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In Vitro Antileishmanial Activity of *Calotropis procera* Extract on *Leishmania major*

Hadi Mirahmadi[1], **Ahmad Mehravaran**[2*], **Abbas Safdari Adimi**[2]

1. Clinical Immunology Research Center, Zahedan University of Medical Sciences, Zahedan, Iran.
2. Infectious Diseases and Tropical Medicine Research Center, Research Institute of Cellular and Molecular Science in Infectious Diseases, Zahedan University of Medical Sciences, Zahedan, Iran.

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ABSTRACT

Background: Pentavalent antimonial agents remain the primary therapeutic option for leishmaniasis; however, accumulating evidence highlights significant adverse effects, toxicity concerns, and emerging drug resistance associated with these compounds. Accordingly, the exploration of alternative therapeutic agents, particularly from natural sources, has gained increasing attention. The present study aimed to investigate the in vitro antileishmanial activity of *Calotropis procera* extract against both promastigote and amastigote forms of *Leishmania major*.

Materials and Methods: Methanolic extract of *C. procera* flowers was obtained through the maceration technique. The antiproliferative activity of the extract on promastigotes was quantified using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to assess cell viability and growth inhibition.

Results: MTT assay findings demonstrated that *C. procera* extract markedly inhibited the proliferation of *L. major* promastigotes. The calculated IC_{50} value for the extract alone was 387.91 µg/mL, whereas the combination of *C. procera* extract with meglumine antimoniate exhibited enhanced inhibitory activity, yielding an IC_{50} value of 211.26 µg/mL.

Conclusion: The observed inhibitory effects suggest that *C. procera* possesses promising antileishmanial properties and may represent a potential natural candidate for the development of alternative therapeutic strategies against leishmaniasis.

*** Corresponding Author:**
Ahmad Mehravaran, Associate Professor.
Address: *Infectious Diseases and Tropical Medicine Research Center, Research Institute of Cellular and Molecular Science in Infectious Diseases, Zahedan University of Medical Sciences, Zahedan, Iran.*
Phone: *+98 (913) 3975876*
E-mail: *ahmadmehravaran55@gmail.com*

Introduction

Leishmaniasis is a zoonotic parasitic disease and a major vector-borne public health problem worldwide. It disproportionately affects disadvantaged populations and remains endemic in many low-resource regions, where approximately 700,000–1,000,000 new cases are reported annually. Consequently, the disease causes substantial morbidity and mortality [1]. Clinically, leishmaniasis manifests in multiple forms, including cutaneous leishmaniasis (CL), often presenting as self-healing dermal lesions, and the more severe visceral leishmaniasis, which can be life-threatening if left untreated [2]. Despite extensive efforts to identify safer and more effective therapeutic options, pentavalent antimonials remain among the most widely used treatments and have been implemented for more than five decades [3]. However, current antileishmanial drugs are associated with important limitations, including the emergence of drug resistance, prolonged treatment duration, and adverse effects with considerable toxicity. In addition, commonly used synthetic agents may cause clinically relevant complications, such as injection site pain, anorexia, gastrointestinal disorders, and resistance development [4].

These challenges highlight the need for alternative therapeutic candidates, particularly those with high efficacy and improved safety profiles. In recent years, increasing attention has been directed toward bioactive natural products, including herbal extracts and other nature-derived approaches, for the management of infectious diseases [5]. Many medicinal plants with ethnobotanical use have been evaluated using experimental methods, and several candidates have shown promise against a range of pathogens. Reported examples include *Blighia sapida*, *Morus alba, Spondias monbin, Treculia africana*, and *Calotropis procera* [6].

C. procera (giant milkweed) is a perennial, greyish-green, woody shrub with broad fleshy leaves that grows widely in tropical regions and warm temperate climates [7]. Across different geographical regions, it is known by various common names, reflecting its broad distribution and traditional relevance [8, 9]. The plant is primarily valued for its medicinal properties and has been used traditionally for pain, inflammation, and digestive disorders. In particular, the latex has been reported to exhibit diverse biological activities, including traditional uses related to antimicrobial and anti-leishmanial effects, and its application has been described in several therapeutic contexts, such as respiratory ailments and skin conditions [10, 11]. Moreover, pharmacological studies have reported anti-inflammatory, analgesic, antimicrobial, antifungal, anticancer, and insecticidal activities for different parts of the plant, including the flowers and latex [12-14].

Given this background, the present study was designed to evaluate the in vitro antileishmanial activity of *C. procera* flower extract against *Leishmania major*.

Materials and Methods

Plant material

The flowers of *C. procera* were collected in 2024 from Sistan and Baluchestan province, Iran, and identified and authenticated at the Department of Botany, University of Sistan and Baluchestan, Iran. A voucher specimen (voucher numbers: CP/1404, 235) was deposited in the university herbarium.

Preparation of plant extracts

Fresh flowers of *C. procera* were collected, sun-dried, and washed with water to remove all contaminants. They were dried at room temperature and grounded into powder. After washing and shade drying at room temperature, the samples (500 mg) were powdered and extracted by maceration with 10 mL of 80% methanol (Merck, Darmstadt, Germany), followed by shaking incubation (325 rpm) at 25±2 ˚C for 72 hours. The extract was filtered through paper, and the residue was re-macerated twice more: the second time with 80% methanol for 48 hours, and the third time with 80% methanol for 24 hours. The concentrated residue was frozen at −20 ˚C. The dried powders were dissolved in phosphate-buffered saline (PBS) and diluted to prepare test concentrations of the extract.

Parasite culture

The Iranian standard reference strain of *L. major* promastigotes (MRHO/IR/75/ER) was provided by the Department of Medical Parasitology and Mycology, Zahedan University of Medical Sciences, Zahedan, Iran. The promastigotes were cultivated in RPMI-1640 medium (Biosera, France) complemented with penicillin-streptomycin antibiotics and 10% fetal bovine serum (Sigma, USA). The cultures were placed in an incubator shaker (120 rpm) at 25±1 ˚C and grown until reaching the stationary growth phase [15, 16].

Macrophage culture

After being acquired from the Pasteur Institute in Tehran, Iran, the THP-1 human macrophage cell line was cultured in DMEM medium (Sigma, USA) enhanced with penicillin, 0.5% streptomycin (Biosera, France), and 10% FBS (Sigma, USA). The cells were maintained at 37 ˚C in an atmosphere containing 5% CO_2.

Anti-promastigote activity

To determine the growth-inhibitory effect of *C. procera* extract, promastigotes of *L. major* (1×10^6 parasites/mL) at the stationary phase were added in triplicate to a 96-well plate and treated with extract concentrations ranging from 0 to 400 µg/mL. Wells containing culture medium without promastigote as blank samples; glucantime-treated promastigotes (Glucantime®: Sanofi, France) were used as positive controls, and PBS-treated promastigotes were used as negative controls. Following treatment, the plates were incubated for 72 hours at 25±1 ˚C with varying drug concentrations as previously described. Ten microliters of MTT solution at concentrations of 25, 50, 100, 200, and 400 µg/mL were added to individual wells and incubated for 3 hours. The 50% inhibitory concentration (IC_{50}) was calculated based on the optical density (OD) absorbance measured at 530 nm [17].

Anti-amastigote activity

To convert the promastigote form into amastigotes, THP-1 cell lines (100,000/mL) were covered in 24-well plates and incubated at 37 ˚C in 5% CO_2 for 24 h. Following this incubation, the macrophages were infected with *L. major* stationary phase promastigotes at a concentration of 1×10^6 per milliliter, at a 10:1 ratio (10 parasites to 1 macrophage), allowing the parasites to transform into the amastigote form over another 24-hour period. After this step, the culture medium was washed to remove free promastigotes, and treatment with the extract was started. In the next step, various concentrations of *C. procera* (CP) alone (50, 100, 200, and 400 µg/mL) and combined with meglumine antimoniate (MA) at the same concentrations (50, 100, 200, and 400 µg/mL) were added, and the mixture was incubated for 72 hours. Following incubation, the samples were methanol-fixed and stained with Wright-Giemsa. The IC_{50} values for each concentration against amastigotes were calculated by averaging the mean number of intracellular amastigotes inside 100 cells [17].

Cytotoxic effects

To evaluate the cytotoxic effect of hydroalcoholic extract CP and its combination with CP, MA, Tween solution (TS), and CP+MA at varied concentrations (50, 100, 200, and 400 µg/mL) on THP-1 cells was investigated. The cells were then incubated for 72 h at 37 ˚C in a 5% CO_2 atmosphere within 96-well plates. Afterward, 10 µL of MTT solution was added to each well, allowing for an additional 3 h of incubation. Following this, 100 µL of DMSO was included, and the OD was measured at 530 nm using a Bio-Tek ELISA reader. The drugs' safety was assessed through the selectivity index (SI), which was determined using the Equation 1:

1. $SI=CC_{50}/IC_{50}$

an SI greater than 1 indicates minimal toxicity.

Statistical analyses

The obtained data were analyzed using SPSS software, version 22.0 and GraphPad Prism software, version 10.0. Non-parametric tests, such as the Mann-Whitney test, were employed to assess the results. A $P<0.001$ was considered statistically significant.

Results

Anti-promastigote effects of CP, MA, and TS or their combination

Figure 1 illustrates the effects of CP extract, MA, TS, and their combination (CP+MA) on inhibiting the promastigote forms of *Leishmania* at different drug concentrations over 72 hours (from 0 to 400 µg/mL). The combination of CP and MA exhibits a significantly higher inhibition percentage (approximately 75%) at the highest concentration. The MA extract also demonstrated a notable effect, while the TS extract appeared to be less effective. These results affirm the potential therapeutic impact of plant extracts in controlling *Leishmania* infections.

Anti-amastigote assessment

The result in Table 1 show that using CP, MA, TS, and their combination (CP+MA) led to a significant decrease in the number of intra-macrophage amastigotes during the clinical stage, especially when compared to untreated control groups, with a statistical significance of $P<0.001$. Overall, the CP+MA combination demonstrated the highest efficacy, achieving complete inhibition (100 µg/mL residual activity) at the highest tested concentration

Table 1. Evaluation of different concentrations of CP, MA, TS and their combination on amastigotes

Concentration (µg/mL)	TS	MA	CP	CP+MA
0	69.6	59.1	64.3	55.8
50	58.2	48.2	55.3	36.8
100	49.4	32.4	42.4	14.2
200	41.3	19.1	29.7	5.7
400	39.2	0	21.3	0

of 400 µg/mL, significantly outperforming individual treatments at lower doses. MA alone also proves highly effective, reaching residual activity at 400 µg/mL, whereas CP shows a dose-dependent but less potent effect until higher concentrations are reached. In contrast, TS showed the least inhibitory effect at all concentrations tested, suggesting that its role is likely to be negligible or merely as a transporter-controlling agent. These findings strongly suggest a synergistic potential when CP is used in conjunction with the standard drug, MA.

Cytotoxic effects

Table 2 presents the analysis of inhibitory concentration (IC_{50}) values against *L. major*, revealing that the combined formulation of CP+MA exhibits the most potent antileishmanial activity. This combination achieves the lowest IC_{50} values against both amastigotes (34.25 µg/mL) and promastigotes (211.26 µg/mL). Notably, the combination demonstrated enhanced effectiveness, as evidenced by its superior SI (18.25), which was significantly higher than that of the individual agents: MA (SI=10.9), CP (SI=8.74), and the TS (SI=9.36).

Discussion

The findings of this study demonstrated that CP extract possesses significant in vitro antileishmanial activity, both when administered alone and when combined with the standard antileishmanial drug MA. The combination of CP and MA (CP+MA) produced approximately 75% inhibition of *Leishmania* promastigotes at a concentration of 400 µg/mL. These results are noteworthy, as they suggest enhanced activity upon co-administration, warranting further studies to validate the magnitude and nature of this interaction. Consistent with this observation, the data in Table 1 indicated that CP+MA significantly reduced the number of intramacrophage amastigotes, particularly relative to the untreated control group, with statistical significance ($P<0.001$). Collectively, these results support the hypothesis that the CP+MA combination may represent a promising therapeutic strategy for controlling *Leishmania* infection. In addition, the extract alone exhibited a dose-dependent effect, underscoring the importance of achieving adequate exposure levels in potential treatment regimens.

Further confirmation of the enhanced antileishmanial potential is provided by the IC_{50} analysis shown in Table 2. Notably, the combined formulation exhibited the most potent activity, achieving IC_{50}=34.25 µg/mL for amastigotes and IC_{50}=211.26 µg/mL for promastigotes. When assessed using the selectivity index SI, CP+MA demonstrated superior performance (SI=18.25) compared with MA alone (SI=10.9) and CP alone (SI=8.74). These findings collectively suggest a favorable interaction between CP constituents and MA, reflected in both potency and

Table 2. Determination of IC_{50} values for CP, MA, TS, and their combination against *L. major* promastigotes and amastigotes

Formula	IC_{50} Amastigotes	P	IC_{50} Promastigotes	P	CC_{50} Macrophages	SI
MA	72.34	-	268.03	-	788.6	10.9
TS	349.68	<0.001	1245.6	<0.001	3274.5	9.36
CP	99.42	<0.001	387.91	<0.001	869.2	8.74
CP+MA	34.25	<0.001	211.26	<0.001	625.3	18.25

SI=CC_{50} macrophages/IC_{50} amastigotes.

Note: Significant statistical differences compared to the control (MA) were observed at the $P<0.001$ level.

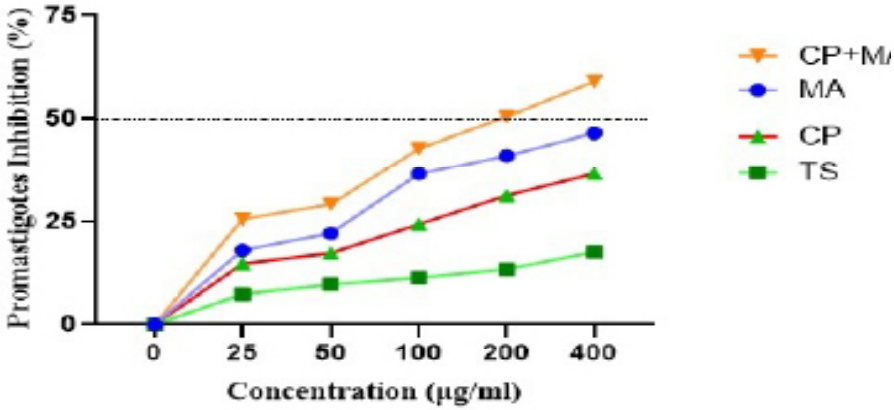

Figure 1. In vitro anti-promastigote activity of different treatments against *L. major* over 72 h

selectivity. Importantly, the relatively favorable SI values from the cytotoxicity assessment indicate an acceptable safety margin for therapeutic consideration (Table 2). This observation is particularly relevant in light of the established toxicity limitations associated with currently available antileishmanial therapies [18]. The reduced cytotoxic potential may be attributable to multiple factors, including the biocompatibility of the extraction/solvent system, the extraction procedure, the final concentration, the chemical composition of the extract, and possible stabilizing effects of plant-derived phytochemicals [19].

In agreement with the present results, a previous study reported that *C. procera* leaf extract exhibits significant antileishmanial activity against *L. major* promastigotes and amastigotes. The proposed mechanism involved modulation of parasite oxidative stress, including increased reactive oxygen species (ROS) generation in promastigotes, as well as upregulation of IFN-γ, TNF-α, and iNOS expression in PBMCs harboring amastigotes [20]. Moreover, studies assessing Calotropis gigantea against *L. major* indicated that non-polar fractions display promising leishmanicidal activity, and these fractions should be evaluated further, particularly against amastigote forms [21].

Phytochemical evidence further supports the plausibility of antileishmanial activity for CP. FTIR and HPLC analyses from prior work identified major constituents, including catechin, rutin, p-coumaric acid, caffeic acid, luteolin, and kaempferol. These compounds have been associated with antioxidant capacity and broad antimicrobial activity, including activity against Gram-positive bacteria [22]. Additionally, enzymatic studies on *C. procera* leaf extracts as inhibitors of key carbohydrate-digestion enzymes in diabetes have shown that the ethanolic extract inhibits α-amylase most effectively, whereas the aqueous extract shows the highest inhibition of α-glucosidase in vitro, with a non-competitive inhibition pattern [9]. Traditional uses of CP leaf extracts in Saudi Arabia and Yemen have also been documented for the management of filariasis and leprosy [23].

Beyond antileishmanial effects, CP has been reported to contain a diverse phytochemical profile, including triterpenoids, flavonoids, cardiac glycosides/cardenolides, anthocyanins, α-amyrin, β-amyrin, lupeol, β-sitosterol, flavanols, as well as additional compounds, such as mudarine and plant-associated bioactive enzymes (e.g. calactin and calotropin) [24]. Such chemical diversity may contribute to multifunctional biological effects, including antimicrobial and immunomodulatory properties, although the precise contribution of each constituent to antileishmanial activity remains to be determined. Despite the encouraging results, several limitations should be acknowledged. First, although the current in vitro evidence is promising, in vivo studies are required to clarify pharmacokinetics, bioavailability, and long-term safety of the extract and the combination. Second, the molecular mechanisms underlying immune modulation and parasite killing require deeper investigation, including pathway-level characterization.

Conclusion

Considering that, despite their high efficacy, chemical anti-leishmanial drugs are associated with severe side effects, toxicity, some drug resistance, and high costs, efforts to find effective, safe, and cost-effective natural agents against *Leishmania* are encouraged *C. procera* can be considered a promising new source of natural anti-leishmanial agents. Certainly, to better understand the effect of *C. procera* extract on leishmaniasis infection,

its active and non-toxic components should be further studied utilizing an experimental murine model.

Ethical Considerations

Compliance with ethical guidelines

This study protocol was approved by the Research Ethics Committee of Zahedan University of Medical Sciences, Zahedan, Iran (Code: IR.ZAUMS.REC.1404.090).

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Authors contribution's

All authors contributed equally to the conception and design of the study, data collection and analysis, interception of the results and drafting of the manuscript. Each author approved the final version of the manuscript for submission.

Conflict of interest

The authors declared no conflict of interest.

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