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Chemical Composition and *In Vitro* Antiprotozoal Properties of *Cephaelis ipepacuanha*

Composição Química e Propriedades Antiprotozoárias In Vitro da *Cephaelis ipepacuanha*

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ABSTRACT
Background: *Cephaelis ipecacuanha* is a medicinal plant used in folk medicine for the treatment of amebiasis. However, the pharmacological properties of this species remain poorly understood.
Objective: Characterize chemical composition and in vitro antiprotozoal activity of *Cephaelis ipecacuanha.*
Method: The aqueous extract was lyophilized using the Chisrt Alpha apparatus and transferred to 96-well plates at concentrations ranging from 62.5 to 1000 µg / mL. The antiprotozoal activities against *Trypanosoma cruzi, Leishmania brasiliensis,* and *L. infantum* were evaluated after 72h incubation. Fibroblasts incubated at the same conditions for 24h were used as a cytotoxicity control. The readings were performed by spectrophotometry after staining with resazurin. The chemical composition of the extract was analyzed by High-Performance Liquid Chromatography (HPLC-DAD).
Results: The results demonstrated that *C. ipecacuanha* had moderate antiprotozoal activity against *T. cruzi.* However, its cytotoxicity against the fibroblasts was significantly higher.
Conclusion: This finding suggests that the use of this plant by the population, besides not having significant benefits, can cause associated health risks.

Keywords: *Cephaelis ipecacuanha. L. brasiliensis. L. Infantum. T. Cruzi***.** Secondary metabolites. Antiprotozoal activity.

RESUMO
Antecedentes: Cephaelis ipecacuanha é uma planta medicinal utilizada na medicina popular para o tratamento da amebíase. No entanto, as propriedades farmacológicas desta espécie permanecem pouco conhecidas.
Objetivo: Caracterizar a composição química e a atividade antiprotozoária in vitro de Cephaelis ipecacuanha.
Método: O extrato aquoso foi liofilizado usando o aparelho Chisrt Alpha e transferido para placas de 96 poços em concentrações que variam de 62,5 a 1000 µg / mL. As atividades antiprotozoárias contra Trypanosoma cruzi, Leishmania brasiliensis e L. infantum foram avaliadas após 72h de incubação. Fibroblastos incubados nas mesmas condições por 24h foram usados como controle de citotoxicidade. As leituras foram realizadas por espectrofotometria após coloração com resazurina. A composição química do extrato foi analisada por Cromatografia Líquida de Alta Eficiência (HPLC-DAD).
Resultados: Os resultados demonstraram que C. ipecacuanha apresentou atividade antiprotozoária moderada contra T. cruzi. No entanto, sua citotoxicidade contra os fibroblastos foi significativamente maior.

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Conclusão: Esse achado sugere que o uso dessa planta pela população, além de não apresentar benefícios significativos, pode acarretar riscos à saúde associados.

Palavras-chave: Cephaelis ipecacuanha. L. brasiliensis. L. Infantum. T. Cruzi. Metabólitos secundários. Atividade antiprotozoária.

1 INTRODUCTION

The World Health Organization (WHO) has actively encouraged the development of studies aimed at evaluating the use of medicinal plants and herbal medicines with health benefits described historically [1,2]. In this context, Brazil stands out for its rich biodiversity, which is still poorly exploited. Therefore, studying the Brazilian flora is an excellent opportunity to discover bioactive species, which may contribute to the development of pharmacological therapies for numerous diseases [3].

Cephaelis ipecacuanha belongs to the family Rubiaceae, which is formed by 450 genera and 6500 species with a wide distribution in countries such as Colombia and Brazil, especially in the Amazon region and in the Brazilian Atlantic Fores [4, 5]. Popularly known as *poaia, ipeca, papaconha* or ipecacuanha, this species is used in folk medicine for treating diarrhea, amoebic dysentery, chronic lung secretion, hemorrhages, asthma [6, 7] cough, influenza and worm infections [8]. As its widespread use for the treatment of fever became known, an intense extractivism lead to the rising of deforested areas and a decrease in this species [4,9].

Regarding the botanical characteristics, poaia has two distinct subtypes. While the variation predominating in Brazil has small annular protrusions, that found in Chile exhibits larger rings involving the roots [10]. The medicinal use of *C. ipecacuanha* in the treatment of infections such as amebiasis is justified by the presence of the alkaloid emetine, the active principle of a drug used to treat amoebic infections in the hospital setting [11].

Trypanosoma cruzi and *Leishmania* spp. are the causative agents of Chagas disease and leishmaniasis, respectively. These diseases mostly affect low-income populations, especially those who live in areas where health and financial resources are minimal. Furthermore, drugs used in the treatment of these diseases are toxic, and drug resistance has been reported, justifying the search for novel and safe therapeutic agents. According to the WHO, the development of therapeutics to avoid drug resistance, including exploration of combinations of approved anti-kinetoplastid drugs, repurposing of existing approved drugs, and development of new medications are research priorities for Chagas disease and leishmaniasis [12].

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Therefore, the objective of this study was to evaluate the chemical composition and the antiprotozoal activity of *C. ipecacuanha* against *Trypanosoma cruzi*, *Leishmania brasiliensis*, and *Leishmania infantum*.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

The species *C. ipecacuanha* was collected in the municipality of Patos-Paraíba and the voucher specimen was deposited in the herbarium Geraldo Mariz of the Federal University of Pernambuco under registration number 35844.

2.2 PREPARATION OF THE LYOPHILIZED POWDER

The roots of *C. ipecacuanha* were crushed and used for decoction extraction following the protocol of the Brazilian Pharmacopoeia (2010). The decoction (DCi) was then filtered, taken to refrigeration and submitted to lyophilization using apparatus model Chisrt Alpha.

2.3 REAGENTS

Sodium resazurin was obtained from Sigma-Aldrich (St. Louis, MO) and stored at 40 ° C protected from light. A solution of resazurin was prepared in 1% phosphate buffer, pH 7, and sterilized before use. Chlorophenol red-β-D-galactopyranoside (CPRG; Roche, Indianapolis, IN) was dissolved in 0.9% Triton X-100 (pH 7.4). Penicillin G (Ern, SA, Barcelona, Spain) and streptomycin (Reig Jofré SA, Barcelona, Spain) were used to prevent bacterial contamination.

2.4 CELL LINES

In vitro studies with *T. cruzi* were carried out using the clone CL-B5 of parasites transfected with the β-galactosidase (lacZ) gene from *Escherichia coli*. Dr. F. Buckner provided these microorganisms through the Gorgas Memorial Institute (Panama). The epimastigotes were grown at 280 °C in liver infusion broth (Difco, Detroit, MI) with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA), penicillin (Ern, SA, Barcelona, Spain) and streptomycin (Reig Jofré SA, Barcelona, Spain), and harvested during the exponential growth phase. The cultures of *L. brasiliensis* and *L. infantum* were obtained from the Institute of Health Sciences Research, Asunción, Paraguay - IICS. Promastigote inhibition assays were performed using the *L. braziliensis* and *L. infantum* strain (MHOM / BR / 75 / M2903), grown at 220 °C in Schneider's Drosophila medium supplemented with 20 % FBS. For the cytotoxicity assays, NCTC929

fibroblasts cultured in Minimum Essential Medium (Sigma) were used. The culture medium was supplemented with heat-inactivated FBS (10%), penicillin G (100 U / ml) and streptomycin (100 μg / ml). Cultures were maintained at 37 ºC in a humidified atmosphere with 5% CO_2. The viability of these strains was evaluated by colorimetric assay using resazurin as an indicator [13,14].

2.5 *IN VITRO* EPIMASTIGOTE SUSCEPTIBILITY TEST

Epimastigote forms of *T. cruzi* (before reaching the stationary phase) were seeded at $1x10^5$ cells /m in 200 μL broth culture medium in 96-well plates. The plates were incubated at 28 °C with the drugs at concentrations ranging from 0.1 to 50 μg / mL) and 50 μL of a CPRG solution at 200 μM. After 72h, the plates were incubated at 37 °C for 6h, and then, the readings were performed using a spectrometer with a wavelength of 595 nm. Each experiment was performed twice, independently, and in triplicate. The efficacy of each compound was estimated by calculating the percentage of epimastigote mortality [15].

2.6 *IN VITRO* LEISHMANICIDAL ASSAY

Promastigotes of *L. brasiliensis* and *L. infantum* were adjusted to 10^6 cells/mL and transferred to 96-well plates. The extract was dissolved in DMSO at the same concentrations described above and moved to the wells. The leishmanicidal activity was evaluated after 72 h by direct counting of the cells and comparing each treatment with the untreated control.

2.7 ANALYSIS OF CYTOTOXITY AGAINST FIBROBLASTS

Fibroblasts from the NCTC929 were adjusted to $3x10^4$ cells / well, transferred to 96-well microplates and cultured at 37 °C under 5% CO_2. After that, the culture medium was removed, and the extract (dissolved in medium) was added to the concentrations previously established in a final volume of 200 μL for 24 h. After this incubation, 20 μL of 2 mM resazurin was added to each well. Plates were incubated for 3 h, and resazurin reduction was measured using double absorption at 490 and 595 nm wavelengths, discounting the value of the control. Each concentration was tested in triplicate.

2.8 CHEMICALS, APPLIANCES AND GENERAL PROCEDURES

This study used only analytical grade chemicals. Acetonitrile, phosphoric acid, gallic acid, ellagic acid, and caffeic acid were purchased from Merck (Darmstadt, Germany).

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Catechin, rutin, luteolin, and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). High-Performance Liquid Chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with alternating Shimadzu LC-20AT pumps connected to a DGU 20A5 degenerator with a CBM 20A integrator, SPD-M20A diode array detector, and LC 1.22 SP1 solution software.

2.9 CHEMICAL ANALYSIS BY HPLC-DAD

The aqueous extract of *Cephaelis ipecacuanha* at a concentration of 12 mg / mL was introduced using a Shimadzu Auto Auto Model SIL-20A self-injector. Separations were performed using the Phenomenex C18 column (4.6 mm x 250 mm x 5 μm particle size). The mobile phase was water with 1% phosphoric acid (v / v) (solvent A) and HPLC grade acetonitrile (solvent B) with a flow rate of 0.7 mL/min and the injection volume of 50 μL. The composition gradient was: 5% solvent B reaching 15% at 10 min; 30% solvent B at 25 min, 65% solvent B at 50 min and 98% solvent B at 65 min. At 70 min, the gradient reached the initial conditions again [16]. The sample and the mobile phase were filtered through a 0.45 μm membrane (Millipore) and then sonicated by ultrasonic bath before use. Standard stock solutions were prepared in the mobile phase of HPLC at a concentration range of 0.030 to 0.300 mg / mL. Quantifications were performed by integrating the peaks using the standard external method, at 254 nm for gallic acid; 280 nm for catechin; 327 for caffeic acid and ellagic acid; and 356 nm rutin, quercetin, and luteolin. The chromatography peaks were confirmed by comparing the retention time with the reference standards and the DAD spectra (200 to 600 nm). All chromatography operations were performed at room temperature and in triplicate. Calibration curve for gallic acid: $Y = 11954x + 1268.3$ ($r = 0.9999$); caffeic acid: $Y = 13047x + 1268.5$ ($r = 0.9999$); ellagic acid: $Y = 12790x + 1176.9$ ($r = 0.9998$); catechin: $Y = 11856x + 1374.2$ ($r = 0.9999$); routine: $Y = 13165x + 1349.8$ ($r = 0.9999$); luteolin: $Y = 12659x + 1327.4$ ($r = 0.9996$) and quercetin: $Y = 11842x + 1257.1$ ($r = 0.9999$).

2.10 LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope of three independent analytical curves. The LOD and LOQ were calculated as 3.3, and 10 σ / S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve [17].

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2.11 STATISTICAL ANALYSIS

The differences between the groups were evaluated by the analysis of variance model and Tukey's test. The level of significance for the analyzes was set at $p < 0.05$. The analyses were performed using software R version 3.1.1[18].

3 RESULTS

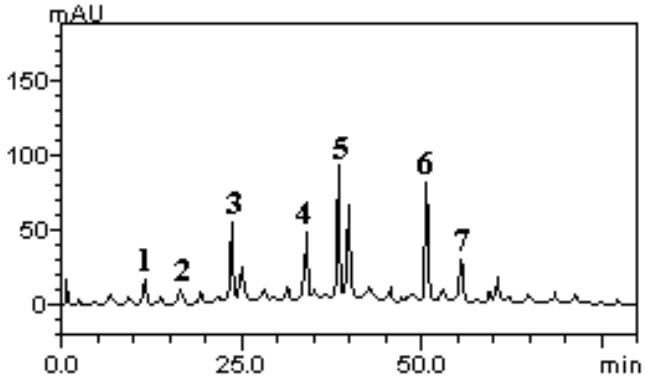
The HPLC profile of the aqueous extract of *Cephaelis ipacacuanha* is shown in figure 1 and table 1. We identified the presence of the following secondary metabolites: gallic acid (retention time - RT = 11.27 min; peak 1), catechin (RT = 15.64 min; peak 2), caffeic acid (RT = 23.81 min, peak 3), ellagic acid (RT = 34.05 min, peak 4), rutin (RT = 38.29 min, peak 5), quercetin (RT = 50.37 min, peak 6) and luteolin (RT = 55.12 min; peak 7).

Table 1. Chemical composition of the *Cephaelis ipacacuanha* extract.

Components	***Cephaelis ipacacuanha***	**LOD**	**LOQ**
	mg/g	**μg/mL**	**μg/mL**
Gallic acid	1.13 ± 0.02 a	0.008	0.027
Catechin	0.84 ± 0.01 b	0.019	0.063
Caffeic acid	4.79 ± 0.01 c	0.017	0.059
Ellagic acid	4.05 ± 0.03 d	0.021	0.070
Rutin	6.37 ± 0.01 e	0.023	0.075
Quercetin	5.19 ± 0.02 f	0.015	0.048
Luteolin	2.08 ± 0.04 g	0.007	0.023

These results are expressed as mean ± standard deviation (SD) of three determinations. Means followed by distinct letters differ statistically according to the Tukey's test ($p < 0.05$). LOD: Limit of detection; LOQ: Limit of quantification.

Figure 1 – Representation of the high-efficiency liquid chromatography profile of the *Cephaelis ipacacuanha* extract.



Gallic acid (peak 1), catechin (peak 2), caffeic acid (3) elagic acid (peak 4), rutin (peak 5), quercetin (peak 6) and luteolin (peak 7).
Source: original

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The antiprotozoal activity of *Cephaelis ipecacuanha* against promastigotes of *L. brasiliensis*, and *L. infantum* and epimastigotes of *T. cruzi*, as well as its cytotoxicity against fibroblasts, is shown in Table 2. Incubation of *T. cruzi* epimastigotes with different concentrations of the *C. ipecacuanha* extract caused significant mortality of this protozoan from 62.5 µg/ mL reaching maximum activity at 1000 µg/ mL. However, no significant antiprotozoal effect of the extract was observed against *L. infantum* and *L. brasiliensis*. On the other hand, all concentrations of the extract caused cytotoxicity above 50% against fibroblasts, suggesting that these cells are more susceptible to the toxic effects of the plant than the cells of the protozoans evaluated by the study. Taken together, the findings of the present research suggest that the use of this plant by the population, besides not having significant benefits, can cause associated health risks.

Table 2 – Antiprotozoal activity and cytotoxicity of the *Cephaelis ipacacuanha* extract

C. ipacacuanha **(µg/mL)**	***T. cruzi*** ***(% mortality)***	***L. brasiliensis*** ***(% mortality)***	***L. infantum*** ***(% mortality)***	**Fibroblasts** ***(% mortality)***
1000	32.83	0.00	0.00	54.98
500	9.53	0.00	0.00	52.16
250	18.64	0.00	0.00	53.02
125	12.91	0.00	0.00	53.98
62.5	12.94	0.29	0.00	53.51

4 DISCUSSION

Species of the family Rubiaceae produce a large number of secondary metabolites such as alkaloids, terpenes, quinones, flavonoids, and steroids [19]. The alkaloid emetine, found in Cephaelis ipecacuanha is known for having astringent, expectorant, anti-inflammatory, and antiparasitic actions [20]. Nevertheless, this constituent has not been identified in the sample analyzed by this study, which had rutin, quercetin, and caffeic acid identified as major constituents.

An *in vitro* study investigated the activity of a large number of flavonoids and other phenolic compounds against *T. brucei*, *T. cruzi*, and *L. donovani*. Among the compounds tested were rutin, quercetin, and luteolin, which were identified as chemical constituents of the extract

analyzed by the present study. The authors demonstrated that the overall activity against *T. cruzi* was moderate, although the majority of the metabolites tested displayed remarkable leishmanicidal potential, including luteolin, and quercetin as the most potent substances. In addition, the caffeic acid, also found in the species under study, showed activity against *T. cruzi* trypomastigotes [21, 22].

Furthermore, the leishmanicidal and trypanosomicidal effects of this species have not been reported in the literature, although ethnobiological studies suggest that it has a vermicide activity. In this context, studies indicated that *Cephaelis ipacacuanha* has been used by communities of the caatinga of the state of Pernambuco (Brazil) to treat worm infections humans and animals. This is corroborated by the studies of who reported the use of this plant in the treatment of both and worm infections by the people Pernambuco and Piauí, respectively [23-25].

However, our data suggest that the use of this plant by the population may cause toxic effects. This is in accordance with the literature, since the dichloromethane and methanolic extracts of species belonging to the same family of *Cephaelis ipacacuanha* also presented considerable cytotoxicity against *Artemia salina* [26].

Therefore, in view of the cytotoxic profile against the fibroblasts, as well as the weak antiprotozoal activity, especially against *Leishmania* strains, we discourage the use of the extract of *Cephaelis ipacacuanha* as an alternative therapy for infections caused by protozoa.

5 CONCLUSION

The chemical composition of *Cephaelis ipecacuanha* is characterized by the presence of phenolic acids and flavonoids such as quercetin, whose anti-inflammatory and antiprotozoal activity were previously reported. However, the extract of this plant was significantly more toxic against fibroblasts that against promastigotes of *L. brasiliensis* and *L. infantum* and epimastigotes of *T. cruzi* and thus, its use may represent a risk to the health of the population.

Therefore, we conclude that although the plant extract is not a promising alternative for the treatment of protozoal infections, *Cephaelis ipecacuanha* may serve as a source of compounds useful for the development of novel drugs.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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