

ANTILEISHMANIAL AND ANTIOXIDANT POTENTIAL OF THE ETHANOL EXTRACT OF *CROTON ARGYROPHYILLOIDES* MUELL. ARG.

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Abstract

Leishmaniasis is a severe and potentially fatal chronic disease caused by *Leishmania* spp. The treatment primarily based on antimony is not always effective and have toxic effects. Secondary metabolites of plants are considered promising agents with antileishmanial action. The objective of this study was to evaluate the *in vitro* antileishmanial activity of the *Croton argyrophyloides* leaves ethanol extract (CALEE), popularly known as marmeleiro prateado, as well determine the main classes of metabolites present, the antioxidant activity and toxicity in *Artemia salina* using the BST method. The antileishmanial test was performed *in vitro* against promastigotes forms of *Leishmania infantum* in a 96-well plate (10⁶ parasites per well). The CALEE was dissolved in DMSO and tested at five concentrations from 100 µg/ml on. Pentamidine was used as a standard drug. The effective concentration that kills 50% of the culture (EC₅₀) was determined using the MTT colorimetric test, and the measurement was performed in a microplate reader at 570 nm. The CALEE presented superior antileishmanial activity to pentamidine, with EC₅₀ 5.63 and 23.71 µg/ml respectively, moderate antioxidant activity, as well as activity against *A. salina* in BST test (EC₅₀ 266.34 µg/ml). Therefore, the CALEE is presented as a potential source for the extraction of compounds with antileishmanial activity.

Keywords: *Croton argyrophyloides*, Antileishmanial, Ethanol extract.

Potencial anti-leishmania e antioxidante do extrato etanólico de *Croton argyrophyloides* MUELL. ARG.

Resumo

A leishmaniose é uma doença crônica grave e potencialmente fatal causada por *Leishmania* spp. O tratamento, principalmente à base de antimônio, nem sempre é efetivo e apresenta efeitos tóxicos. Metabólitos secundários de plantas são considerados promissores agentes com ação leishmanicida. O objetivo deste estudo foi avaliar *in vitro* a atividade leishmanicida do extrato etanólico das folhas do *Croton argyrophyloides* (EEFCA), popularmente conhecido como marmeleiro prateado, bem como determinar as principais classes de metabolitos presentes, a atividade antioxidante e a toxicidade em *Artemia salina* pelo método BST. O teste leishmanicida foi realizado *in vitro* contra a forma promastigota de *Leishmania infantum* em placa de 96 poços (10⁶ parasitos por poço). O EEFCA foi dissolvido em DMSO e testado em cinco concentrações a partir de 100µg/mL. A pentamidina foi usada como droga padrão. A concentração efetiva que mata 50% da cultura (CE₅₀) foi determinada por meio do teste colorimétrico do MTT e a leitura foi realizada em um leitor de microplacas a 570 nm. O EEFCA apresentou atividade antileishmanial superior à pentamidina, com CE₅₀ 5,63 e 23,71 µg/mL respectivamente, moderada ação antioxidante, assim como atividade frente à *Artemia salina* pelo teste BST (EC₅₀ 266,34 µg/ml). Portanto, o EEFCA se apresenta como uma potencial fonte para extração de compostos com atividade anti-leishmania.

Palavras-chave: *Croton argyrophyloides*, Anti-Leishmania, Extrato etanólico.

Potencial anti-leishmania y antioxidante del extracto de etanol de *Croton argyrophyloides* MUELL. ARG.

Resumen

La Leishmaniasis es una enfermedad crónica grave y potencialmente fatal causada por *Leishmania* spp. El tratamiento basado, principalmente, en antimonio, no siempre es eficaz y presenta efectos tóxicos. Metabolitos secundarios de la planta se consideran agentes promisorios con acción leishmanicida. El objetivo de este estudio fue evaluar *in vitro* la actividad leishmanicida del extracto de etanol de las hojas de *Croton argyrophyloides* (EEFCA), popularmente conocido como *marmeleiro prateado*, y determinar las principales clases de metabolitos presentes, la actividad antioxidante y la toxicidad en *Artemia salina* por el método BST. La prueba anti-leishmania se realizó *in vitro* contra la forma promastigote de *Leishmania infantum* en placa del 96 pocillos (10⁶ parásitos por pozo). El EEFCA se disolvió en DMSO y se probó en cinco concentraciones desde 100 µg/mL. La pentamidina se usó como fármaco estándar. La concentración eficaz que mata 50% de la cultura (EC₅₀) se determinó utilizando el ensayo colorimétrico MTT y la medición se realizó en un lector de microplacas a 570 nm. El EEFCA presentó actividad anti-Leishmania superior a la de la pentamidina, con CE₅₀ 5,63 y 23,71 µg/mL respectivamente, actividad antioxidante moderada, así como actividad frente a la *Artemia salina* mediante la prueba de BST (EC₅₀ 266,34 µg/mL). Por lo tanto, el EEFCA, se presenta como una fuente potencial para la extracción de compuestos con actividad anti-leishmania.

Palabras-clave: *Croton argyrophyloides*. Anti-Leishmania. Extracto de etanol.

INTRODUCTION

Leishmaniasis is a parasitic disease caused by several species of *Leishmania*, which are transmitted by the bite of the female sandfly Diptera, which belongs to the Psychodidae family, subfamily Phlebotominae. It is endemic in 98 countries, with a risk group with more than 350 million people. It is estimated that each year 2 million new cases occur, being 500,000 cases of visceral leishmaniasis and 1.5 million of cutaneous and mucocutaneous leishmaniasis⁽¹⁾. The leishmaniasis in conjunction with other parasitic diseases such as malaria and Chagas disease is considered by the World Health Organization the six major tropical diseases in our planet⁽¹⁾, being considered an international public health problem. Depending on the species, leishmaniasis can develop in cutaneous (CL), mucocutaneous (MCL) or visceral form (VL), the latter being the most severe form of the disease in the Americas caused by *Leishmania infantum* Nicolle⁽²⁾.

One of the clinical changes in patients with Leishmaniasis is the elevation of oxidative process. The oxidation is a process that occurs naturally, or by some biological dysfunction, as in the case of diseases. Oxidation occurs through the generation of free radicals, which have an unpaired electron in oxygen or nitrogen atom, which can be classified as reactive oxygen species (ROS) or reactive nitrogen species (RNS)⁽³⁾. Free radicals have essential functions in the body; however, the excess of these can cause various undesirable actions such as peroxidation of membrane lipids, proteins and enzymes to attack, as well as carbohydrates and nucleic acids. This series of actions can result in several injuries to the body, as in the case of patients with leishmaniasis. The excess of free radicals is contained by antioxidant molecules. Phenolic compounds such as flavonoids derived from plants stand out due to their good inhibitive action of these radicals⁽⁴⁾.

Moreover, the currently available drugs for the treatment of Leishmaniasis do not have full efficacy and are highly toxic⁽¹⁾. Plants are important sources of substances with biological activities, mainly antiparasitic activity⁽⁵⁾. Among these, we can highlight species of the Euphorbiaceae family, which has about 300 genera and 7,600 species, being *Croton* the most representative⁽⁶⁾. Today there are more than 1,300 known species of *Croton* which are distributed in all tropical and subtropical regions of the globe⁽⁷⁾.

Croton argyrophyloides Muell Arg. is an endemic species in northeastern Brazil, known popularly as “marmeleiro prateado”, because the underside of its leaves looks silvery. Its chemical composition is pretty varied and terpenoids are the predominant secondary metabolites in their extracts(8). The essential oil from leaves of *C. argyrophyloides* shows activity against *Aedes aegyti* larvae (LC_{50} : 102 $\mu\text{g/ml}$) and its compound has over twenty constituents, being the α -Pinene, β -Pinene 1,8-Cineole, E-caryophyllene and spathulenol the most abundant ones(9). This species is used in folk medicine to treat cancer, diabetes, digestive problems, dysentery, fever, hypercholesterolemia, hypertension, inflammation, intestinal problems, malaria and ulcer. Phytochemical tests have shown in plants of this kind the presence of alkaloids, flavonoids, triterpenoids, steroids and a number of diterpenoids(10).

Alkaloids, flavonoids, diterpenes, triterpenes and steroids are cited in the literature as molecules with anti-leishmania action(11). Considering this, we aimed at developing a phytochemical screening of ethanol extract oc *C. argyrophyloides* (CALEE) and test the activity of this against *A. saline*, *L. infantum* and antioxidant.

MATERIAL AND METHODS

Plant used

In this study, the species *C. argyrophyloides* (Euphoraceae), popularly known as *marmeleiro prateado*, was used. Plants were collected in the herbarium of the Universidade Estadual do Ceará. A voucher specimen was made and deposited in the Herbarium Prisco Bezerra from the Universidade Federal do Ceará, under the number 46719.

Preparation of the *Croton argyrophyloides* leaf ethanol extract (CALEE)

The *C. argyrophyloides* leaves ethanol extract (CALEE) was prepared by maceration: the dry leaves (2.5 kg) were ground in an industrial shredder and placed in commercial ethanol 70% (Coperalcool®) for seven days. After this period, the solution was filtered and the ethanol solvent was removed on a rotary evaporator (Quimis®) to produce the CALEE (79.26g).

Phytochemical tests

Qualitative phytochemical tests for the presence of phenols, steroids, triterpenes, flavonoids and alkaloids were performed based on Matos (2009)(12). These tests were based on visual observation of the color change or precipitate formation after addition of specific reagents.

Phenol content and flavonoid content

The determination of total phenols was made by spectroscopy in the visible region by the Folin-Ciocalteu method(13). 7.5 mg of the CALEE extract was dissolved in methanol. The mixture was transferred to a volumetric flask of 25 ml, and its final volume was completed with methanol. An aliquot of 100 μl of this solution with 500 μl of Folin-Ciocalteu reagent was mixed for 30 seconds. Then, 6 ml of distilled water and 2 ml of Na_2CO_3 (15%) were added. The mixture was stirred again for 1 minute and filled up the volume of 10 ml with distilled water (1.4 ml). After 2 hours, the absorbance of samples at 750 nm in spectrophotometer UV-Vis, using buckets, was determined, the first bucket containing the background (entire mixture without the sample) and the second filled with the sample. The quantification of the phenolic compounds was found by a calibration curve of gallic acid.

The flavonoid content was analyzed by the method of Funari e Ferro (1989)⁽¹⁴⁾. 1 ml of the extract CALEE (10 g/l) was mixed with 1 ml of aluminum chloride in ethanol (20 g/l) and diluted with ethanol to 25 ml. The blank samples were prepared with 1 ml of plant extract and one drop of glacial acetic acid and diluted to 25 ml. After 30 min, it was determined the absorbance of samples at 415 nm in spectrophotometer UV-Vis, using buckets. The quantification of the flavonoid compounds will be found by a calibration curve using rutin as a reference compound.

Antioxidant activity

The antioxidant activity was determined using two techniques. The technique of inhibiting free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) was performed according to Brand-Williams (1995). In the DPPH solution at the concentration $6,5 \times 10^{-5} M$, 0.1 ml of methanolic solution was added at concentrations of CALEE 10,000, 5,000, 1,000, 500, 100, 50, 10 e 5 $\mu g/ml$. The test was done in triplicate. The absorbance was determined after a 60 min incubation in the dark at room temperature in a spectrophotometer Thermo brand, model Biomate 5, at a wavelength of 515 nm and used to calculate the sweep rate of the sample in percent (IV%). The IV is equivalent to the amount of sample required to inhibit free radical DPPH. The IV values were found in the Origin 7.0 software applied to calculate the concentration that inhibits 50% of the free radical solution (IC_{50}). It used quercetin as standard in the same concentration of CALEE.

The other technique used was the Trolox Equivalent Antioxidant Capacity (TEAC), developed in accordance with Re et al. (1999)⁽¹⁵⁾. It used the ABTS^{••} radical cation (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid). The reading of the absorbance was performed at 750 nm in a spectrophotometer Thermo brand, model Biomate 5. The analysis was performed in triplicate. The trolox was used as a standard drug at the same concentration of CALEE.

Brine shrimp test (BST)

The brine shrimp lethality test (BST), according to the method proposed by Meyer et al. (1982)⁽¹⁶⁾ with some modifications, was used. *A. salina* eggs were incubated in artificial brine at room temperature for 48 hours in a small aquarium. With the help of a light source, the larvae were attracted to light and collected with Pasteur pipette and transferred to a beaker with saline water. The CALEE was dissolved in dimethylsulfoxide (DMSO 14.08 mol/l) and saline water in concentrations of 1,000, 100, 10 e 1 $\mu g/ml$. 10 metanauplius were transferred to test tubes containing 5 ml with each concentration tested. A control group with brine and larvae was prepared containing only DMSO (2%). The positive control used was potassium dichromate. Assays were performed in triplicate. The count of the number of dead larvae was done after 24 hours of exposure, and this number was used to calculate the lethal concentration (LC_{50}) using the MicroCal Origin 4.1 program.

In vitro tests against *L. infantum* promastigotes

L. infantum IOCL2272 was obtained from a culture maintained in the Departamento de Patologia e Medicina Forense, Faculdade de Medicina, Universidade Federal do Cear , Brazil. *L. infantum* were grown in Schneider medium (Sigma[®]) supplemented (gentamicin 40 mg/ml, 5% male-sterile human urine, 10% fetal bovine serum (FBS) (Cultilab[®]) and 10% PBS). The experiments were performed in 96-well plates, according to Silva et al. (2014)⁽¹¹⁾. The CALEE was dissolved in dimethylsulfoxide (DMSO) and diluted in Schneider medium (Sigma[®]). The final concentration of DMSO did not exceed 1%. *L. infantum* promastigotes were used at a concentration of 10^6 parasites per well. The reference product used was pentamidine isothianate (pentacarinat[®]). Negative controls were performed with 10^6 parasites per well in Schneider (Sigma[®]) supplemented, without CALEE and pentamidine isothianate. The parasites were incubated at 26°C for 48 hours.

After this period, cell viability was assessed by the conversion of soluble tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide) (Sigma®) in insoluble formazan by mitochondrial enzymes. Twenty milliliters of MTT [5 mg/ml] was added per well and the culture maintained for 4 hours at 26°C. Subsequently, 100 µl of a solution of sodium dodecyl sulfate (SDS) at 10%: isopropyl alcohol (1:1) was added. After stirring for 15 minutes, the optical density reading at 570 nm in a spectrophotometer was made. Assays were performed in triplicate.

The EC₅₀ values (concentration of compound able to inhibit 50% of the parasite), with 95% confidence intervals were calculated using a nonlinear regression curve on the statistical software GraphPad Prism 5.0.

RESULTS AND DISCUSSION

The plants have been an important source for new compounds with antileishmanial activity, and Brazil has excellent prospects for the discovery of new compounds with therapeutic properties since there are many medicinal plants distributed in abundance in tropical region⁽¹⁷⁾. The phytochemical screening of the *Croton argyrophylloides* leaf ethanol extract (CALEE) indicated the presence of condensed tannins, flavones, flavonols, flavonoids, xanthonenes, free steroids and saponins. The quantitative analysis of total phenols and flavonoids indicated 8.64 (± 0.77) and 3.74 (± 0.11)% respectively. The literature has already registered several leishmanicide phenolic compounds such as rutin and quercetin⁽¹⁸⁾.

In general, phenols and flavonoids are known molecules with good antioxidant activity⁽⁵⁾. Parallel to this, in the case of leishmaniasis⁽⁹⁾, it is known that the individual probably presents an imbalance in redox mechanisms, favoring an intense oxidative stress in the body. Oxidative stress can cause serious tissue damage⁽¹⁹⁾. Therefore, antioxidants may improve the clinical condition of the patient with leishmaniasis by reducing oxidative stress. The CALEE showed moderate inhibition on the free radicals by the method of Re et al. (1999)⁽¹⁵⁾ (Table 1).

This activity can be justified by the presence of phenols and flavonoids in its composition.

Table 1 - Antioxidant activity of the of *Croton argyrophylloides* leaves ethanol extract

Droga	IC ₅₀ [µg/ml] (CI95%)*	
	DPPH.	ABTS**
CALEE	> 1.000	220,0 ± 70,0
Quercetin	10,0 ± 1,0	–
Trolox	-	28,0± 4,0

*IC₅₀: Concentration able to inhibit 50% of the free radicals; IC95%: confidence interval of 95%.

In anti-Leishmania assays, CALEE presented effective action against *L. infantum* promastigotes with EC₅₀ below the standard drug (pentamidine), with values of 5.63 and 23.71 µg/mL respectively (see Table 2). This activity can be justified by the compounds found in CALEE, such as phenolic compounds and steroids, as described with antileishmanial activity^(17,13). Drugs that have antileishmanial and antioxidant activity are promising for studies and development herbal and/or plant protection products for the treatment of leishmaniasis, as well as acting directly on the parasite, acting on the symptoms of the disease by reducing oxidative stress and improving the general condition of the patient.

Table 2 - Antileishmanial activity and brine shrimp toxicity of the *Croton argyrophyloides* leaves ethanol extract

Drug	IC ₅₀ [mg/ml] (IC95%)*	
	EC ₅₀ <i>Leishmania infantum</i>	LC ₅₀ <i>Artemia salina</i>
CALEE	5.63 ± 1.18 ^a	266.34 ± 88.80
Pentamidine	23.71 ± 1.18 ^b	-

*IC₅₀: Concentration able to inhibit 50% of the culture of *Leishmania infantum*; IC95%: confidence interval of 95%.

The test of acute toxicity against the crustacean *Artemia salina* is used in research to determine the cytotoxicity of several medicinal plants and their chemical compounds. The plant extracts are considered bioactive when LC₅₀ is below 1000 µg/mL. When an extract shows activity in the test, it is believed that it also presents other biological activities such as anticancer and antileishmanial⁽²⁰⁾.

CONCLUSION

The evaluation of CALEE potential against *L. infantum* showed better antileishmanial activity than pentamidine in MTT Colorimetric test, and also showed antioxidant activity and activity against *A. salina* in BST test. Therefore, this research has opened possibilities for studying anti-*Leishmania* activity and *in vivo* toxicity evaluation for possible development of a herbal medicine using *C. argyrophyloides* extracts for the treatment of Leishmaniasis due to the fact that the occurrence of drugs used to combat the parasitic infection are not effective, and have high toxicity.

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