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Comparing in vitro antileishmanial activity of Scrophularia bavanatica hydroalcoholic extract against Leishmania major and Leishmania tropica promastigotes

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Article

Keywords: Scrophularia bavanatica, Leishmania major, Leishmania tropica, herbal medicine

Posted Date: March 6th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-8826035/v1

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Additional Declarations: No competing interests reported.

Abstract

Cutaneous leishmaniasis (CL), a neglected tropical disease, presents a significant therapeutic challenge due to the toxicity and emerging resistance of first-line drugs like Glucantime. Medicinal plants offer a promising reservoir for novel antileishmanial agents. *Scrophularia bavanatica* is a traditionally-used plant in Iran; yet, its efficacy against different Leishmania species remains unexplored. This study compared the *in vitro* antileishmanial efficacy of *Scrophularia bavanatica* hydroalcoholic extract (SBE) against promastigotes of *Leishmania major* and *Leishmania tropica*. Two parallel *in vitro* studies were conducted. Promastigotes of *L. major* [MRHO/IR/75/ER] and *L. tropica* [MHOM/IR/NADIM3] were exposed to various concentrations of SBE (0.01–100 mg/mL and 0.2–125 mg/mL, respectively) and standard Glucantime for 24, 48, and 72 hours. Parasite viability was assessed using the XTT cell proliferation assay. LC_{50} values and survival percentages were calculated and statistically compared. SBE exhibited a time- and concentration-dependent antileishmanial effect against both species. However, *L. tropica* demonstrated significantly higher resistance. The LC_{50} of SBE for *L. major* in the dynamic phase (47.1 µg/mL) was significantly lower than that for *L. tropica*, for which the LC_{50} exceeded the highest tested concentration (> 125 µg/mL). A significant reduction in *L. tropica* promastigote survival was only observed after 72 hours of exposure (P = 0.000), whereas *L. major* showed significant susceptibility at 24 and 48 hours. Glucantime was effective against both species by 72 hours, but required higher concentrations for *L. tropica*. *Scrophularia bavanatica* extract possesses potent antileishmanial activity, but its efficacy is highly species-specific. *L. tropica* is inherently more resistant to both SBE and Glucantime compared to *L. major*, necessitating longer exposure times and higher doses for effective treatment. This highlights the critical importance of species identification in CL management and puts SBE forward as a promising candidate for further development against *L. major*.

Introduction

Leishmaniasis comprises a group of neglected tropical diseases caused by protozoan parasites of the genus *Leishmania*, transmitted to humans through the bite of infected phlebotomine sand flies. The disease manifests in several clinical forms, of which cutaneous leishmaniasis (CL) is the most prevalent, characterized by ulcerative skin lesions that may result in permanent scarring, psychological distress, and social stigma. Despite being largely non-fatal, CL represents a substantial public health burden, particularly in low- and middle-income countries. According to the World Health Organization, an estimated 600,000 to 1 million new CL cases occur annually worldwide, with transmission documented in more than 90 endemic countries (WHO 2023; WHO 2024).

Iran remains one of the major endemic foci of CL in the Eastern Mediterranean region. Two *Leishmania* species are predominantly responsible for transmission: *Leishmania major*, the agent of zoonotic CL commonly associated with rural settings, and *Leishmania tropica*, which causes anthroponotic CL and is mainly reported from urban and peri-urban areas (Sharifi et al. 2018; Alvarez-Romero JG, Adams M 2020). These species differ not only in epidemiology and reservoirs, but also in clinical presentation and therapeutic response, complicating disease control strategies (Reithinger et al. 2020).

Pentavalent antimonials, particularly meglumine antimoniate (Glucantime), have remained the first-line treatment for CL for decades. However, their clinical utility is increasingly compromised by severe systemic toxicity (including cardiotoxicity, hepatotoxicity, and pancreatitis), painful parenteral administration, prolonged treatment regimens, and emerging parasite resistance, especially in *L. tropica*–endemic regions (Sundar & Chakravarty 2015; Hadighi et al. 2007; Ponte-Sucre et al. 2017). These limitations, combined with the lack of effective vaccines, have intensified the search for safer, affordable, and locally accessible alternative therapies.

In this context, medicinal plants have gained renewed scientific attention as promising sources of antileishmanial agents. Plant-derived compounds offer notable advantages, including chemical diversity, multi-target mechanisms of action, and a long history of empirical use in traditional medicine systems (Sen & Chatterjee 2011; Ioset & Kaur 2017). Recent studies have emphasized their potential not only for direct antiparasitic activity, but also for immunomodulatory, anti-inflammatory, and wound-healing effects, which are particularly relevant for CL management (Torres-Santos et al. 2021).

Scrophularia bavanatica (local name: *Bender*) is a perennial herb endemic to the mountainous regions of Fars Province, Iran. In Iranian traditional medicine, a raw and caustic poultice prepared from its aerial parts is topically applied to CL ulcers to accelerate healing. Although *S. bavanatica* itself has received limited phytochemical characterization, extensive studies on related *Scrophularia* species have identified iridoid glycosides, phenylpropanoids, flavonoids, and phenolic acids, compounds well known for their anti-inflammatory, antimicrobial, antioxidant, and wound-healing properties (Giner et al. 2000; Bahramikia & Yazdanparast 2018; Tasneem et al. 2021).

Picture 1

No direct comparative evaluation of species-specific susceptibility has been conducted so far. Such comparisons are critical, as inter-species variation in drug sensitivity is a well-recognized clinical challenge in CL treatment and a key contributor to therapeutic failure (Dowlati 1996; Hendrickx et al. 2020; Ponte-Sucre et al. 2021).

Accordingly, the present study compared the effects of SBE against *L. major* and *L. tropica* on Leishmania promastigotes, aiming to elucidate potential species-dependent differences in susceptibility. By addressing this gap, the study contributes to the growing paradigm of species-oriented and personalized therapeutic strategies for CL. To our knowledge, this is the first report to directly compare the *in vitro* susceptibility of *L. major* and *L. tropica* to *Scrophularia bavanatica* alcoholic extract, providing a scientific framework for the rational development of this traditional remedy as a complementary antileishmanial intervention.

Methodology

Plant Material and Extract Preparation

For both studies, aerial parts of *Scrophularia bavanatica* were collected from Bavanat mountainous region of Fars Province. This herb is abundantly present in the plains and gardens of the region and is not at risk for extinction. Yet, the authors complied with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora and the required permission was obtained from Bavanat Natural Resources Organization, Iran in May 2023. Subsequently, the sample was dried and macerated in 80% methanol for seven days. The plant was identified by a plant taxonomist, Ali Mohammad Ranjbar, and a voucher specimen was deposited at the Herbarium of Yazd Medical University, Iran with voucher ID number SSU0130. The solvent was evaporated, and the crude extract was lyophilized. The resulting powder was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 20 mg/mL for use in bioassays. The concentrations were selected based on prior cytotoxicity assays. DMSO concentration in final assays were < 1%.

Parasite Cultures

Two standard Iranian strains were used: a) Leishmania major [MRHO/IR/75/ER] and b) Leishmania tropica [MHOM/IR/NADIM3]. Promastigotes were cultured axenically at 26°C in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin, and 100 IU/mL penicillin.

Experimental Design and Anti-leishmanial Assay

Parasites (1×10^6 promastigotes/mL) were incubated with various concentrations of SBE and Glucantime (positive control) for 24, 48, and 72 hours.

-For L. major: SBE concentrations: 0.01, 0.1, 1, 10, 100 µg/mL; Glucantime: 25 µg/mL.

-For L. tropica: SBE concentrations: 0.2, 1, 5, 25, 125 mg/mL; Glucantime: 25 & 125 µg/mL.

After incubation, parasite viability was assessed using the XTT cell proliferation assay. The absorbance was measured at 450 nm (reference 630 nm) using an ELISA reader. All experiments were performed in triplicate.

Statistical Analysis

Data from both studies were analyzed for consistency using SPSS26. Survival percentages were calculated relative to untreated controls. LC_{50} values were determined from dose-response curves. Statistical significance was assessed with SPSS23 using Student's t-test and repeated measures ANOVA where appropriate ($P \leq 0.05$).

Results

Comparative LC_{50} of SBE and Glucantime

The LC_{50} values revealed a stark contrast in susceptibility between the two species (Table 1). SBE was drastically more potent against L. major, with an LC_{50} of 47.1 mg/mL in the dynamic phase, compared to L. tropica, which required a much higher concentration. Similarly, Glucantime was more effective against L. major (LC_{50}=368.2 µg/mL) than against L. tropica (LC_{50}>125 µg/mL).

Table 1
Comparative LC_{50} values of SBE and Glucantime against *L. major* and *L. tropica* promastigotes

Parasite Species	Stage	SBE LC_{50}	Glucantime LC_{50}
L. major	Dynamic	47.1 mg/mL	368.2 µg/mL
L. major	Stationary	53.2 mg/mL	377.6 µg/mL
L. tropica	Dynamic	> 125 mg/mL	> 125 µg/mL
L. tropica	Stationary	> 125 mg/mL	> 125 µg/mL

Table 1

Phase-Dependent Susceptibility

Both species showed a consistent pattern where promastigotes in the stationary (infective) phase were more resistant to both SBE and Glucantime than those in the dynamic (logarithmic) phase (Table 1). Nonetheless, this difference in resistance between phases was more marginal compared to the vast difference in resistance between species.

Time and Concentration-Dependent Effects

The response to SBE over time was fundamentally different between the two species (Fig. 1). For L. major, a significant antileishmanial effect was observed early. At 24 h, all tested concentrations of SBE (0.01-10 µg/mL) and Glucantime significantly reduced parasite survival in the logarithmic phase compared to the control (P = 0.000). This significant effect persisted at 48 h (P = 0.007). For L. tropica, no significant effect was observed at 24 h or 48 h at any concentration (P > 0.05).

Figure 1

A statistically significant reduction in promastigote survival was only achieved after 72 hours of continuous exposure (P = 0.000), demonstrating a pronounced delayed effect. L. major showed high susceptibility even at low µg/mL concentrations, while L. tropica requires high mg/mL concentrations for a significant effect.

Discussion

This comparative analysis provided compelling evidence that the hydroalcoholic extract of Scrophularia bavanatica (SBE) possesses significant *in vitro* antileishmanial activity, but its efficacy is profoundly species-dependent. The central and most critical finding is the stark differential susceptibility, wherein Leishmania tropica exhibits inherent and significantly higher resistance to both the natural product (SBE) and the standard chemotherapeutic agent (Glucantime) compared to Leishmania major.

The quantitative disparity in potency is striking. The LC_{50} value of SBE for *L. major* in the dynamic phase (47.1 mg/mL), while already high, was markedly lower than the value for *L. tropica*, which exceeded 125 mg/mL. This orders-of-magnitude difference in required concentration is further compounded by a pronounced temporal disparity. *L. major* demonstrated significant susceptibility within 24 hours of exposure, whereas a statistically significant reduction in *L. tropica* promastigote survival was achieved only after prolonged 72-hour incubation. This delayed effect against *L. tropica* is not merely a kinetic delay, but likely reflects a fundamental resilience that requires sustained chemical pressure to overcome. These *in vitro* observations align robustly with well-documented clinical realities, where infections caused by *L. tropica* (anthroponotic cutaneous leishmaniasis) are frequently associated with more chronic, therapeutic-recalcitrant lesions and higher rates of treatment failure compared to those caused by L. major (Dowlati 1996).

The convergence of resistance patterns, where *L. tropica* is less susceptible to both SBE and Glucantime, suggests that the underlying resistance mechanisms may target fundamental cellular processes or general stress response pathways that are perturbed by both antimonials and the bioactive components of SBE. This cross-resistance pattern highlights the complexity of treating anthroponotic CL. Several intrinsic factors in *L. tropica* could account for this robust defense profile:

a) Enhanced Drug Efflux Systems: Members of the ATP-Binding Cassette (ABC) transporter family, such as P-glycoprotein and MRPA, are frequently overexpressed in resistant Leishmania strains and can actively efflux a wide range of structurally unrelated compounds, including natural products (Mandal et al. 2009). It is plausible that *L. tropica* possesses a more constitutive or inducible efflux system that efficiently removes SBE's bioactive components before they reach their intracellular targets.

b) Robust Antioxidant Defense Machinery: Many plant-derived antileishmanial compounds, including flavonoids and phenolic acids (known constituents of Scrophularia species (Giner et al. 2000; Steven et al. 2002), exert their effect through pro-oxidant mechanisms, generating reactive oxygen species (ROS) that overwhelm the parasite's antioxidant defenses. *L. tropica* may possess a more efficient antioxidant system, including higher levels of trypanothione, tryparedoxin peroxidase, and superoxide dismutase, effectively neutralizing the oxidative assault initiated by SBE (Singh & Kumar 2015). This would render a pro-oxidant extract less effective against this particular species.

c) Metabolic and Structural Differences: Differences in surface molecule composition, such as lipophosphoglycan (LPG), can alter membrane permeability and drug uptake. A thicker or differently

structured glycocalyx in *L. tropica* could impede the entry of SBE's active principles. Furthermore, intrinsic differences in metabolic flexibility, such as the ability to utilize alternative energy sources or repair mechanisms, could contribute to its ability to withstand longer periods of metabolic stress induced by the extract.

The pronounced difference in susceptibility between *L. major* and *L. tropica* to SBE may be rooted in the chemical composition of the extract and species-specific parasite biochemistry. *Scrophularia* species are known to produce bioactive iridoid glycosides (e.g., harpagoside, aucubin), flavonoids (e.g., quercetin, apigenin derivatives), and phenolic acids, which have demonstrated antiprotozoal activities through mechanisms such as membrane disruption, inhibition of key enzymes, and induction of oxidative stress (Giner et al. 2000; Steven et al. 2002). It is plausible that one or more of these compounds selectively target metabolic or structural vulnerabilities in *L. major*. For instance, *L. major* may possess a less efficient efflux system for certain flavonoids, or its redox homeostasis may be more easily overwhelmed by pro-oxidant phenolics present in SBE. Conversely, *L. tropica* may express higher levels of antioxidant enzymes like tryparedoxin peroxidase or possess a thicker surface glycocalyx, limiting the uptake of these bioactive molecules. Future chemical fractionation of SBE, followed by testing of individual compounds against both species, would help identify the specific agent(s) responsible for the observed selective activity and clarify the underlying biochemical and molecular mechanisms of species-specific sensitivity.

The traditional use of a crude SBE poultice is a fascinating context for these findings. The application of mashed plant material directly onto a CL ulcer likely achieves extremely high local concentrations of bioactive compounds, potentially high enough to surmount the resistance mechanisms of even *L. tropica* in a superficial, accessible lesion. Our study provided a robust scientific rationale for this centuries-old practice. More importantly, it refines this traditional knowledge by suggesting that its efficacy would be predictably superior against *L. major* infections, for which much lower concentrations are effective. The potent activity of SBE against *L. major* at low µg/mL concentrations is highly promising and positions this extract as a particularly attractive candidate for the development of a standardized topical treatment for zoonotic CL, potentially reducing reliance on painful and toxic systemic antimonials.

The observed phase-dependent susceptibility, where stationary-phase (metacyclic) promastigotes were more resistant than logarithmic-phase promastigotes, is a common phenomenon in antileishmanial drug testing. Metacyclic promastigotes are naturally quiescent and biochemically primed for survival within the mammalian host macrophage, making them inherently more resilient to chemotherapeutic agents. However, the marginal nature of this phase-dependent difference when compared to the vast interspecies difference in resistance further highlights that the species identity is a far more critical determinant of treatment outcome than the parasite's *in vitro* growth phase in the context of SBE.

Limitations of the Study

While this study provides valuable comparative data on the species-specific antileishmanial activity of *Scrophularia bavanatica* extract, its findings should be interpreted within the context of several inherent limitations.

First, as an *in vitro* investigation, this study utilized the promastigote form of the parasite. While promastigotes are the standard and accepted model for initial anti-leishmanial screening, they are not the clinically relevant stage found in the infected host. The amastigote, which resides and replicates within mammalian macrophages, possesses distinct physiological and metabolic characteristics that can significantly influence drug susceptibility. Therefore, the potent activity observed against *L. major* promastigotes may not fully translate to efficacy against the intracellular amastigote form, which is the ultimate target for therapeutic intervention.

Second, the simplified *in vitro* system cannot replicate the complex biological environment of a dermal lesion. Critical *in vivo* factors such as the bioavailability of the extract's active compounds at the infection site, their potential metabolism by host tissues, and the pivotal role of the host's immune system in parasite clearance are not accounted for. The interplay between a drug and the host's immune response is often a key determinant of treatment success in leishmaniasis, and this dimension is absent from our model.

Finally, the use of a crude hydroalcoholic extract, while relevant for validating traditional use, presents a limitation for mechanistic interpretation. The observed antileishmanial effect is likely the result of synergistic or additive interactions between multiple bioactive compounds (e.g., iridoids, flavonoids). Consequently, the precise molecular mechanism(s) of action and the specific compound(s) responsible for the species-specific activity remain unidentified. Further bioassay-guided fractionation is necessary to isolate and characterize the active principles.

Suggestions for Further Research

Future research should focus on:

1. Bioassay-guided fractionation of SBE to identify the active antileishmanial principle(s).
2. *In vivo* validation of efficacy in BALB/c mice models infected with both species.
3. Investigating the mechanism of action of SBE and the basis of *L. tropica*'s resistance.
4. Formulation studies, such as embedding SBE in nano emulsions or liposomes, to enhance delivery and efficacy, especially for the more resistant *L. tropica*.

Conclusion

This comparative study demonstrated that Scrophularia bavanatica extract can be a highly effective *in vitro* agent against *L. major* promastigotes, showing activity at low concentrations. In contrast, *L. tropica* exhibits significant natural resistance, requiring much higher concentrations and longer exposure times for a similar effect. This mirrors the differential sensitivity seen with Glucantime. These findings have critical implications for the treatment of CL:

-They emphasize the necessity of species identification before initiating treatment.

-They may validate the traditional use of SBE, particularly for zoonotic CL caused by *L. major*.

-They position SBE as a promising lead candidate for the development of a standardized, effective, and safer topical treatment for *L. major* infections, potentially reducing reliance on systemic antimonial.

Abbreviations

ANOVA
Analysis of Variance
CL
Cutaneous Leishmaniasis
DMSO
dimethyl sulfoxide
LPG
lipophosphoglycan
MRPA
Multidrug-Resistant Pseudomonas aeruginosa
ROS
reactive oxygen species
SBE
Scrophularia bavanatica extract
WHO
World Health Organization
XTT
2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide

Declarations

Acknowledgements The authors would like to express their sincere gratitude to Dr. Ali Mohammad Ranjbar for his valuable assistance in extract preparation.

Author contributions Conceptualization: KB; Methodology: AFB; Investigation and formal analysis: AFB; Writing– original draft: KB; Writing– review & editing: KB; Resources: KB; Supervision: AFB; *In vitro* experimentation guidance: AFB; Statistical consultation: AFB; Pharmacological interpretation: AFB; All authors read and approved the final manuscript.

Funding This study was supported by Shahid Sadoughi University of Medical Sciences, Yazd, Iran (Project No. 4651). The funding body had no role in the design of the study, data collection, analysis, interpretation, or manuscript writing.

Data availability The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics approval The study proposal was approved by Committee of Ethics at School of Medicine, Shahid Sadoughi University of Medical Sciences (SSU), Yazd, Iran (approval code: R.SSU.MEDICINE.REC.1395.176**.** This study did not involve human subjects; thus, no national and institutional guidelines for the ethical principles outlined in the Declaration of Helsinki were required.

Consent for publication Not applicable.

Clinical trial Not applicable.

Competing interests The authors declare no competing interests.

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Table

Table 1: Comparative LC_{50} values of SBE and Glucantime against *L. major* and *L. tropica* promastigotes

Parasite Species	Stage	SBE LC_{50}	Glucantime LC_{50}
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L. major	Stationary	53.2 mg/mL	377.6 µg/mL
L. tropica	Dynamic	>125 mg/mL	>125 µg/mL
L. tropica	Stationary	>125 mg/mL	>125 µg/mL

Picture 1

Picture 1 is available in the supplementary files section.

Figures

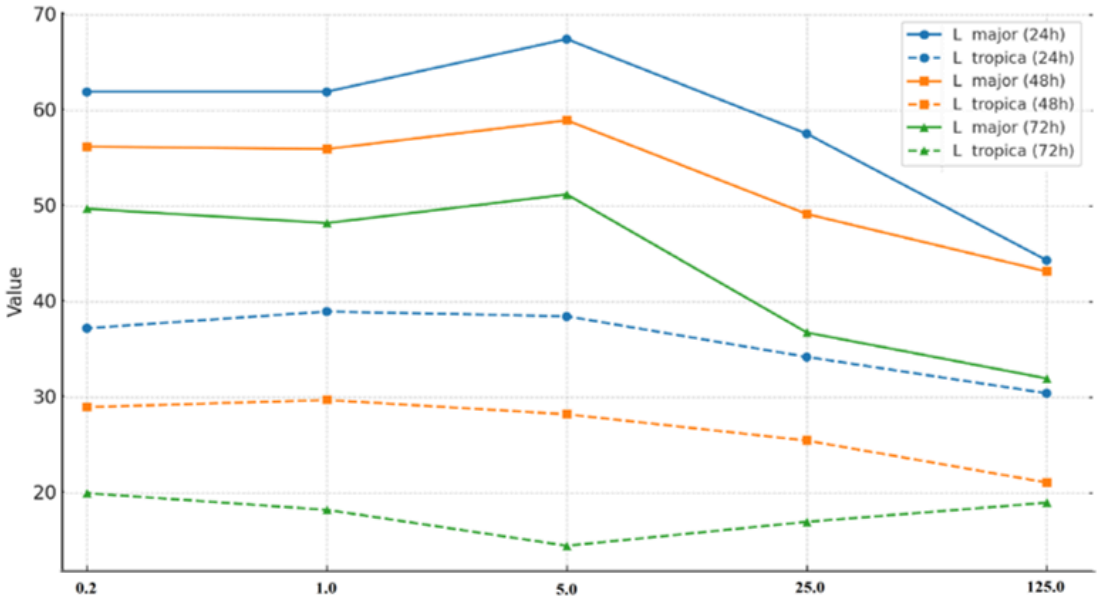


Figure 1

Comparative survival of *L. major* (A) and *L. tropica* (B) promastigotes after 24 h, 48 h, and 72 h exposure to different concentrations of SBE

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Scrophularia.jpg