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Assessment of Leishmanicidal Properties and Cell Toxicity of the Hydroalcoholic Extract from Thymus kotschyanus In Vitro

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ABSTRACT

Existing therapies for leishmaniasis face challenges including adverse reactions, reduced effectiveness, and emerging resistance. Plants from the Thymus genus are recognized for their therapeutic properties. This research investigated the potential antileishmanial activity and toxic effects of a hydroalcoholic extract derived from Thymus kotschyanus through laboratory-based tests. The above-ground portions of T. kotschyanus were processed via maceration using 70% ethanol to obtain the extract. Its impact was assessed on both promastigote and amastigote forms of Leishmania major by enumerating parasites directly within a macrophage system. Toxicity was measured using the MTT method. Rates of macrophage infection and counts of intracellular amastigotes were compared against untreated controls and glucantime-treated groups (standard drug). The extract suppressed proliferation of promastigotes and amastigotes, with peak inhibition at 48 hours. IC50 values against non-infected macrophages reached 381.8 µg/L at 48 hours and 392.9 µg/L at 72 hours. Doses of 300 and 350 µg/mL markedly lowered the proportion of infected macrophages ($p=0.042$ and $p=0.001$, respectively) and the quantity of amastigotes inside macrophages ($p<0.05$ and $p=0.001$, respectively) relative to controls. These doses outperformed 5 µg/mL glucantime in reducing amastigote counts across time points ($p<0.05$ and $p<0.01$, respectively), while showing comparable efficacy to 10 and 15 µg/mL glucantime. The hydroalcoholic extract from Thymus kotschyanus could serve as a promising and relatively non-toxic natural agent against L. major. Additional studies are required to establish it as a novel plant-based treatment.

Keywords: Amastigote, Cytotoxicity, Leishmania major; Promastigote, Thymus kotschyanu

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Introduction

Leishmaniasis ranks among the most overlooked vector-transmitted parasitic illnesses that are resurging, posing significant health concerns in numerous tropical and subtropical areas, particularly Iran. The disease manifests in various forms, such as cutaneous, mucocutaneous, and visceral [1, 2]. It affects 98 nations globally, with annual new cases ranging from 900,000 to 1,600,000, and approximately 350 million individuals vulnerable to infection worldwide [3]. Caused by protozoans of the Leishmania genus, the infection spreads via bites from infected phlebotomine sandflies [1, 3]. The predominant type is cutaneous leishmaniasis, appearing as zoonotic (wet) or anthroponotic (dry) variants, linked to Leishmania major and L. tropica, respectively [1].

The parasite exhibits two morphological stages: flagellated promastigotes in the sandfly vector's digestive tract and non-flagellated amastigotes within mammalian host macrophages [2, 4]. Since amastigotes reside inside host macrophages and drive disease symptoms and progression, they serve as key targets for evaluating antileishmanial agents in laboratory settings.

Although extensive efforts have aimed at discovering new antileishmanial agents, primary treatments for cutaneous forms remain pentavalent antimonials like glucantime, associated with severe adverse effects, inconsistent effectiveness, prolonged regimens, and increasing resistance [5, 6]. Alternatives such as amphotericin B, pentamidine, and miltefosine also carry risks including muscle pain, fever, loss of appetite, vomiting, red blood cell breakdown, and damage to organs like the liver, kidneys, and spleen [6-8]. No human vaccine is currently approved [6, 7]. Thus, exploring natural or synthesized compounds offers potential alternatives. Medicinal plants have long been employed for various ailments, including infectious conditions like cutaneous leishmaniasis [9, 10]. Among these, Thymus kotschyanus Boiss. & Hohen (Lamiaceae family; locally called "Avishan" or "Azorbe") is native to certain Iranian regions, such as mountainous areas in Ardabil, West Azerbaijan, Hamedan, and Kurdistan provinces. Traditionally, its aerial parts, especially fragrant leaves, are prepared as infusions, used as seasonings, or applied medicinally [11, 12], often for managing chronic gastritis and digestive issues [8, 13]. Key components in its essential oil include carvacrol, thymol, α-terpineol, borneol, linalool, 1,8-cineole, p-cymene, γ-terpinene, isomenthone, α-pinene, and others [12, 14-16].

Prior research has documented various benefits of T. kotschyanus, including antioxidant [12, 15], antispasmodic [13], antibacterial, and antifungal properties [14]. However, no prior studies have examined its impact on intracellular amastigotes of Old World Leishmania species causing cutaneous disease. Accordingly, this work evaluated the leishmanicidal potential of T. kotschyanus hydroalcoholic extracts against promastigotes and intracellular amastigotes of L. major, along with its toxicity toward RAW264.7 macrophage cells in laboratory conditions.

Materials and Methods

Ethical approval

The research protocol received approval from the Ethics Committee at Shahid Beheshti University of Medical Sciences (code: IR.SBMU.RETECH.REC.1400.011).

Reagents

Materials included RPMI-1640 medium and fetal bovine serum (Gibco™), glucantime (meglumine antimoniate; Sanofi Aventis, France), MTT reagent (Sigma-Aldrich, USA), penicillin/streptomycin (Sigma-Aldrich, Germany), dimethyl sulfoxide (DMSO; Sigma Chemical), DMEM, and Novy–MacNeal–Nicolle (NNN) medium.

Plant collection and extraction

Thymus kotschyanus Boiss. et Hohen was gathered from the former route between Ardabil and Sarein in Ardabil province, northwestern Iran (July 2020). A reference specimen was archived at the Pharmacognosy Department herbarium, School of Pharmacy (Mashad, Iran), under accession 13580. The above-ground parts were cleansed, shade-dried, pulverized, and placed in a percolator. One hundred grams of powdered material underwent successive maceration with 70% aqueous ethanol (100 mL) for a minimum of 72 hours with occasional stirring at ambient temperature. The resulting extract was passed through a 0.22 µm sterile filter (Sartorius, Germany), concentrated via rotary evaporation under reduced pressure at 40°C, and preserved at −20°C until use.

Parasite and cell culture

A reference strain of Leishmania major (MRHO/IR/75/ER) was sourced from Tehran University of Medical Sciences (Tehran, Iran), initially grown in NNN medium at 25±1°C. For larger yields, promastigotes were transferred to RPMI-1640 supplemented with 10% inactivated fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cultures were kept at 25±1°C without media refresh for 10±1 days to obtain stationary-phase metacyclic promastigotes. For toxicity assessments, RAW264.7 macrophages were acquired from the Iranian Biological Resource Center (IBRC, Iran). This cell line features loosely attached or rounded forms capable of phagocytosis and pinocytosis [16], offering a reliable macrophage model with consistent traits and straightforward maintenance. Cells were propagated in DMEM with the specified supplements at 37°C in 5% CO2 humidified conditions.

Cytotoxicity evaluation

To assess cytotoxicity, 100 µL of RAW264.7 macrophage suspension in complete DMEM medium (at a density of 5×10^5 cells/mL) was added to each well of a 96-well plate. Subsequently, 100 µL of T. kotschyanus extract at varying concentrations (ranging from 37 to 1500 µg/mL) was introduced into the wells. Plates were maintained at 37°C with 5% CO_2, and all experiments were conducted in triplicate. Wells containing culture medium alone (without extract) or medium with 1% DMSO served as controls. For the MTT procedure, 10 µL of MTT solution (5 mg/mL) was pipetted into each well, followed by a 4-hour incubation at 37°C in the dark. After removing the medium and MTT, 100 µL of DMSO was added, and plates were held for 30 minutes to dissolve formazan crystals. Absorbance was measured at 570 nm using an ELISA reader. Cell viability was computed using the formula [17]: Viability percentage = 100 × (Absorbance of treated cells - Absorbance of the blank/Absorbance of negative control cells - Absorbance of the blank)

Here, untreated macrophages in complete RPMI-1640 served as the negative control, while medium lacking cells and treatments acted as the blank. Viability percentages were plotted against extract concentrations, and the IC_{50} (concentration causing 50% growth inhibition) was determined via GraphPad Prism 8 software.

Assessment of anti-promastigote activity

The impact of T. kotschyanus hydroalcoholic extract on L. major promastigotes was examined. Briefly, 100 µL of promastigotes in logarithmic phase (10^6 cells/mL) was dispensed into wells of a 96-well plate. An equal volume of extract at concentrations from 37 to 1500 µg/mL was then added. Plates were incubated at 25±1°C, with evaluations at 24, 48, and 72 hours. Cultures without extract or containing DMSO were included as controls. Post-incubation, viable parasites were enumerated directly under a light microscope and compared to controls. The inhibition percentage was derived as: Inhibition rate (%) = 100 − [(Number of viable parasites in treated wells / Number of viable parasites in untreated control) × 100]. Values represent means from triplicate experiments.

Impact on infected macrophages and intracellular amastigotes

Sterile coverslips were placed in 6-well plates, followed by addition of 500 µL of macrophages (10^5 cells/mL) and incubation at 37°C with 5% CO_2 for 4–5 hours. Non-adherent cells and medium were removed, and fresh medium with stationary-phase L. major promastigotes (parasite-to-macrophage ratio of 10:1) was introduced to allow infection. After 24 hours, extracellular promastigotes were washed away with RPMI-1640. Infected macrophages were then exposed to four extract doses (150, 250, 300, and 350 µg/mL) at 37°C with 5% CO_2 for 24, 48, and 72 hours. Untreated infected macrophages served as negative controls, while those treated with glucantime (5, 10, and 15 µg/mL) acted as positive reference controls. At completion, supernatants were discarded, slides were fixed in methanol, stained with Giemsa, and examined microscopically. Treatment effects were quantified by assessing the proportion of infected macrophages (percentage of at least 100 cells harboring amastigotes) and amastigote survival (percentage within 100 macrophages), relative to controls and the reference drug. All assays were performed in triplicate.

Statistical analysis

Data are expressed as means from three independent replicates. IC_{50} values were derived using GraphPad Prism 8. Comparisons of means employed the Wilcoxon signed-ranks test and Student's t-test. The Wilcoxon test was applied to differences in macrophage infection rates and intracellular amastigote counts between treated and control groups. Statistical significance was set at $P < 0.05$.

Results and Discussion

Rising parasite resistance to standard treatments, coupled with high costs, extended treatment periods, and toxicities (including liver and kidney damage), underscores the need for new therapeutic options [6, 18]. T. kotschyanus emerges as a candidate natural agent with potential antileishmanial properties. Cytotoxicity of T. kotschyanus hydroalcoholic extract at multiple doses against RAW264.7 macrophages was determined via MTT assay at 24, 48, and 72 hours, with comparisons to controls **(Figure 1).** IC_{50} values on uninfected macrophages were 381.8 µg/mL at 48 hours and 392.9 µg/mL at 72 hours. Toxicity increased with concentration, but overall, the extract exhibited minimal cytotoxicity toward uninfected macrophages. These observations align with findings by Machado *et al*., who noted that essential oil from Thymus capitellatus lacked toxicity against mammalian cells [10].

The antileishmanial effect on L. major promastigotes is illustrated in **Figure 2**, where mortality rates rose with extract concentration ($p<0.05$). Maximal inhibition occurred at 48 hours compared to other time points, with significant differences observed ($p<0.05$).

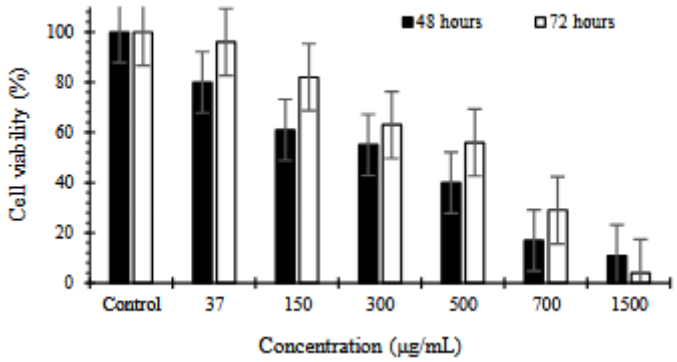

Figure 1. Cell viability percentages for non-infected RAW264.7 macrophages treated with varying doses of the hydro-ethanolic extract from Thymus kotschyanus over 48 and 72 hours of exposure, relative to the untreated control; data are presented as means ± SEM (n = 3).

In the current investigation, varying doses of the plant extract induced mortality in L. major promastigotes ranging from 31% at 37 µg/mL to no less than 86% at 1500 µg/mL **(Figure 2),** with the inhibition exhibiting a dose-dependent pattern ($p<0.05$). These antileishmanial observations align with findings from Najm *et al*., demonstrating that ethanolic extracts derived from three Artemisia species (A. persica, A. spicigera, and A. fragrance) suppressed the proliferation of L. major [19]. Furthermore, the study revealed that the leishmanicidal potency of different concentrations of the T. kotschyanus extract was most pronounced against promastigote stages at the 48-hour mark relative to other durations evaluated. These outcomes are in agreement with prior reports from other investigators [10, 19]. The deadly impact of the T. kotschyanus extract likely stems from various biochemical and morphological disruptions observed in earlier research [10, 19]; for instance, treatment of promastigotes with essential oil from Thymus capitellatus resulted in irregularly shaped cells, mitochondrial swelling, and the formation of autophagosome-like structures [10].

The leishmanicidal properties of various doses of the T. kotschyanus hydroalcoholic extract against L. major-infected macrophages were assessed at 24, 48, and 72 hours post-treatment, compared to untreated **controls (Table 1).**

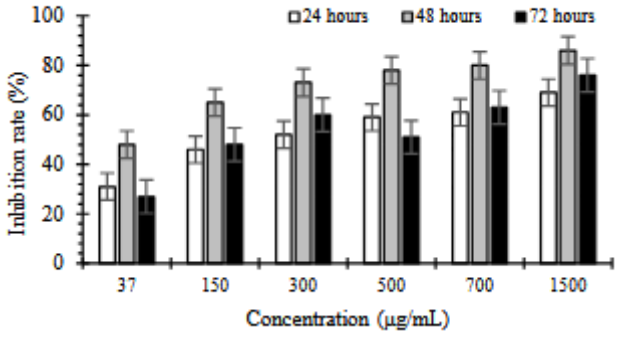

Figure 2. Inhibition percentages of L. major promastigotes exposed to various concentrations (ranging from 37 to 1500 µg/mL) of Thymus kotschyanus extract following 24, 48, and 72 hours of incubation; values are expressed as mean ± SEM from triplicate experiments.

The results indicated that the leishmanicidal activity of the extract exhibited a clear concentration-dependent pattern, with greater efficacy observed as the extract concentration increased across all incubation periods. Notably, at concentrations of 300 and 350 µg/mL, the plant extract led to a statistically significant decrease in the

proportion of RAW264.7 cells infected with L. major ($p = 0.042$ and $p = 0.001$, respectively) throughout the 24- to 72-hour period when compared to untreated infected macrophages serving as controls. These observations are consistent with the data presented by Najm *et al*. [19].

In a manner comparable to its impact on infection rates in macrophages, the anti-amastigote activity of the T. kotschyanus crude extract against the mean number of amastigotes per macrophage also followed a concentration-dependent trend **(Table 2).** The study demonstrated a marked decrease in the average count of intracellular amastigotes at 300 µg/mL ($p < 0.05$) and 350 µg/mL ($p = 0.001$) relative to control groups at 24, 48, and 72 hours after treatment. Moreover, the extent of amastigote reduction was greatest at the 48-hour time point across all tested concentrations of the extract, ranging from 11.7% reduction at 150 µg/mL to 83.52% at 350 µg/mL **(Table 2)**. **Figure 3** presents the infection rates in macrophages treated with T. kotschyanus extract in comparison to glucantime (the standard reference drug) at 24, 48, and 72 hours post-incubation.

Table 1. Leishmanicidal effects of *Thymus kotschyanus* extract on infected macrophages with *Leishmania major*

Concentration (µg/mL)	Percentage of infected macrophages			p-Value	Reduction in % infected macrophages
	Time (h)	Treated	Control		
150	24	50.00 ± 1.00	58.00± 1.00	0.575	13.79
	48	65.67± 1.53	69.67±1.53	0.26	5.74
	72	71.67± 1.53	75.33± 1.53	0.476	4.86
250	24	49.33± 1.53	58.00± 1.00	0.075	14.95
	48	59.00 ± 1.53	69.67±1.53	0.066	15.32
	72	64.00 ± 1.53	75.33± 1.53	0.057	15.04
300	24	35.33± 1.15	58.00± 1.00	0.042	39.09
	48	44.33± 1.53	69.67±1.53	0.01	36.37
	72	49.33± 1.53	75.33± 1.53	0.035	34.51
350	24	22.33± 1.53	58.00± 1.00	0.001	61.5
	48	30.67± 1.53	69.67±1.53	0.001	55.98
	72	32.00± 1.00	75.33± 1.53	0.001	57.52

Data represent mean ± SEM of experiments done in triplicate

Table 2. Leishmanicidal activities of *Thymus kotschyanus* extract on the mean number of amastigotes in macrophages

Concentration (µg/mL)	No. of amastigotes per 100 Mϕ			p-Value	Reduction of amastigotes per 100 Mϕ
	Time (h)	Treated	Control		
150	24	23.67 ± 1.53	25.33± 1.53	0.15	6.55
	48	25.00 ± 1.00	28.33± 1.15	0.086	11.75
	72	29.00 ± 1.00	32.67± 1.53	0.1	11.23
250	24	20.33 ± 0.58	25.33± 1.53	0.07	19.74
	48	21.33 ± 1.53	28.33± 1.15	0.065	24.71
	72	24.67 ± 1.53	32.67± 1.53	0.084	24.49
300	24	11.00 ± 1.00	25.33± 1.53	0.001	56.57
	48	6.67 ± 1.53	28.33± 1.15	0.001	76.46
	72	14.33 ± 1.53	32.67± 1.53	0.05	56.14
350	24	8.00 ± 1.00	25.33± 1.53	0.001	68.42
	48	4.67 ± 0.58	28.33± 1.15	0.001	83.52
	72	9.33 ± 1.53	32.67± 1.53	0.001	71.44

Data represent mean ± SEM of experiments done in triplicate

In this investigation, no statistically significant difference was observed in the rates of infected macrophages between treatment with 350 µg/mL of the crude extract and 5 µg/mL of the standard drug glucantime ($p > 0.05$). However, at higher glucantime concentrations of 10 and 15 µg/mL, a notable inhibitory effect on infected macrophages was evident when compared to the 350 µg/mL extract concentration in terms of L. major proliferation **(Figure 3).** Previous research on T. kotschyanus has highlighted that its substantial content of phenolic compounds, including thymol and carvacrol, confers potent antimicrobial activity against Candida albicans yeast as well as both Gram-positive and Gram-negative pathogenic bacteria [11, 14, 20, 21]. It is plausible

that these very compounds are responsible for suppressing the development and multiplication of L. major promastigotes and amastigotes.

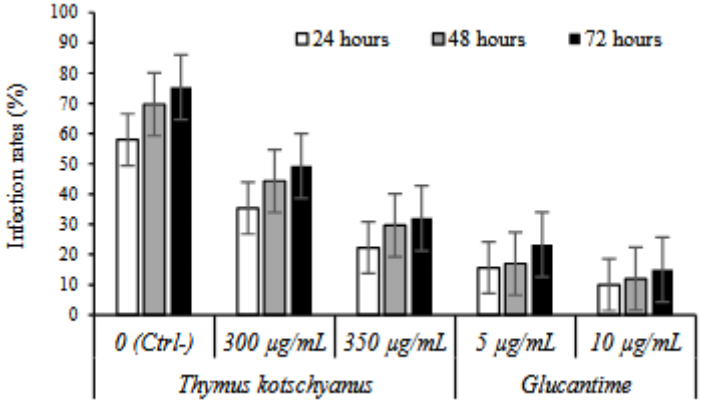

Figure 3. Infection rates in macrophages following treatment with Thymus kotschyanus extract versus glucantime (the reference drug) at 24, 48, and 72 hours post-incubation; values are shown as mean ± SEM from experiments performed in triplicate.

As illustrated in **Figure 4**, the anti-amastigote activity of the T. kotschyanus crude extract on the mean number of amastigotes per macrophage was evaluated at 24, 48, and 72 hours after treatment and compared to glucantime as the standard drug.

Extract concentrations of 300 and 350 µg/mL demonstrated significantly greater efficacy against amastigote forms compared to 5 µg/mL glucantime at all tested time points ($p < 0.05$ and $p < 0.01$, respectively), while no statistical differences were observed between these extract concentrations and 10 or 15 µg/mL glucantime.

The viability percentage of amastigotes in macrophages treated with 350 µg/mL of the plant extract was comparable to that achieved with 10–15 µg/mL glucantime across the three time points examined; specifically, the average number of amastigotes per 100 macrophages was 4.67 ± 0.58 for this extract concentration, compared to 7.33 ± 1.53 for 10 µg/mL glucantime and 2.67 ± 0.58 for 15 µg/mL glucantime at the 48-hour incubation period.

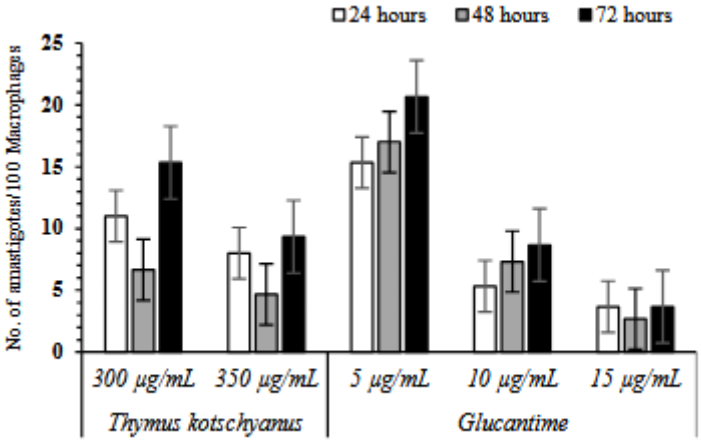

Figure 4. Viability percentages of amastigotes in macrophages treated with Thymus kotschyanus extract versus glucantime (the reference drug) at 24, 48, and 72 hours post-incubation; data are presented as mean ± SEM from triplicate experiments.

The investigation demonstrated that the hydroalcoholic extract of this plant exhibited comparable efficacy against L. major to that of glucantime ($p > 0.05$), achieving inhibitory effects within a concentration range of 300–350 µg/mL. Based on the outcomes of this in vitro evaluation, the T. kotschyanus extract could be regarded as a promising and safe natural agent against L. major, representing a viable alternative particularly in light of the

potential side effects associated with glucantime. However, further research is required to establish the plant as a novel herbal treatment option.

Conclusion

The results highlighted substantial leishmanicidal activity of T. kotschyanus extracts against L. major, underscoring the potential of this plant as a natural resource for developing a new antileishmanial agent. Nonetheless, additional studies are essential to validate these observations in animal models and human clinical trials. Given the pronounced leishmanicidal properties combined with minimal or negligible cytotoxicity toward mammalian cells, T. kotschyanus emerges as a promising natural candidate for the formulation of a novel drug with leishmanicidal potential.

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Conflict of Interest: None

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Ethics Statement: None

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