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Anti-leishmanial activity of *Hypericum Scabrum* extract against *Leishmania major*

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Abstract
Leishmaniasis is a vector-borne disease and one of the most significant neglected tropical diseases. Current anti-leishmanial treatments are often ineffective over extended periods and are associated with toxic side effects, highlighting the urgent need for new, effective, and safe alternative treatments for this infectious disease. The objective of this study was to evaluate the anti-leishmanial effects of a hydroalcoholic extract of *Hypericum scabrum* (*H. scabrum*), comparing its efficacy to that of the control drug glucantime against the standard strain of *Leishmania major*. The *H. scabrum* plants were collected from the western regions of Iran. A hydroalcoholic extract was prepared from the flower and stem of the plant using a maceration method. High-performance liquid chromatography analysis was conducted to identify the chemical compounds present in the extract. Promastigotes of *L. major* were cultured, and the anti-leishmanial activity of the extracts was assessed at concentrations ranging from 12.5 to 800 µg/ml using the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay. The half-maximal inhibitory concentration (IC50) values for the *H. scabrum* plant extract at 24, 48, and 72 h were 245.47, 141.25 and 85.11 µg/ml, respectively. The IC50 values for glucantime (the control drug) at 24 h, 48 h, and 72 h were 30.19, 21.37, and 12.58 µg/ml, respectively. While the *H. scabrum* extract exhibited a lower effect compared to the control drug, it still demonstrated a significant inhibitory effect on the promastigote form of *L. major*. Given that the plant extract of *H. scabrum* has demonstrated promising anti-leishmanial effects against *L. major* promastigotes, further studies are warranted to evaluate the efficacy of these extracts in animal models of *leishmaniasis*.

Keywords Hydroalcoholic extract, *Hypericum scabrum*, Leishmaniasis, *Leishmania major*, MTT assay

Introduction

Leishmaniasis, a parasitic disease affecting both humans and animals, is caused by protozoan parasites of the genus *Leishmania*, (Kinetoplastida; Trypanosomatidae) (Torres-Guerrero et al. 2017). This disease has a long history of affecting humanity, with an estimated 350 million individuals at risk of infection (de Vries and Schallig 2022). The World Health Organization (WHO) reports an annual incidence of 1.5–2 million new cases globally (Alvar et al. 2012). Leishmaniasis manifests in three clinical forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL) (Abadías-Granado et al. 2021). CL, the most common clinical manifestation of disease, is characterized by the

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presence of skin lesions (Gurel et al. 2020). The current gold standard treatment for most CL lesions is meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostam) (Blum and Hatz 2009). However, their clinical application is limited by several reasons, including: prolonged treatment duration, high cost, lack of efficacy, and severe toxicity. Therefore, the development of alternative treatments with improved efficacy, lower cost, and reduced toxicity is crucial (Singh and Sivakumar 2004; Sundar et al. 2019). Furthermore, the emergence of drug resistance to current treatments, particularly in *Leishmania* species that are resistant to pentavalent antimony compounds (Glucantime and Pentostam), presents a serious threat to global health efforts (Ponte-Sucre et al. 2017; Sundar et al. 2019). This resistance manifests as persistent and often recurrent lesions, which may present as erythematous nodules, plaques with minimal scaling, or papules in areas previously affected by the disease (Madusanka et al. 2022).

The wide collection of medicinal plants, with their diverse chemical constituents, offers a promising avenue for exploring new therapeutic options. Historically, humans have relied on plant-based remedies to combat diseases, and the wealth of untapped plant diversity provides a rich source for discovering novel therapeutic agents (Asfaw et al. 2022). Encouragingly, recent research has demonstrated that extracts from a multitude of traditional medicinal plants exhibit inhibitory and even lethal effects against various microorganisms, including *Leishmania* species (Gharirvand Eskandari et al. 2020; Passero et al. 2021; Saberi et al. 2021).

The genus *Hypericum* (Guttiferae or Hypericaceae) comprises over 400 species of plants and shrubs (Kowa et al. 2020). Phytochemical research has revealed a wide array of biological activities associated with members of this genus, including antimicrobial, cytotoxic, anticancer, antidepressant, antiviral, anthelmintic, wound healing, diuretic, antioxidant, and antifungal effects (Keser et al. 2020). *Hypericum scabrum* (*H. scabrum*) has a long history of use in traditional medicine for the treatment of various disease (Kmail 2022). Its flowers are known to contain a rich phytochemical profile, including: Flavonoids: Catechin (in higher concentrations than other flavonoids), quercetin, hyperin, and phloroglucinol, Phenolic acids: Primarily vanillic acid, Vitamins: Notably vitamin K and Phytosterols: Ergosterol being the most prevalent. Furthermore, *H. scabrum* flowers contain several antimicrobial compounds, including hyperforin, α-pinene, hypericin, cetophonen, and paracimen (Khorshidi et al. 2020). Therefore, the exploration of *H. scabrum* as a source of therapeutic agents offers promising prospects for the development of novel treatments for a range of diseases, particularly those with emerging drug resistance. This work attempted to examine the lethal effect of *H. scabrum* extract on *Leishmania major* in In-vitro conditions.

Material and methods

Preparation of *H. scabrum* extract

The stem and flower of *H. scabrum* was collected from mountainous regions of Ilam province and the plant was identified (herbarium code: 327) by the Biotechnology and Medicinal Plants Research Center, Ilam University of Medical sciences, Ilam, Iran.

The flowers and stems of *H. scabrum* were thoroughly washed with distilled several times to remove dust and soil and then dried in a shaded area at room temperature. In next step, the dried plant material was ground into a fine powder using an electric mill (IKA ®, Germany) and sieved through a 10 mm mesh. Subsequently, 100 g of the prepared powder were placed in a specialized cartridge and immersed in a volumetric flask containing 500 mL of solvent, which consisted of 250 mL methanol 70% (Merck, Germany) mixed with 250 mL of distilled water. The solution was agitated every 30 min to facilitate the extraction of active ingredients. To separate the solvent, the solution was subjected to vacuum filtration and heat (aratajhiz.co, Iran). The obtained extract was placed in an oven at 50–60 °C for 24 h to evaporate any remaining solvent. The final extract was stored at 4 °C for further analysis and processing (Karimi et al. 2021).

Parasite culture

The study utilized a standard strain of *L. major* (MRHO/IR/75/ER), which is cataloged under Accession number KU680836.1 in GenBank. This strain was obtained from the Leishmaniasis Laboratory at the School of Public Health, Tehran University of Medical Sciences, Iran. These promastigotes were grown in the Roswell Park Memorial Institute (RPMI) 1640 media (Gibco, Life Technologies GmbH, Germany) supplemented with 10–15% heat inactivated fetal calvin serum (FCS) (Gibco, Germany), 100 u/ml Penicillin, and 100 µg/ml Streptomycin (Gibco, Germany) (Saberi et al. 2018).

Effect of *H. scabrum* extract against *L. major*

To assess the effects of the *H. scabrum* extract, the promastigotes (1×10^6 per well) were added in 96-well plates. Different concentration of the *H. scabrum* extract, ranging from 12.5 to 800 µg/ml, were added to the wells. The plates were then incubated at a controlled temperature of 25 °C for varying lengths of time (24, 48, and 72 h). Additionally, glucantime served as the positive control, while phosphate-buffered saline (PBS, pH=7.2) acted as the negative control. Each experiment was repeated three times to ensure reliable results (Ghaderian et al. 2024).

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) test

The effect of *H. scabrum* extract on the viability of *L. major* cells was determined using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay (Ghaderian et al. 2024). After the incubation period (24, 48, or 72 h), 20 µL of MTT solution was added to each well containing the cells. The cells were allowed to absorb the MTT solution for 5 h at a controlled temperature (25 °C). The media was then removed, and 100 µL of Dimethyl sulfoxide (DMSO, Sigma–Aldrich) solution was added to each well. After 15 min of incubation at 25 °C, the absorbance was read with an ELISA reader at a wavelength of 570 nm. The results were analyzed to determine the IC50 values. This value represents the concentration of the extract that reduced cell growth by 50% compared to untreated cells. The relative quantities of *L. major* cells were assessed by measuring the optical absorbance of both treated and untreated samples, as well as blank wells, using the formula (Ghaderian et al. 2024):

$$\text{Viable cells } (\%) = \text{OD of treat cells/ OD of control cells } \times 100$$

Examining morphological changes of promastigotes

To investigate the morphological changes of promastigotes induced by the presence or absence of the extract, approximately 10 µL of culture media were carefully removed from each well using a sampler. These samples were then examined under a light microscope at 100×magnification. The focus was on observing changes in the shape and motility of the promastigotes (Khademvatan et al. 2011).

High-performance liquid chromatography (HPLC)

To identify and determine the amount of hypericin in the extracted extract, a high-performance liquid chromatography (HPLC) (Platin blue, Knauer, Germany) method was used. First, a standard curve was generated for concentrations of 12.5, 25, 50, 75, 100, and 200 µg of hypericin/1 ml of methanol. Then, concentrations of 1–50 and 1–100 µg/ml of hypericin extract were prepared in HPLC-grade methanol. The extracts were centrifuged and filtered (using a syringe head filter with a 0.22 µm pore size) to remove impurities. All prepared concentrations were injected separately into the HPLC device. A mixture of methanol and water in a 50:50 ratio, with a flow rate of 1 ml/min and an injection volume of 1 µl, was used as the mobile phase. The samples were detected and measured at a wavelength of 280 nm after passing through the stationary phase (a C18 column (Knauer, Germany)). Their chromatogram curves were then generated. The curves related to the flavonolignan compounds in the methanolic extract of silymarin were compared and calculated against the standard silibinin (Jaimand et al. 2014).

Statistical analysis

The research data were analyzed using SPSS version 22 and GraphPad Prism version 5.01 (San Diego, CA) statistical software, employing ANOVA and T-test for statistical evaluation. All the tests were done in triplicate.

Results

Anti-leishmanial activity of *H. scabrum* extract

The findings from the current study indicate that *H. scabrum* extract demonstrates significantly greater efficacy at all tested concentrations compared to the negative control (PBS).The maximum anti-leishmanial activity of the *H. scabrum* extract was observed at a concentration of 800 µg/ml after 72 h of exposure. Conversely, the minimum activity was associated with a concentration of 12.5 µg/ml after 24 h. The survival rate results from this study are summarized in Table 1.

In addition, the findings of this study indicate that the *H. scabrum* extract exhibited the highest anti-leishmanial efficacy at 72 h, with an IC50 value of 245.47 µg/ml. Conversely, the extract demonstrated the lowest anti-leishmanial efficacy at 24 h, with an IC50 value of 85.11 µg/ml. As presented in Table 2, the IC50 values for various concentrations of glucantime reveal that the highest efficacy was observed at 72 h, with an IC50 of 12.58 µg/ml. In contrast, the lowest efficacy was recorded at 24, with

Table 1 Survival rate of *L. major* promastigotes exposed to glucantime and *H. scabrum* extract at varying concentrations and time points

***H. scabrum* extract**				**Glucantime (control)**			
Concentrations	**Time**			**Concentrations (µg/ml)**	**Time**		
	24 h (%)	**48 h (%)**	**72 h (%)**		**24 h (%)**	**48 h (%)**	**72 h (%)**
12.5 µg/ml	85	83	79	12.5	66	66	53
25 µg/ml	79	71	64	25	54	48	46
50 µg/ml	68	66	61	50	37	28	26
100 µg/ml	62	61	47	100	25	20	15
200 µg/ml	55	38	38	200	10	6	4
400 µg/ml	35	30	27	400	3	3	0
800 µg/ml	29	21	18	800	0	0	0

Table 2 Effect of IC50 of *H. scabrum* extract and glucantime on *L. major* promastigotes over time. The p-value of less than 0.05 was considered statistically significant

Drugs	Time			*P*-value
	24 h (µg/ml)	48 h (µg/ml)	72 h (µg/ml)	
H. scabrum extract	245.47	141.25	85.11	$p < 0.001$
Glucantime	30.19	21.37	12.58	$p < 0.001$

an IC50 of 30.19 µg/ml. Overall; the data suggest that both the duration of exposure and the concentration of the *H. scabrum* extract statistically significant influence its inhibitory effects on *Leishmania* ($p < 0.001$).

In the 24, 48 and 72 h evaluation, the *H. scabrum* extract exhibited the highest average OD at a concentration of 12.5 µg/ml, indicating the greatest number of viable cells, with a value of 0.754, 0.640 and 0.559 respectively. In contrast, the lowest average OD was observed at a concentration of 800 µg/ml, with a value of 0.253, 0.162, and 0.126 respectively. The glucantime exhibited the highest average OD at a concentration of 12.5 µg/ml, with a value of 0.585, 0.512 and 0.374 respectively. Conversely, the lowest average OD was observed at a concentration of 800 µg/ml, with a value of 0.007, 0.007 and 0.002 respectively (Fig. 1). Overall, the data indicate that both the extract and glucantime exhibit varying effects on cell viability across different concentrations and time points, with significant statistical differences observed throughout the experiments ($p < 0.001$).

Morphological changes induced by the *H. scabrum* extract

In addition to effectively eliminating the promastigote, the extract also induced notable morphological alterations in these organisms. Among the observed changes were shrinkage and rounding of the promastigote, which indicate significant alterations in cellular structure (Fig. 2).

Identification of hypericin in the *H. scabrum* extract

The data obtained from HPLC indicated that hypericin was the principal compound identified in the *H. scabrum* extract. Additionally, the quantity and percentage of hypericin were quantified and are presented in Fig. 3.

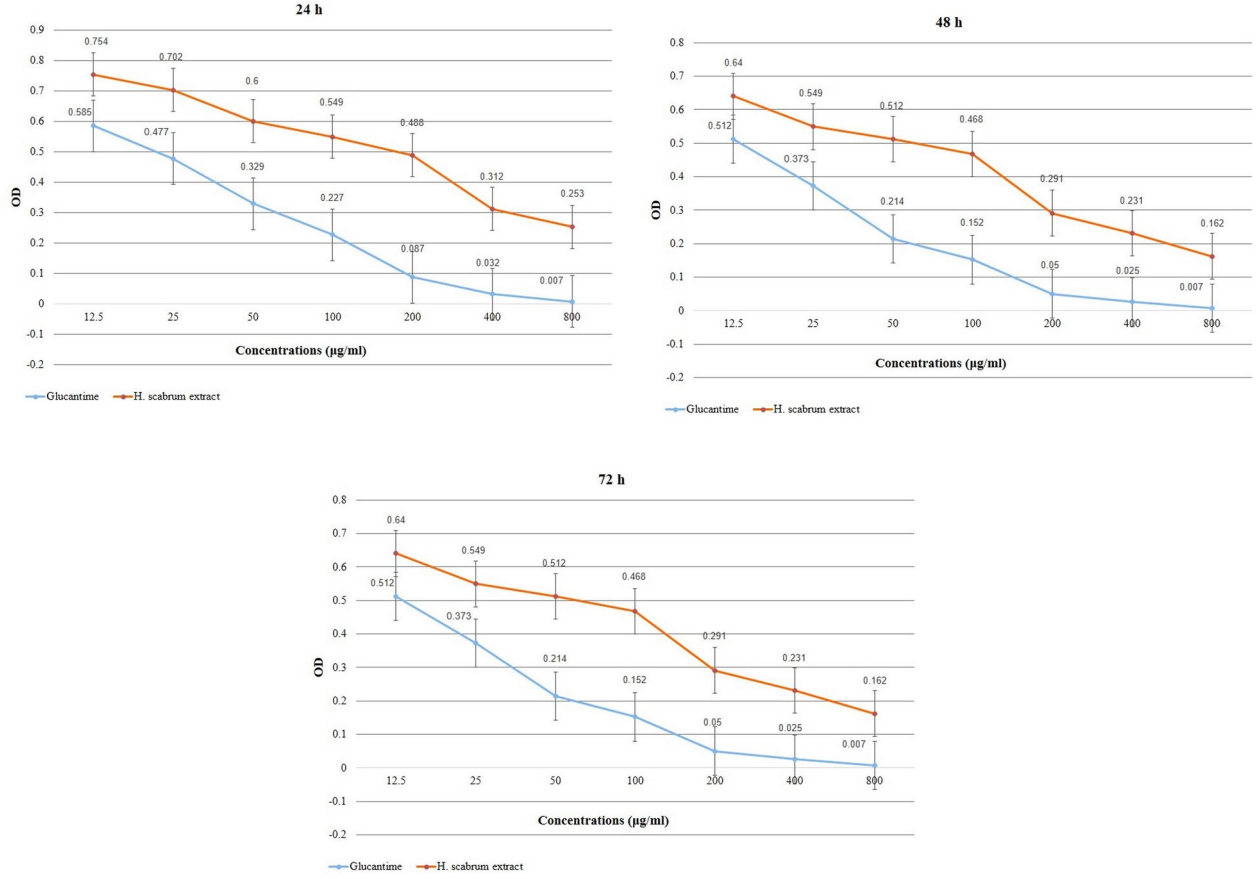


Fig. 1 Comparison of the average OD obtained from the anti-leishmanial activity of the *H. scabrum* extract and Glucantime at different concentrations (12.5–800 µg/ml) and times (24, 48 and 72 h). The error bars represent standard error

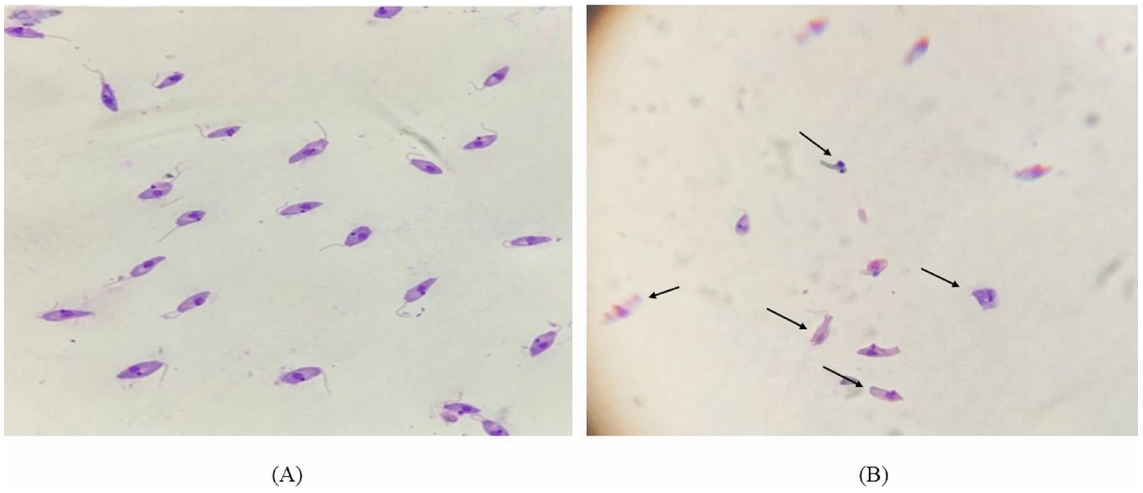
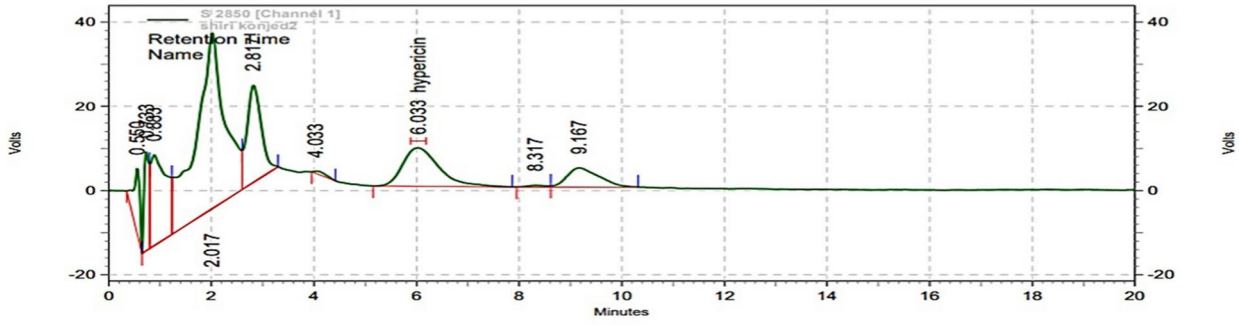


Fig. 2 Comparison of morphological changes of *L. major* promastigotes in the group without drug (**A**) and the group treated with *H. scabrum* extract (**B**)

(A).



(B).

S 2850 [Channel 1] Results

Pk #	Name	Retention Time	Area	Area %	Height	Height %	Width
1		0.550	130698	3.73	15161	10.86	0.30
2		0.733	156974	4.48	23396	16.77	0.15
3		0.883	453543	12.95	21543	15.44	0.43
4		2.017	1641694	46.87	41716	29.89	1.37
5		2.817	450843	12.87	23094	16.55	0.70
6		4.033	12382	0.35	536	0.38	0.47
7	hypericin	6.033	448805	12.81	9104	6.52	2.72
8		8.317	10226	0.29	436	0.31	0.67
9		9.167	197360	5.63	4566	3.27	1.70
Totals			3502525	100.00	139552	100.00	

Fig. 3 Section (**A**) represent the Chromatography results, and section (**B**) show the Hypericin amounts in *H. scabrum* extract

Discussion

Leishmaniasis is a growing global health concern, and to date, no definitive vaccine exists for this disease (Mann et al. 2021). Although CL is often considered a self-limiting disease, the lesions associated with it can lead to secondary infections, which may slow the healing process (de Vries and Schallig 2022). In addition, numerous reports highlight issues such as relapse, incomplete treatment, and the emergence of drug resistance of Leishmaniasis (Domagalska et al. 2023). As a result of the challenges associated with current treatments, many researchers are exploring the development of cost-effective drugs that offer higher efficacy and fewer side effects (Altamura et al. 2022). One approach recommended by the World Health Organization (WHO) is the utilization of plants and natural products in the treatment of Leishmaniasis (Cortes et al. 2020; Gervazoni et al. 2020). In recent years, there has been a growing trend among people in society towards the use of herbal medicines. This increased acceptance, combined with numerous studies confirming the antimicrobial properties of plants and their extracted compounds, has led researchers to explore the potential of herbal medicines as viable treatment options for various diseases, including Leishmaniasis (Cortes et al. 2020; Gharirvand Eskandari et al. 2020).

The study by Keser et al. (2020) found that water and ethanol extracts of *H. scabrum* flowers showed stronger antimicrobial activity when combined with other plant extracts against various bacterial strains. These findings suggest that *H. scabrum* extract can enhance the antimicrobial activity of other natural extracts, making it a promising candidate for future research in the development of synergistic natural remedies (Keser et al. 2020).

The present study investigated the anti-leishmanial activity of the *H. scabrum* extract, revealing significant findings. The *H. scabrum* extract exhibited the highest anti-leishmanial activity at 72 h, with an IC50 value of 245.47 µg/ml. Conversely, the *H. scabrum* extract demonstrated the lowest anti-leishmanial efficacy at 24 h, with an IC50 value of 85.11 µg/ml. The results of this study are consistent with those of other studies that have evaluated the anti-leishmanial activity of various plant extracts. A similar study has investigated the effects of extracts from *H. scabrum* on different *Leishmania* species, including *L. major*, *L. tropica*, and *L. infantum/donovani*. Extracts from *H. scabrum* demonstrated significant leishmanicidal activity, with no living *Leishmania* parasites found at concentrations of 100 µg/mL or higher. This suggests a potent inhibitory effect on the parasites, particularly those that are resistant to standard treatments (Ozpinar et al. 2024). The cytotoxicity of these extracts was assessed using the XTT method on human fibroblast cell lines (WI-38). Importantly, the concentrations effective against *Leishmania* did not exhibit cytotoxic effects on human cells, indicating a selective action against the parasites (Ozpinar et al. 2024). While the precise mechanisms by which *H. scabrum* extracts exert their effects on *Leishmania* are still under investigation, several hypotheses can be drawn from existing literature, including the disruption of metabolic pathways and the induction of apoptosis (Ozpinar et al. 2024).

The study by Naserifard et al. (2013) found that the aqueous extract of *Scophularia straiata* had a proper anti-leishmanial activity, eliminating amastigotes within macrophages and killing promastigotes in the medium at a concentration of 25 µg/mL (Naserifard et al. 2013). In another study, Gharirvand Eskandari and Doudi (2016) evaluated the anti-leishmanial effects of the extract and essential oil of *Medicago lupulina* leaves on *L. major* promastigotes. They reported that the IC50 of glucantime was equal to 19 µg/mL after 48 h, while the IC50 values for the alcoholic extract and essential oil of this plant after 48 h were equal to 130 and 340 µg/mL, respectively (Gharirvand Eskandari and Doudi 2016). This suggests that the anti-leishmanial activity of *H. scabrum* extract is comparable to that of other plant extracts. The potential synergistic effects of combining *H. scabrum* extract with other natural extracts or compounds warrant further investigation to enhance its therapeutic efficacy and reduce the risk of drug resistance. In the study conducted by Soudi et al. (2017), it was found that the root extract of *Echinacea purpurea* has a significant and irreversible leishmanicidal effect on *L. major* promastigotes in vitro, with effective concentrations around 50 mg/mL, suggesting its viability for clinical applications (Sooudi et al. 2007). Additionally, previous studies highlighted that both *Allium hirtifolium* and *Ziziphus spina-christi* extracts possess strong inhibitory effects on the growth of *L. major* promastigotes, with IC50 values of 87 µg/mL and 112 µg/mL, respectively. These findings underscore their potential as effective herbal treatments for leishmaniasis (Amanzadeh et al. 2006). The systematic review of herbal extracts as antileishmanial agents highlights a promising strategy for developing effective treatments for leishmaniasis. Key findings regarding specific extracts include: *Artemisia aucheri* extract exhibited an IC50 value of approximately 5.9 µg/mL against *L. major*. The *Ferula assa-foetida* extract demonstrated comparable efficacy with an IC50 of around 7.5 µg/mL. Also, *Gossypium hirsutum* extract showed the highest potency among the evaluated extracts, achieving an IC50 of 3.6 µg/mL. These findings underscore the potential of these herbal extracts as viable therapeutic options for combating leishmaniasis, warranting further investigation into their mechanisms and clinical applications (Gharirvand Eskandari et al. 2020). The findings showed that the *H. scabrum* extract exhibited anti-leishmanial efficacy and hypericin was principal compound, which is consistent

with a similar study accomplished by Singh et al. (Singh et al. 2015). In the mentioned study, researchers examined the effects of hypericin, an active compound derived from *Hypericum perforatum*, which belongs to the same family as *H. scabrum*, on *L. donovani*. The hypericin was identified as a natural compound with inhibitory properties against spermidine synthase (LdSS), an enzyme associated with *L. donovani*. This compound demonstrated the ability to inhibit the activity of the recombinant LdSS enzyme, leading to the death of *L. donovani* at an IC50 of 18 µM under laboratory conditions. In vivo assessments revealed that treatment with hypericin resulted in a significant reduction in spermidine levels in *Leishmania* promastigotes. Specifically, spermidine levels decreased from a normal value of 1±34 µM to 0.1±1 µM after 24 h of hypericin treatment, indicating the target of the inhibitory action. Furthermore, hypericin treatment was associated with a decrease in trypanothione levels, which dropped from 54±317 µM in the normal state to 9±39 µM after 24 h of treatment. In contrast, glutathione levels increased from 206±17 µM in the normal state to 249±12 µM following hypericin treatment. Additionally, elevated levels of reactive oxygen species were observed in *L. donovani* following hypericin exposure (Singh et al. 2015).

The findings of this study highlight the potential of the *H. scabrum* extract as a promising anti-leishmanial agent, particularly when used at higher concentrations and for longer periods. The extract's ability to inhibit *Leishmania* sp. growth and survival, along with its ability to induce morphological changes, suggests that it could be valuable anti-leishmanial treatments. In vivo studies are essential to validate these findings and to fully explore the therapeutic potential of *H. scabrum* extracts in the effective treatment of leishmaniasis. Furthermore, comprehensive investigations into the specific mechanisms of action are critical for the development of innovative treatment strategies utilizing these natural products.

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Author contributions
Conceptualization: AM. Formal analysis: EK. Methodology: ZJ, RS. Project administration: AM, EK, JA, and RN. Writing of original draft: RS. Design the figures: RS, ZJ. Writing-review & editing: RS, AM. All authors reviewed the manuscript.

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All data generated or analyzed during this study are included in this published article.

Declarations

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Consent to participate
Not applicable.

Consent for publication
All authors of this article agree to submit the article to AMB Express Journal.

Conflict of interest
The authors declare there is no conflict.

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