

Original Research Article

Identification and quantification of flavonoids extracted from *Calotropis procera* and evaluation of their antileishmanial activity

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Abstract

Purpose: To identify and quantify flavonoids from *Calotropis procera* fruit extract, and evaluate the antileishmanial activity of the extract.

Method: Reflux technique was used to extract flavonoids from the fruits at 60 °C. Phytochemical screening of the methanol extract was done using high-performance liquid chromatography (HPLC). To assess its therapeutic potential, the inhibitory effect on *Leishmania tropica* promastigotes at extract concentrations of 31.5 to 1,000 µg/mL was quantified after 24 h using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Sodium stibogluconate (100 mg/mL) injection was used as positive control, while 2.5 µL of Dimethyl sulfoxide (DMSO) was used as negative control.

Results: Phytochemical analysis revealed that methanolic extract of *Calotropis procera* fruit contains flavonoid and phenolic compounds. Examples include: rutin (90.5 ppm), quercetin (84.9 ppm), apigenin (74.9 ppm), kaempferol (70.4 ppm), luteolin (69.8 ppm), caffeic acid (41.6 ppm) and gallic acid (88.7 ppm). Extract exhibited significant dose-dependent reduction in promastigote viability when compared to the negative control ($p < 0.05$). The most pronounced inhibitory effect was recorded at 1000 µg/mL. The inhibitory concentration (IC_{50}) for *C. procera* methanolic fruit extract was 101.4 µg/mL.

Conclusion: These findings suggest that the fruits of *Calotropis procera* hold potential as a promising source of novel antileishmanial agents.

Keywords: *Calotropis procera*, *Leishmania tropica*, Flavonoids, HPLC, Anti-leishmaniasis

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INTRODUCTION

Leishmania tropica is a Zoonotic protozoan parasite belonging to the *Leishmania* genus. An estimated 350 million people are vulnerable to infection by this parasite across the endemic regions, such as urban areas of the Middle East, Mediterranean region, India, Pakistan, Central Asia and Western China [1]. Nearly one million

newly diagnosed cases of leishmaniasis are reported annually by the World Health Organization (WHO) [1]. Leishmaniasis manifests either as mucocutaneous (MCL), cutaneous (CL), or visceral forms. The most prevalent form is cutaneous leishmaniasis. It is characterized by a localized skin ulcer or nodule [1]. Since there is no approved vaccine for leishmaniasis, anti-parasitic drugs such as

pentavalent antimonials (PVA; meglumine-antimoniate and sodium-stibogluconate) are used as first-line drugs for the treatment, while miltefosine, paromomycin, amphotericin B, and pentamidine are used as alternatives [2]. Side effects associated with PVA range from common gastrointestinal issues to serious cardiovascular and organ damage. Other limitations to its use include poor tolerability due to the injection site [2]. Second-line anti-leishmaniasis agents such as amphotericin B, pentamidine, paromomycin, and azoles are also influential, but their extended therapeutic regimen and prohibitive cost limit their use, especially in poor countries [2]. Finally, there are reports of increased resistance to these antileishmanial drugs [3].

Due to relatively low toxicity, low cost, and availability of medicinal plants, interest in their use as sources of anti-leishmanial agents has increased in recent years. Studies have shown that phytochemicals like flavonoids, alkaloids, triterpenoids, quinones, steroids, and terpenes from certain plants may have anti-leishmanial activity [4]. *Calotropis procera*, commonly known as Apple of Sodom, Giant Milkweed, or Calotrope, is a perennial, soft-wooded, evergreen xerophytic shrub that belongs to the family Apocynaceae and the subfamily Asclepiadaceae. It is generally found in arid and semi-arid environments [5]. The leaf extract has been used to treat fungal infections, provide pain relief, and act as an antipyretic. Many ethnic groups have utilized *C. procera* leaves and flowers to treat skin disease, respiratory diseases, rheumatoid diseases and leprosy [6]. Some metabolites, such as tannins, glycosides, flavonoids and phenolics that are reported in this plant, are distributed among flowers, fruits,

stems, latex, leaves, roots, and bark. The diverse pharmacological effects associated with using extracts from these plant parts include anti-inflammatory, antidiarrheal, anticancer, insecticidal, larvicidal, acaricidal, anthelmintic, and schizonticidal activities [7]. This study aims to identify and quantify flavonoids extracted from *Calotropis procera* fruits and determine their antileishmanial activity.

EXPERIMENTAL

Preparation of extract from *Calotropis procera* fruits

Fruits of *C. procera* were collected in May 2024 from a garden in Al-Zubair district in Al Basrah, Southern Iraq. Al Al-Zubair-global positioning system (GPS) coordinates are located at latitude 30° 23' " North and longitude 47° 42' " East. The plant was authenticated by Prof. Abdulridha Al-Mayah, a taxonomist, from the College of Science, Department of Biology, University of Basrah, southern Iraq (BSRA herbarium no. 1719). Collected fruits were dried under shade, pulverized and stored in a dark glass container (Figure 1).

Extraction process

Thirty grams (30 g) of powdered sample was mixed with 500 mL of methanol (80 % v/v) in a Reflux apparatus and extraction was carried out for 2 h at 60 °C [8]. After extraction, the methanolic extract was filtered and evaporated to dryness in a Rotary evaporator at 45 °C under reduced pressure.



Figure 1: *Calotropis procera* leaves and fruits from Basrah governorate, Southern Iraq (Al-Zubair district)

HPLC analysis

High-performance liquid chromatography (HPLC; SYKA, Germany) was used to identify the phenolics and flavonoids in *C. procera* crude extract. Reference samples containing rutin, apigenin, quercetin, luteolin, kaempferol, caffeic acid and gallic acid obtained from Hyper Chem® (China) were used to prepare stock solution (1 mg/mL) using methanol as a solvent. These prepared solutions were subsequently diluted serially to 2 - 10 ppm solutions for the determination of the standard calibration curve. Methanolic extract of *C. procera* fruit at 1 mg/mL was prepared and filtered through 0.2 µm membrane filter. A 100 µL aliquot of filtered solution was then analyzed using HPLC. A mixture of methanol, distilled water, and formic acid at the ratio of 70:25:5 was used as mobile phase. Sample migration was carried out in a C18-ODS (25 cm x 4.6 mm) column at 1.0 mL/min, flow rate and UV detector at 280 nm. The total run time was 25 min. Samples were subsequently injected through HPLC system using a Hamilton microliter syringe with 20 µL injection volume.

Evaluation of antileishmanial activity

Parasite preparation

The *Leishmania tropica* (*L. tropica*) strain was obtained from the animal cell culture laboratory at the Center for Biotechnology, Al-Nahrain University. The promastigotes were grown in RPMI-1640 media containing 10 % fetal bovine serum (FBS; Gibco) that had been heat-inactivated, and the mixture was incubated at 25 °C. Parasites in the late logarithmic phase previously cultured at a concentration of 3×10^6 promastigotes/mL were transferred to fresh media to establish a new culture at a cell density of 4×10^6 parasites/mL and incubated at 25 °C for subsequent testing.

Inhibitory effect of *C. procera* extract on *L. tropica*

To evaluate the anti-leishmanial activity of *C. procera* methanolic extract, 96-well plates containing promastigote cell density of 1×10^4 per RPMI-containing well were used. A 100 µL of 31.25, 62.5, 250, 500 and 1000 µg/mL of *C. procera* extract was added to each well. Incubation was carried out at 25 °C for 24 h or longer, if necessary, until approximately 60 % confluency was achieved. Sodium stibogluconate (100 mg/mL) injection was used as positive control. The positive control was also serially diluted to achieve an initial concentration of 1000

µg/mL. A 6 µL sample from this solution was then added to each well and inoculated with *L. tropica* promastigotes. Dimethyl sulfoxide (DMSO; Sigma-Aldrich, Germany) was used as negative control. All experiments were performed in triplicate [9].

MTT assay

The influence of *C. procera* methanolic extract on survival of *L. tropica* cells was assessed through a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [9]. Following a 24-h incubation, 20 µL of the MTT solution was added to every well containing the cultured cells. The cells were then cultured at 25 °C for 4 h to facilitate the uptake of the MTT dye. After this period, the supernatant phase was gently removed, and 100 µL of DMSO was added to solubilize the formazan crystals formed within the cells. At this stage, the plates were incubated for 20 min at 25 °C on a shaker and shielded from light. Subsequently, the intensity of formazan formation was determined using a spectrophotometric plate reader (DNM-9602G) at 570 nm. Proportional cell counts of *L. tropica* were estimated by measuring the absorbance for both samples (treated and untreated), along with samples in negative control. The IC₅₀, which represents the concentration of extract required to reduce cell viability by 50 % relative to negative control, was calculated using Eq 1.

$$\text{Viable cells (\%)} = (A_t/A_c)100 \dots\dots (1)$$

Where A_t is absorbance of treated cells, and A_c is absorbance of control cells

Statistical analysis

Data were evaluated using GraphPad Prism 6.0 software. Triplicate determinations were performed, and data are presented as mean ± standard deviation (SD). Statistical significance was determined at $p < 0.05$.

RESULTS

HPLC chromatogram

The HPLC-generated chromatograms of the extract and reference compounds are presented in Figure 2 and Figure 3. Measurement was performed using a standard calibration curve. The concentrations of the identified compounds from the analyzed sample are summarized in (Table 1). In the methanolic extract of *C. procera*, rutin (90.5 ppm) was the most abundant flavonoid, while caffeic acid (41.6 ppm) was the least abundant (Table 1).

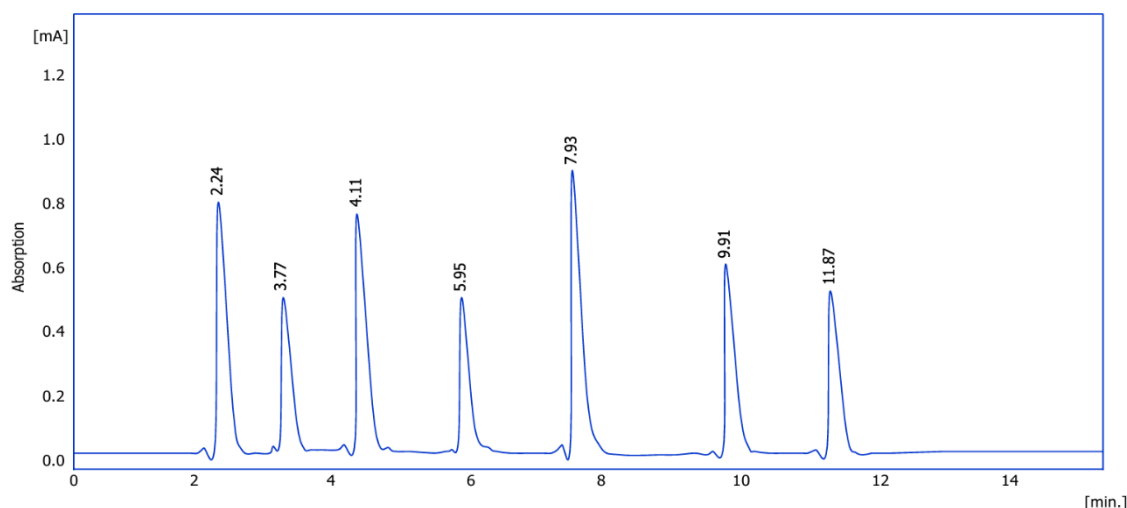


Figure 2: HPLC Chromatogram of methanol fruit extract of *Calotropis procera*

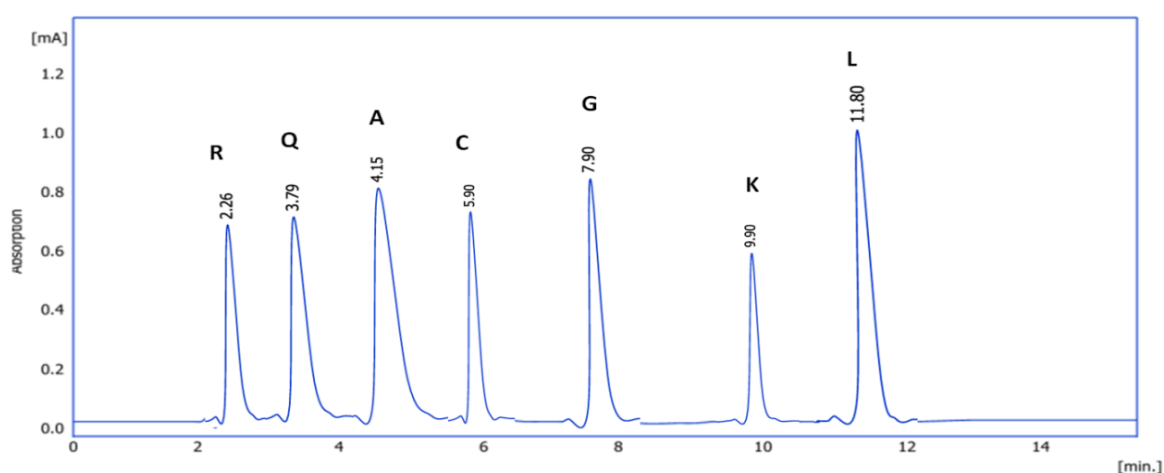


Figure 3: HPLC Chromatogram of standards R: Rutin, Q: Quercetin A: Apigenin C: Caffeic acid G: Gallic acid K: Kaempferol L: Luteolin

Table 1: Retention time and peak area of HPLC chromatogram of methanol fruit extract of *Calotropis procera*

Flavonoids	Retention time (min)	Area (mAU.s)	Quantity (ppm)
Rutin	2.24	7850.15	90.5
Quercetin	3.77	5624.99	84.9
Apigenin	4.11	7125.99	74.9
Caffeic acid	5.95	5441.08	41.6
Gallic acid	7.93	9854.07	88.7
Kaempferol	9.91	4562.08	70.4
Luteolin	11.87	3698.88	69.8
Total		44157.49	

Effect of extract on parasite viability

The methanolic fruit extract of *C. procera* exhibited markedly greater anti-leishmanial activity at all concentrations when compared to negative control ($p < 0.05$). Significant inhibitory effect was obtained at 1000 $\mu\text{g/mL}$ of extract after 24 h, yielding an IC_{50} of 101.4 $\mu\text{g/mL}$. The

least activity was observed at 31.5 $\mu\text{g/mL}$ under the same conditions. A summary of the parasite survival rate across various concentrations is presented in Figure 4 and Table 2. The number of Leishmania cells decreased as the extract concentration was increased.

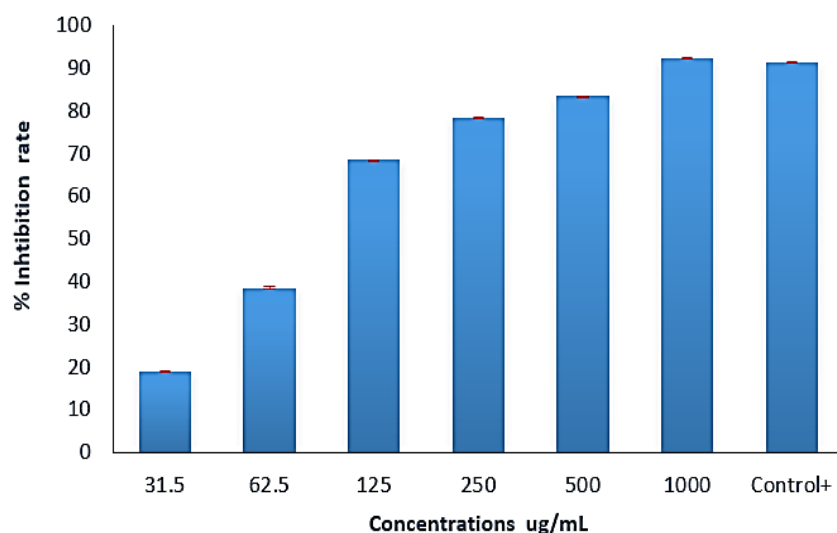


Figure 4: Inhibition rate of *L. tropica* against different concentrations of *Calotropis procera* extracts and control

Table 2: Effect of changes in concentration of methanol fruit extract of *Calotropis procera* on percentage viability and mean inhibition of *L. tropica*

<i>C. procera</i> extract ($\mu\text{g/mL}$)	Mean $\pm$ SD	Viability (%)
31.5	1.62 $\pm$ 0.105	81
62.5	1.23 $\pm$ 0.365	61.5
125	0.63 $\pm$ 0.070	31.5
250	0.42 $\pm$ 0.045	21.5
500	0.33 $\pm$ 0.020	16.5
1000	0.153 $\pm$ 0.025	7.65
Control+	0.180 $\pm$ 0.0125	9
Control-	2 $\pm$ 0.030	0

DISCUSSION

The HPLC is commonly used to quantify and identify phenolic compounds in plants [10]. In this study, methanol has been used as solvent to extract phenolic compounds from the plant due to its high polarity and the preferential solubility of flavonoid and phenolic compounds in methanol [11]. The compounds identified have been previously reported to have notable therapeutic properties. For instance, kaempferol has shown strong antioxidant, antimicrobial, and cytotoxic properties [12], while quercetin is recognized for its anti-inflammatory, vasodilatory, antihypertensive, anti-obesity, anti-hypercholesterolemic, and anti-atherosclerotic effects [14]. Similarly, luteolin and rutin have demonstrated anti-oxidant, anti-inflammatory, antibacterial, and antiproliferative actions [9,15]. Several studies have indicated that phenolic compounds derived from medicinal plants are useful in drug development, due to their diverse pharmacological properties, including antileishmanial activity [13-17]. Keshav *et al* [17] further demonstrated the effectiveness of gallic acid against murine visceral leishmaniasis.

Leishmaniasis. Currently, no vaccine has been developed to prevent this disease. Many researchers are investigating cost-effective therapeutic alternatives with improved efficacy and reduced side effects [18].

Interest in herbal medicines has grown markedly in recent years. This shift in perception, supported by numerous studies highlighting the antimicrobial efficacy of plant-derived bioactive compounds, has promoted interest among scientists and researchers toward herbal therapies as a substitute for disease management, including Leishmaniasis [19].

Alghamdi *et al* [20] had demonstrated that *C. procera* leaf aqueous extract has significant activity against various strains of bacteria. However, evidence from this current study suggests that *C. procera* fruit methanolic extract could be a promising candidate for further studies aimed at developing new therapeutic options against leishmaniasis parasites. *In vitro*, the extract showed inhibitory effect on *L. tropica* after 24 hours of incubation, with an IC_{50} of 101.4 $\mu\text{g/mL}$. This activity was evident through significant inhibition of parasite growth, reduced survival, and distinct morphological alterations.

The high flavonoid content identified in the extract is likely a key contributor to this effect [20-22].

CONCLUSION

This investigation reveals that the methanolic extract of *Calotropis procera* fruit possesses strong activity against *Leishmania tropica*. These results suggest that extract of *Calotropis procera* fruit may serve as a promising natural therapeutic candidate.

DECLARATIONS

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Ethical approval

Not required.

Use of Artificial intelligence/Large language models

We also declare that we did not use Generative artificial intelligence (AI) and AI-assisted technologies in writing the manuscript.

Availability of data and materials

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

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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