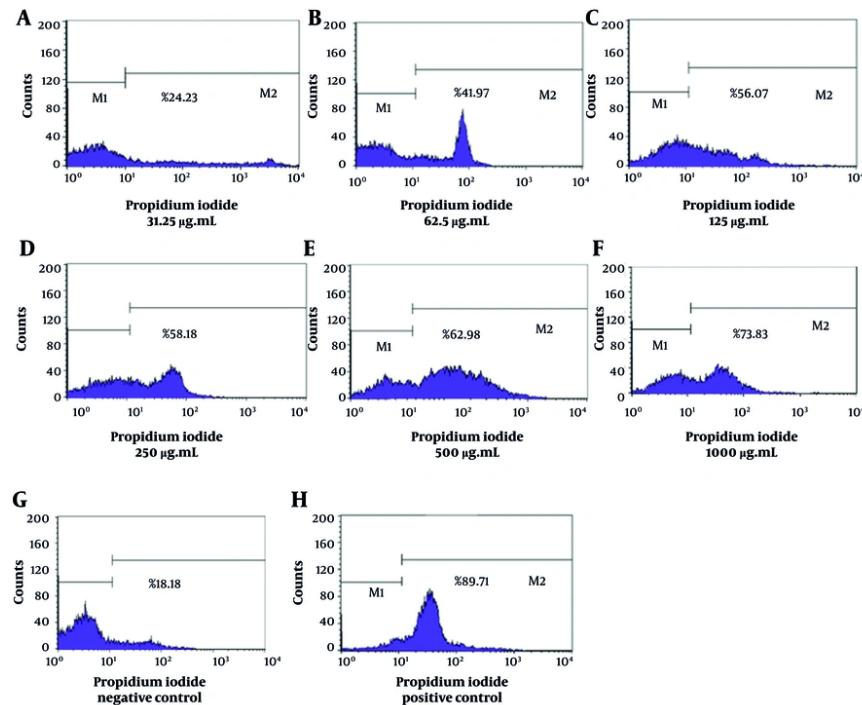
**Figure 1.** The mortality rate of *Leishmania major* promastigotes exposed to different concentrations of *Thymus vulgaris* and *Zataria multiflora* essential oils



**Figure 2.** Flow cytometry analysis of promastigotes treated with *Zataria multiflora* essential oil at indicated concentrations (A-F), negative control (G), and Amphotericin B (20 µg/mL) (H). Cell viability was assessed by propidium iodide (PI) staining; the M2 marker indicates non-viable cells.

in clearing the infection than the PC, amphotericin B. The IC<sub>50</sub> values for *Z. multiflora* and *T. vulgaris* were determined to be 118.4 and 131.2 µg/mL after 24 hours, and 87.28 and 100.68 µg/mL after 48 hours of exposure to the parasite, respectively. The Selectivity Index (SI) for *T. vulgaris* essential oil against promastigotes and

amastigotes (48 hours) was 8.84 and 8.14 µg/mL, respectively. For *Z. multiflora* essential oil, the SI against promastigotes and amastigotes (48 hours) was 13.39 and 9.16 µg/mL, respectively, indicating moderate selective toxicity of both essential oils towards the parasite over host cells (Table 3, Figures 5 and 6).



**Figure 3.** Flow cytometry analysis of promastigotes treated with *Thymus vulgaris* essential oil at indicated concentrations (A-F), negative control (G), and Amphotericin B (20 µg/mL) (H). Cell viability was assessed by propidium iodide (PI) staining; the M2 marker indicates non-viable cells.

**Table 3.** Inhibitory Concentration and CC<sub>50</sub> Values of *Zataria multiflora* and *Thymus vulgaris* Essential Oils Against *Leishmania major* Promastigotes and Amastigotes, and Their Corresponding Selectivity Indices

Characteristic	<i>Z. multiflora</i> (µg/mL)	<i>T. vulgaris</i> (µg/mL)
Promastigote IC <sub>50</sub>	59.69	92.75
Amastigote IC <sub>50</sub> (48 hours)	87.28	100.68
Macrophage CC <sub>50</sub>	799.6	820.4
SI for promastigotes	13.39	8.84
SI for amastigotes (48 hours)	9.16	8.14

Abbreviations: IC<sub>50</sub>, Inhibitory Concentration; SI, Selectivity Index;

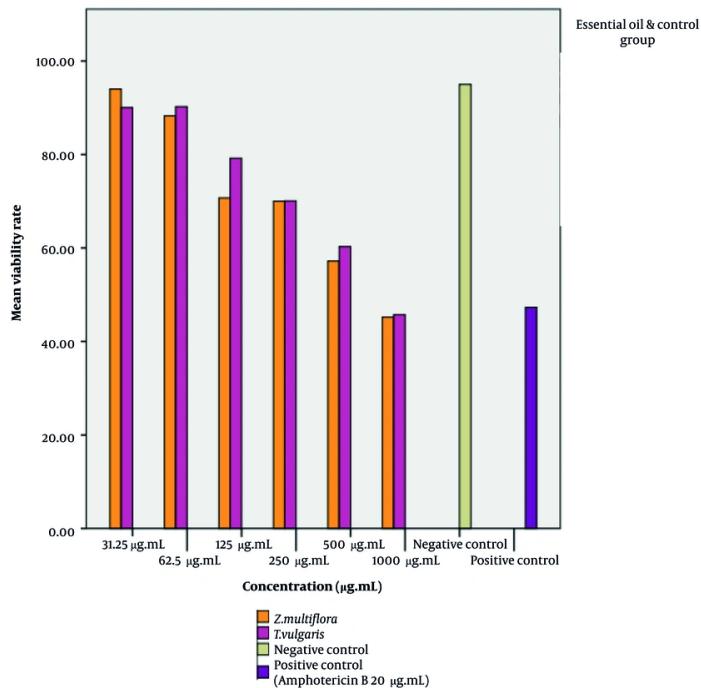
## 5. Discussion

### 5.1. Documented Bioactivities of *Zataria multiflora* and *Thymus vulgaris*

*Zataria multiflora*, native to central and southern Iran, has demonstrated antibacterial, antifungal, and antiparasitic properties (14, 31). Similarly, *T. vulgaris* is known for its antimicrobial activities (32). One study demonstrated that treatment with *Z. multiflora* essential

oil at a concentration of 100 µg/mL exhibited significantly higher toxicity against *Leishmania tropica* parasites, reducing infectivity by more than 86% in macrophages infected with *Leishmania tropica* amastigotes compared to the untreated control group (12). A key finding was that all tested concentrations of *Z. multiflora* essential oil exhibited significant antileishmanial activity against both extracellular promastigotes and intracellular amastigotes of *Leishmania tropica* in infected macrophages under in vitro conditions. Notably, the essential oil demonstrated particularly high antileishmanial efficacy against amastigotes, with a half - maximal IC<sub>50</sub> of 8.3 µg/mL.

Similarly, in a study by Zaki *et al.*, the antileishmanial effects of silver nanoparticles coated with *T. vulgaris* extract were investigated. The obtained IC<sub>50</sub> values were 4.7 µg/mL for *L. major* promastigotes and 3.02 µg/mL for amastigotes, demonstrating potent activity against both parasite stages (18). In a study conducted by Youssefi *et al.* to investigate the effects of thymol and carvacrol on *Leishmania infantum*, the IC<sub>50</sub> values for promastigotes



**Figure 4.** The viability rate of J774 cells after 48 hours of exposure to *Thymus vulgaris* and *Zataria multiflora* essential oils

were determined to be 7.2 and 9.8 µg/mL, respectively (33). These studies demonstrated the efficacy and therapeutic value of *Z. multiflora* and *T. vulgaris* extracts and compounds against other *Leishmania* species.

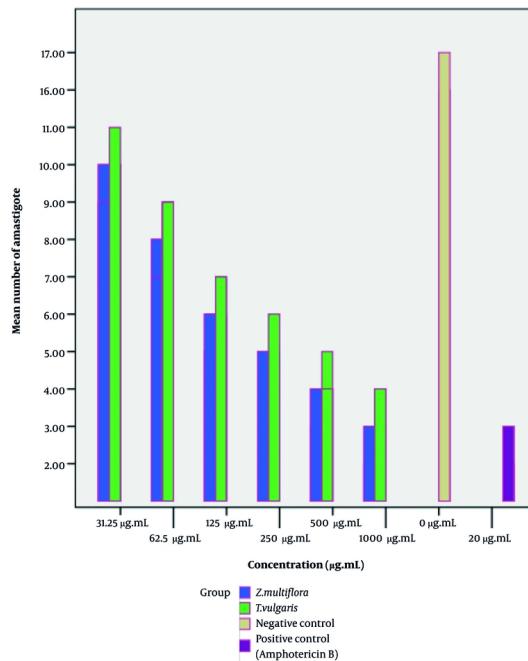
#### 5.2. Key Findings and Chemical Basis of the Present Study

However, despite these encouraging findings, a comprehensive understanding of the direct antileishmanial efficacy of the essential oils of *Z. multiflora* and *T. vulgaris* specifically against *L. major*, a highly relevant species in our region, remains to be fully elucidated. In another study conducted by Barati et al., the antileishmanial effects of the hydroalcoholic extract of *Z. multiflora* against *L. major* were investigated. Their results demonstrated that the extract significantly inhibited the growth of promastigotes, with a half-maximal IC<sub>50</sub> of 7.4 mg/mL (34).

Our study highlights the potential of essential oils from *Z. multiflora* and *T. vulgaris* as promising antileishmanial agents. Gas chromatography-mass spectrometry analysis revealed that thymol and

carvacrol were the major bioactive compounds, known for their antimicrobial and antiparasitic effects. In vitro assays demonstrated significant dose-dependent inhibition of *L. major* promastigotes, with IC<sub>50</sub> values of 59.69 µg/mL for *Z. multiflora* and 92.75 µg/mL for *T. vulgaris*. The cytotoxicity assessments on J774 macrophages further supported the safety profile of these essential oils, with CC<sub>50</sub> values of 799.6 µg/mL for *Z. multiflora* and 820.4 µg/mL for *T. vulgaris*. The findings of this study also revealed that the IC<sub>50</sub> values for the essential oils of *Z. multiflora* and *T. vulgaris* after 48 hours of exposure to *L. major* amastigotes were 87.28 and 100.68 µg/mL, respectively.

In light of the aforementioned study, it can be concluded that the antileishmanial effects of these plants are likely due to the presence of these active compounds. Additionally, their study demonstrated that the tested compounds exhibited the highest growth inhibition at higher concentrations. In our study, the highest inhibition of parasite growth was observed at higher concentrations, which may corroborate previous findings reported in the literature.



**Figure 5.** Mean number of Leishmania amastigotes after 24 hours of incubation following treatment with varying concentrations of *Zataria multiflora* and *Thymus vulgaris* essential oils compared to negative and positive controls. The positive control utilized Amphotericin B at 20 µg/mL.

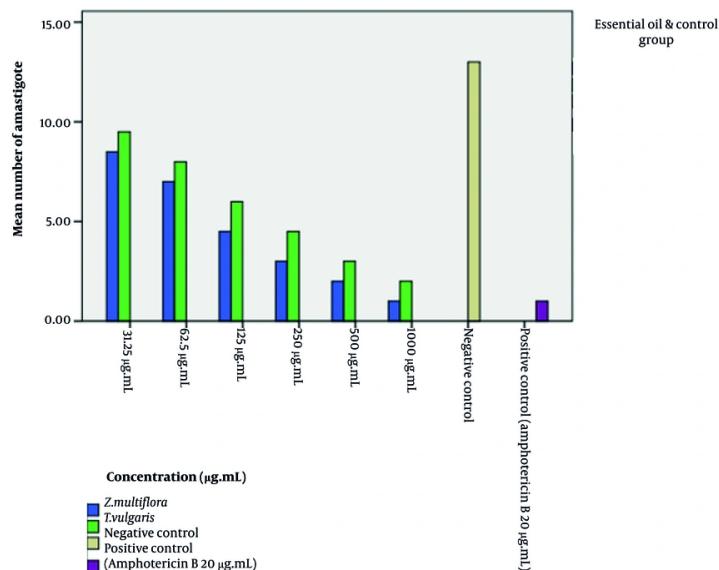
### 5.3. Comparison with Existing Literature and Potential Mechanisms

This indicates a therapeutic window that could be beneficial for further development in clinical settings, aligning with the growing interest in natural products as alternatives to synthetic pharmaceuticals. The observed cytotoxicity levels suggest that while these essential oils exert significant anti-parasitic effects, they also retain the potential for safety in therapeutic applications. The antileishmanial effects of these essential oils may be attributed to their complex chemical compositions, which likely act synergistically (35, 36). This synergy is not limited to the dominant phenols; other major components such as γ-terpinene and p-cymene are known to exert membrane-perturbing and pro-oxidant effects, while terpinene-4-ol can disrupt mitochondrial function. The presence of (E)-caryophyllene may also contribute to a potential immunomodulatory response (37, 38). Previous studies have shown that the combination of multiple bioactive compounds can lead to improved therapeutic outcomes. Thymol and carvacrol, in particular, are

known to disrupt cell membrane integrity and induce oxidative stress in parasites (39-41).

The proposed mechanism involves the interaction of these phenolic compounds with the phospholipid bilayers of the parasite's cell membrane, increasing its permeability and leading to the leakage of vital intracellular components such as potassium ions, proteins, and nucleotides, ultimately causing cell lysis (42, 43). Furthermore, these compounds can disrupt mitochondrial function by collapsing the mitochondrial membrane potential, a critical step for ATP production, thereby depriving the parasite of energy (33). These compounds may also act by inhibiting key enzymes in protozoal metabolism, including dihydrofolate reductase (DHFR).

The inhibition of DHFR leads to the depletion of cellular folate reserves, as this enzyme plays a vital role in producing tetrahydrofolate, a precursor for cofactors essential in the synthesis of purines, dTTP, and amino acids. Ultimately, this process results in the cessation of cell proliferation and cell death (44, 45). Another key mechanism is the induction of an apoptotic-like death pathway in *Leishmania* parasites, characterized by



**Figure 6.** Mean number of *Leishmania* amastigotes after 48 hours of incubation following treatment with varying concentrations of *Zataria multiflora* and *Thymus vulgaris* essential oils compared to negative and positive controls. The positive control utilized Amphotericin B at 20 µg/mL.

phosphatidylserine externalization, DNA fragmentation, and activation of caspase-like proteases (46, 47). The lipophilic nature of thymol and carvacrol facilitates their penetration into the parasite, enabling these multi-faceted attacks. This multi-target mechanism is advantageous as it reduces the likelihood of the development of parasite resistance, a significant limitation of current single-target drugs (48).

#### 5.4. Conclusions

This study demonstrates the anti-leishmanial potential of essential oils from *Z. multiflora* and *T. vulgaris* against *L. major*. In vitro tests demonstrated effective dose-dependent inhibition of *L. major* promastigotes and amastigotes, with low toxicity observed in J774 macrophages, indicating a favorable safety profile. These findings suggest that these essential oils could be promising natural alternatives for leishmaniasis treatment, especially given the challenges of drug resistance and side effects with current therapies. Further in vivo studies are needed to validate their therapeutic potential.

#### 5.5. Limitations and Future Research Directions

Given the ongoing challenges associated with conventional leishmaniasis therapies, including drug resistance and adverse effects, the exploration of these natural alternatives is timely and necessary. However, this study was limited to in vitro experiments, and further in vivo studies are needed to validate these findings. Future research should also explore the formulation of these essential oils for topical or systemic use in clinical settings.

In conclusion, our findings underscore the potential of *Z. multiflora* and *T. vulgaris* essential oils as effective and safe alternatives for leishmaniasis treatment. Continued research into their mechanisms of action and therapeutic applications is essential to address the global burden of this neglected tropical disease. The identification of their bioactive compounds and the elucidation of their mechanisms of action remain critical areas for future exploration, with the ultimate goal of improving health outcomes for affected populations.

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## Footnotes

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