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Original paper

In vitro antileishmanial activity of hydroalcoholic *Thymbra spicata* extract on *Leishmania major* promastigotes

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ABSTRACT. Cutaneous leishmaniosis is a major worldwide public health problem with annual incidence of 1.5 million cases across 98 countries. Treatment still relies on the use of chemical drugs with increasing resistance and side effects. The aim of this study was to investigate the anti-leishmanial effect of the hydroalcoholic *Thymbra spicata* extract on *Leishmania major* (*L. major*) promastigotes. In this study, 1×10^5 *L. major* promastigotes were cultured in 96-well plates and treated with different concentrations of hydroalcoholic *T. spicata* extract (12.5 to 400 µg/ml) then incubated at 25°C for 24, 48 and 72 hours. Lethal percentage of promastigotes in each well was determined. RPMI 1640 medium containing *L. major* promastigotes with glucantime or without any treatment were used as positive and negative controls respectively. The 50% lethal concentration (LC50) of *T. spicata* extract and glucantime was calculated by GraphPad Prism software. The results indicated a significant decrease in the number of promastigotes treated with *T. spicata* extract and glucantime in comparison with negative control ($P<0.0001$). LC50 values for *T. spicata* extract were 18.49, 8.58, and 1.64 µg/ml after 24, 48 and 72 hours, respectively. In addition, anti-leishmanial effect of *T. spicata* extract and glucantime were dependent on concentration ($P<0.0001$). Our study revealed *T. spicata* extract as an herbal product against *L. major* promastigotes. However, more investigations are needed to find its antileishmanial activity *in vivo* and clinical trial studies.

Keywords: leishmaniosis, *Leishmania major*, *Thymbra spicata*, herbal extract, medicinal plants

Introduction

Leishmaniosis is a tropical and subtropical disease caused by parasites of the *Leishmania* genus, which are usually transmitted by the bite of infected female phlebotomine sandflies [1,2]. Clinical manifestations of leishmaniosis are usually divided into cutaneous leishmaniosis, muco - cutaneous leishmaniosis (MCL), and visceral leishmaniosis (VL) [3]. According to the World Health Organization (WHO) report, leishmaniosis is found in 98 countries around the world. Approximately, 12 million people in the world are infected, and 350 million people are at risk of acquiring the disease, with an annual incidence of 1–1.5 million new cases of CL and 500,000 for VL [4]. Since no effective vaccine is available for leishmaniosis; treatment of the disease exclusively depends on chemical drugs [5]. Antimonial compounds constitute the main approach for treatment of the leishmaniosis such as pentavalent antimony (meglumine antimoniate or sodium stibogluconate) as the first-line drugs [6]. Although drug resistance is developing by some parasite species [7]. Pentamidine and amphotericin B are second-line drugs, however these drugs showed toxicity and require long-term parenteral administration [8]. Because of high toxicity and emergence of resistance to some commercial compounds for treatment of leishmaniosis, it is

essential to discover an effective, safe and affordable drug. Some traditional medicines act as a valuable source of bioactive agents that are used for treatment many diseases such as leishmaniosis [9]. Given the benefits of using herbal remedies, these compounds should be tested with appropriate selection of antileishmanial activity in every country of the world. In recent years, traditional herbal medicines have received significant attention and substantiated the effectiveness of these combinations in treatment of leishmaniosis [10].

The genus *Thymbra* belongs to the Lamiaceae family. Seven species and one subspecies of *Thymbra* have been characterized in the Mediterranean region of southern Europe, North Africa, and the Middle East [11]. *T. spicata* is a native plant in the flora of Iran. Due to antimicrobial and antiseptic properties, it has become a popular plant among the people [11]. In traditional medicine, *T. spicata* has been used as antiseptic and anti-helminthic agents also for treating diabetes, stomachache, and common cold [12]. On the other hand, its leaves have been traditionally used as a spice that gives a good flavor and taste to food [12].

To date, it has not been investigated anti-leishmanial activity of *T. spicata* extract against *Leishmania* species. Therefore, the aim of the present study was to examine antileishmanial effect of hydroalcoholic extract of *T. spicata* against *L. major* promastigote forms *in vitro.*

Materials and Methods

Preparation of T. spicata extract

T. spicata plant was collected from rangelands and forests of Ilam province on September 2019. The plant was identified (herbarium code: Voucher specimens No 596) by the Jihad-e Daneshgahi center of medicinal plants and agricultural products, Ilam, Iran.

Plant extraction

The leaves and stems of *T. spicata* plant were harvested and washed with distilled water, then shade dried at room temperature. The dried plants grinded by an electric mill to obtain a fine powder and passed through a 10 mm sieve. The prepared powder was transferred into a special cartridge and placed into a volumetric flask contained 70% (v/v) ethanol in distilled water and connected to an. extraction device. The solution was shaken every 30 minutes to better obtain the active ingredients. To separate the solvent, the obtained solution was connected to a vacuum pump and heat source., The obtained extract was placed into an oven at 50 to 60°C for 24 hours to remove remained solvent. The obtained extract was stored at 4°C for further processing.

L. major culture and maintenance

The standard strain of *L. major* (MRHO/IR /75/ER) was obtained from the School of Public Health, Tehran University of Medical Sciences, Iran. The parasites were cultured in the RPMI 1640 media (Gibco, Life Technologies GmbH, Germany) supplemented with 10–15% heat inactivated fetal bovine serum (FBS) (Gibco, Germany), 100 u/ml penicillin, and 100 µg/ml streptomycin (Gibco).

Effect of T. spicata extract against L. major promastigote

Antileishmanial activity of the *T. spicata* extract was done in 96 well plates. *L. major* promastigotes were counted by a hemocytometer chamber. 1×10^5 promastigotes/well were cultured and treated in presence or absence of different concentrations (400 to 12.5 µg/ml) of the hydroalcoholic extract of *T. spicata* and incubated for 24, 48 and 72 hours at 25°C. All experiments were done in triplicate. After the incubation period, 20 µl of each well was harvested and added to the same volume of Eosin 0.1%. The number of viable promastigotes was counted using light microscope. The LC50 of *T. spicata* extract and glucantime was determined as a concentration that is able to kill 50% of promastigotes. Different concentrations of standard drug of glucantime served as positive control.

Statistical analysis

Table 1. Lethal percentage of different concentrations of *T. spicata* extract on *Leishmania major* promastigotes after 24, 48 and 72 hours of incubation

T. spicata extract	24 hours	48 hours	72 hours
12.5 µg/ml	18%	34%	80%
25 µg/ml	22%	24%	85%
50 µg/ml	40%	69%	96%
100 µg/ml	66%	83%	96%
200 µg/ml	85%	92%	98%
400 µg/ml	96%	98%	100%

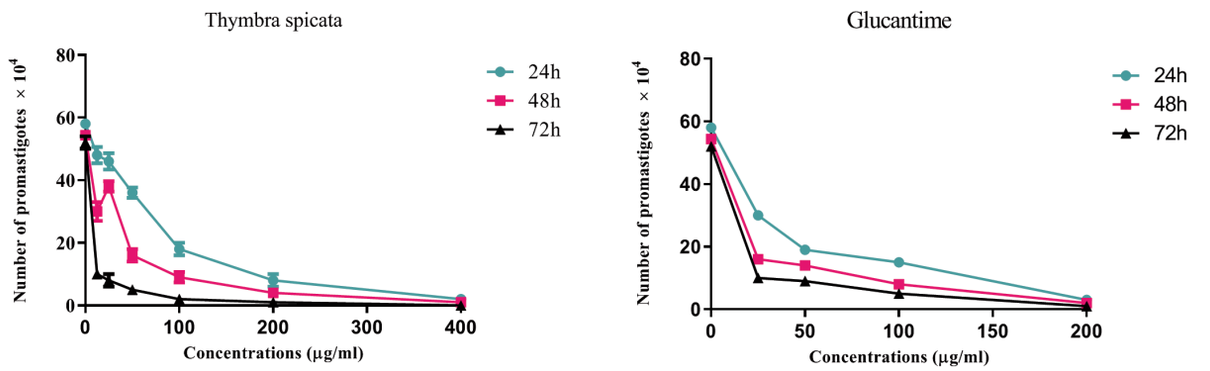

Figure 1. Anti-leishmanial effects of different concentrations of hydroalcoholic extract of *T. spicata* (12.5 to 400µg/ml) and glucantime (12.5 to 200 µg/ml) on the number of *Leishmania major* promastigotes after 24, 48 and 72 hours incubation

The Two-Way Repeated Measure ANOVA was used to evaluate the anti-leishmanial effect of extract and glucantime drug using GraphPad Prism software. Differences were considered statistically significant when P<0.05.

Results

The results from this study have been summarized in Tables 1,2. *T. spicata* extract showed significant effect on killing the promastigotes after 24, 48 and 72 hours in all concentrations as compared to the negative control group (P<0.0001). The maximum antileishmanial activity was found in concentration of 400 µg/ml after 72 hours, and the minimum activity was related to 12.5 µg/ml concentration after 24 hours. LC50 values of hydroalcoholic extract of *T. spicata* against *L. major* promastigotes were 18.49 µg/ml, 8.58 µg/ml and 1.64 µg/ml in 24, 48 and 72 hours, respectively. Additionally, LC50 values for glucantime were 11.54 µg/ml for 24 hours, 5.81 µg/ml for 48 hour, and 3.38 µg/ml after 72 hours of incubation. According to our findings, *T. spicata* extract and glucantime exhibited a dose- and time-dependent manner on killing the promastigotes (Figure 1). Analysis of variance between groups showed that lethal effect of glucantime on *L. major* promastigotes was higher than *T. spicata* extract after 24 and 48 hours (P<0.0001). However, no significant difference was found between *T. spicata* extract and glucantime after 72 hour treatment (P>0.05).

Table 2. Lethal percentage of different concentrations of glucantime on *Leishmania major* promastigotes after 24, 48 and 72 hours of incubation

Glucantime	24 hours	48 hours	72 hours
25 µg/ml	54%	60%	81%
50 µg/ml	66%	75%	84%
100 µg/ml	74%	88%	90%
200 µg/ml	94%	96%	98%

Discussion

Leishmaniosis has been reported as one of the most important neglected diseases [13]. Use of common chemical drugs for treatment of leishmaniosis such as pentavalent antimonials, amphotericin B, paromomycin, miltefosin and liposomal amphotericin B have been limited because of various reasons including low efficacy, high cost, painful injections and side effects [14]. Therefore, some populations have recently tendency to use native herbal medicines to treat leishmaniosis because plant drugs have less side effects and are more accessible especial for low-income people [10,15]. *In vitro* and *in vivo* anti-leishmanial activities of some medicinal plants such as *Pistacia khinjuk* [16], *Artemisia annua* [17], *Maesa balansae* [18], *Thymus vulgaris* [19], *Achillea millefolium* [19], *Berberis vulgaris* [20], *Artemisia essence* [21], *Tephrosia vogelii* [22], *Coriandrum sativum* [23,24], *Aloe vera* [17], *Ricinus communis* [23], *Lippia sidoides* [24], *Copaifera reticulata* [24], curcumin and its bioactive compounds [25] and etc. have been identified. In the present study, antileishmanial activity of hydroalcoholic *T. spicata* extract was examined against *L. major* promastigotes. Our findings showed that the

concentration of 200 and 400 µg/ml of *T. spicata* extract had desirable anti-leishmanial effects so that the highest leishmanicidal activity was related to the concentration of 400 µg/ml of *T. spicata* killed 100 and 96 percentage of *Leishmania* promastigotes after 72 and 24 hours, respectively. Therefore, it was demonstrated that *T. spicata* extract acts on *L. major* promastigotes in a dose and time-dependent manner. In the current study, IC50 values for *T. spicata* extract and glucantime were ranged within 1.64–18.49 µg/ml and 3.38–11.54 µg/ml, respectively. Analysis of variance between groups indicated that anti-leishmanial effect of glucantime on *L. major* promastigotes was significantly higher than *T. spicata* extract after 24 and 48 hours ($P<0.0001$) while, there was no significant difference between those after 72 hours ($P>0.05$). Our finding is consistent to Yousefi et al. [26] which indicated inhibitory effect of *Alkanna tincturia* and *Peganum harmala* extracts on the *in vitro* growth of *L. major* promastigotes. In another study, Badirzadeh et al. [27] revealed that *Urtica dioica* extract significantly reduced the *L. major* promastigotes viability.

In addition, effects of aqueous extract of *Artemisia seiberi* and artemisinin were determined on *L. major* under *in vitro*. The findings showed that both extracts had the leishmanicidal effects and reduced the parasite number in comparison with the control groups. In addition, anti-leishmanial activity of *Artemisia sieberi* was higher compared with artemisinin [28]. The finding of the present study supports the hypothesis that *T. spicata* has an effective antileishmanial activity although it is required to further investigations to determine cytotoxicity and *in vivo* anti-leishmanial effectiveness of this extract.

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