Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

Carajurin: a anthocyanidin from *Arrabidaea chica* as a potential biological marker of antileishmanial activity

João Victor Silva-Silva [a,1], Carla J. Moragas-Tellis [b,1], Maria S.S. Chagas [b], Paulo Victor R. Souza [b,c], Davyson L. Moreira [b,f], Celeste S.F. de Souza [a], Kerolain F. Teixeira [d], Arthur R. Cenci [d], Aldo S. de Oliveira [d], Fernando Almeida-Souza [a,e,*,1], Maria D. Behrens [b,1], Kátia S. Calabrese [a,1]

[a] *Laboratory of Immunomodulation and Protozoology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, RJ, Brazil*
[b] *Laboratory of Natural Products for Public Health, Pharmaceutical Technology Institute – Farmanguinhos, Oswaldo Cruz Foundation, Rio de Janeiro, RJ, Brazil*
[c] *Student on Postgraduate Program in Translational Research in Drugs and Medicines, Farmanguinhos, Oswaldo Cruz Foundation, Rio de Janeiro, RJ, Brazil*
[d] *Department of Exact Sciences and Education. Federal University of Santa Catarina, Blumenau, SC, Brazil*
[e] *Postgraduate Program in Animal Science, State University of Maranhão, São Luis, MA, Brazil*
[f] *Research Directorate of the Rio de Janeiro Botanical Garden Research Institute, Jardim Botânico, Rio de Janeiro, RJ, 22460-030, Brazil*

ARTICLE INFO

ABSTRACT

Leishmaniasis is a group of neglected tropical diseases whose treatment with antimonials bears limitations and has changed little in over 80 years. Medicinal plants have been evaluated as a therapeutic alternative for leishmaniasis. *Arrabidaea chica* is popularly used as a wound healing and antiparasitic agent, especially as leishmanicidal agent. This study examined the leishmanicidal activity of a crude extract (ACCE), an anthocyanidin-rich fraction (ACAF), and three isolated anthocyanidins from *A. chica*: carajurin, 3′-hydroxy-carajurone, and carajurone. We evaluated the antileishmanial activity against promastigote and intracellular amastigote forms of *Leishmania amazonensis* and determined cytotoxicity in BALB/c peritoneal macrophages, as well as nitrite quantification, using the Griess method. Molecular docking was carried out to evaluate interactions of carajurin at the nitric oxide synthase enzyme. All compounds were active against promastigotes after 72 h, with IC_{50} values of 101.5 ± 0.06 µg/mL for ACCE and 4.976 ± 1.09 µg/mL for ACAF. Anthocyanidins carajurin, 3′-hydroxy-carajurone, and carajurone had IC_{50} values of 3.66 ± 1.16, 22.70 ± 1.20, and 28.28 ± 0.07 µg/mL, respectively. The cytotoxicity assay after 72 h showed results ranging from 9.640 to 66.74 µg/mL for anthocyanidins. ACAF and carajurin showed selectivity against intracellular amastigote forms (SI> 10), with low cytotoxicity within 24 h, a statistically significant reduction in all infection parameters, and induced nitrite production. Molecular docking studies were developed to understand a possible mechanism of activation of the nitric oxide synthase enzyme, which leads to an increase in the production of nitric oxide observed in the other experiments reported. These results encourage us to suggest carajurin as a biological marker of *A. chica*.

1. Introduction

Leishmaniasis are a complex of diseases caused by an intracellular protozoan of the genus *Leishmania*, which has more than 20 species, covering a broad clinical spectrum, from self-limited cutaneous forms to the fatal visceral form, depending on the immune response of the host and the *Leishmania* species [1].

Leishmaniasis are a serious public health problem and are classified

as neglected tropical diseases [1,2], being associated with limited investments in diagnosis, treatment, and control, as well as the population's conditions of poverty [3]. For this reason, more than 1 billion people live in endemic areas at risk of infection, with 1 million cases of cutaneous leishmaniasis reported in the last 5 years, 300 thousand cases of visceral leishmaniasis estimated annually, and more than 20 thousand deaths per year [1]. Brazil was among the seven countries responsible for more than 90% of the new cases of visceral leishmaniasis notified to WHO in 2017, and among the nine countries in which most skin cases of leishmaniasis occur [1].

In addition to these circumstances, current chemotherapy for the treatment of leishmaniasis, in all clinical forms, has changed little in over 80 years and still depends on the use of pentavalent antimonials such as sodium stibogluconate (Pentostam) and meglumine antimonate (Glucantime®) [4]. Although there are alternatives in severe cases for those not responding to antimonials, such as amphotericin B liposomal and conventional, pentamidine, paromomycin, and miltefosine, they also involve toxicity problems, high costs, the resistance of parasites, and therapeutic failure [5]. Therefore, there is an urgent need for discovery or development of new and more effective drugs to replace or complement those in use.

The use of plants in the treatment of these diseases appears as an alternative, as it has long been considered a medicinal source to treat different diseases [6]. It is worth mentioning that, of the 16 antiparasitic drugs that were approved by the United States regulatory agency (FDA, USA), between January 1981 and December 2014, 43.75% are natural products or derivatives [7]. As a result, the search for new therapies has grown in recent years, being natural products — mainly those derived from plants — used in the study of therapeutic alternatives for leishmaniasis [8]. In Brazil, the National List of Medicinal Plants of Interest to the Unified Health System (RENISUS) was issued in 2009. In this list of 71 medicinal plants, the species *Arrabidaea chica* stands out, being considered by the Brazilian health authorities as one of the most important medicinal plants used in popular medicine, especially in the Amazon region [9].

Arrabidaea chica (Humb. & Bonpl.) B. Verlot belongs to the Bignoniaceae family and is found in the Cerrado (a sort of Brazilian savanna), Atlantic Forest, and in the Amazon biomes [10]. Pharmacological studies in the literature describe antimicrobial [11], anti-inflammatory, antiangiogenic and antiproliferative activities [12], cytotoxicity [13], genotoxicity [14], healing potential [15], photochemical-protective [16] and antioxidant activities [16–18]. Other studies also report antiparasitic activity against *Trypanosoma cruzi* [19,20] and *Leishmania* spp. [13,21,22]. Recent results by our research network shows that the variation in the anthocyanidin profile in extracts of four morphotypes of *A. chica* affected their leishmanicidal activity [23]. That study implies this activity might be related to the presence of carajurin and that this would, therefore, be a potential biological marker of the species. However, that was the first study on the characterization of the biological marker for the species. To date, there are no data on possible mechanisms of leishmanicidal action for extracts or isolated substances from *A. chica*.

Given that natural products are promising sources of new potential therapeutic agents, the widespread use of *A. chica* in popular medicine, as well as the advances in phytochemical studies of this plant, this study presents the evaluation of the leishmanicidal activity of the crude extract — through bioguided fractionation — of the anthocyanidin-rich fraction, and the isolated compounds, carajurin, 3′-hydroxy-carajurone, and carajurone. Its results contributed to confirm the importance of carajurin as a biological marker of the leishmanicidal activity of *A. chica*.

2. Materials and methods

2.1. Reagents

Brewer thioglycolate medium, RPMI 1640 medium, 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), amphotericin B, Schneider's insect medium, and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), and penicillin were acquired from Gibco (Gaithersburg, MD, USA).

2.2. Plant material

Leaves of *A. chica*, morphotype IV, were collected at Fiocruz Atlantic Forest Campus, Rio de Janeiro city, State of Rio de Janeiro, Brazil (S 22.9406° W 43.4046°) in February 2016. Voucher specimens were identified by Dr. Marcelo Galvão of Botanical Collection of Medicinal Plants (CBPM) of Farmanguinhos/Fiocruz. A sample (CPBM 668) was deposited at the CBPM.

2.3. Extraction, fractionation, and isolation

Leaves of *A. chica* were dried at 60 °C with air circulation and ground in a cutting mill. *Arrabidaea chica* crude extract (ACCE) was obtained by maceration in ethyl alcohol: water 70/30 (v/v) for seven days. The resulting extract was filtered and evaporated to dryness under reduced pressure at 30–40 °C. The reddish residue (15 g) was fractioned by liquid-liquid partition, with *n*-hexane (3 × 200 mL), dichloromethane (3 ×200 mL), ethyl acetate (3 ×200 mL) and *n*-butanol (3 × 200 mL). The dichloromethane fraction (5 g) (ACDF) was chromatographed by column chromatography on Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO, USA) using methanol as eluent to produce an anthocyanidin-rich fraction named ACAF. Successive chromatographic fractionation steps on Sephadex LH-20 led to the isolation of three anthocyanidins: (**1**) carajurin (45 mg), (**2**) 3′-hydroxy-carajurone (12 mg), and (**3**) carajurone (3.5 mg). The identification of isolated compounds was achieved by direct infusion electrospray ionization mass spectrometry (ESIMS) and ^{1}H- and ^{13}C NMR spectrometry.

Carajurin (**1**) ^{1}H NMR (400 MHz-CDCl$_3$-d6) d: 6.98 (d, 1 H, H-3, $J =$ 7.8 Hz); 7.99 (d, 1 H, H-4, $J =$ 7.8 Hz); 7.01 (d, 2 H, H-3′and H-5′, $J =$ 8.8 Hz); 7.89 (d, 2 H, H-2′ and H-6′, $J =$ 8.8 Hz); 6.53 (s, 1 H, H-8); 3.90 (s, 3 H, 4′-O-CH$_3$); 4.10 (s, 3 H, 5-O-CH$_3$). ^{13}C NMR (100 MHz-CDCl$_3$-d6) d: 158.90 (C-2); 102.62 (C-3); 133.76 (C-4); 135.02 (C-5); 139.93 (C-6); 176.82 (C-7); 98.61 (C-8); 156.86 (C-9); 118.16 (C-10); 123.43 (C-1′); 127.68 (C-2′); 114.77 (C-3′); 162.50 (C-4′); 60.42 (C-5′); 55.58 (C-6′). Positive ESIMS m/z: 299.0905 [M + H]$^+$ (calc. m/z of 299.0924; $\Delta =$ −6.35), m/z 284.0671 M −[CH$_3$]$^+$ (calc. m/z of 284.0690; $\Delta =$ −6.68); m/z 269.0441 M −[CH$_3$] −[CH$_3$]$^+$ (calc. m/z of 269.0455; $\Delta =$ −5.20); m/z 256.0722 (M − [CH$_3$] − [C=O]$^+$) (calc. m/z of 256.0741; $\Delta =$ −7.41); 241.0491 M −[CH$_3$] −[CH$_3$] −[C=O]$^+$ (calc. m/z of 241.0506; $\Delta =$ −6.22).

3′-hydroxy-carajurone (**2**) (C$_{16}$H$_{13}$O$_6$) ^{1}H NMR (500 MHz-Methanol-d6) d: 7.27 (d, 1 H, H-3); 8.17 (d, 1 H, H-4, $J=$ 8.0 Hz); 6.50 (s, 1 H, H-8); 7.44 (d, 1 H, H-2′, $J=$ 2 Hz); 7.48 (dd, 1 H, H-5′, $J=$ 8.5, $J=$2 Hz); 6.88 (d, 1 H, H-6′, $J=$ 8 Hz), 4.04 (s, 3 H, 5-O-CH3). ^{13}C NMR (100 MHz-Methanol-d6) d: 166.05 (C-2); 103.41(C-3); 183.49 (C-4); 163.05 (C-5); 95.91(C-6); 166.05 (C-7); 101.31 (C-8); 159.72 (C-9); 104.27 (C-10); 123.43 (C-1′); 113.89 (C-2′); 147.31 (C-3′); 151.65 (C-4′); 116.86 (C-5′); 120.23 (C-6′). Positive ESIMS m/z: 301.0627 m/z [M + H]$^+$ (calc. m/z 301.0717; $\Delta =$ 29.8), m/z 286.0414 [(M)-CH$_3$]$^+$ and at m/z 256.0466 [(M) −CH$_3$ −CO]$^+$.

Carajurone (**3**) (C$_{16}$H$_{13}$O$_5$) ^{1}H NMR (400 MHz-Methanol-d6) d: 7.35 (d, 1 H, H-3, $J=$ 8 Hz); 8.20 (d, 1 H, H-4, $J=$ 8.0 Hz); 6.53 (s, 1 H, H-8); 7.94 (dd, 2 H, H-2′ and H6′, $J=$ 2,0; $J=$7,0 Hz); 6,94 (dd, 2 H, H-3′and H-5′, $J=$ 7,;0; $J=$2 Hz), 4.05 (s, 3 H, 4′-O-CH3). Positive ESIMS m/z: 285.0749 m/z [M + H] (calc. m/z of 285.0749; $\Delta =$ −4.56), m/z 270.0492 [(M − CH$_3$]$^+$ (calc. m/z of 270.0533; $\Delta =$ −15.44) and at m/z 242.0541 [(M − CH$_3$− CO)]$^+$ (calc. m/z of 242.0584; $\Delta =$ −17.76).

2.4. High-performance liquid chromatograph coupled to diode-array UV-vis detector (HPLC-DAD-UV)

Chromatographic analyses were performed on an HPLC-DAD-UV, using a Shimadzu Nexera XR® liquid chromatograph (Shimadzu, Kyoto, Japan) coupled to a UV detector with the diode array SPDM20A, a CBM20A controller, DGU20A degasser, LC20AD binary pump, CTO20A oven, and SILA20A auto-injector (Shimadzu, Kyoto, Japan). A Shimadzu LabSolutions Software Version 5.3 (Shimadzu, Kyoto, Japan) was used to analyze the chromatograms. DAD analysis was applied to select the optimized wavelength of anthocyanidins in this study. In a full-scan experiment, chromatograms at 480 nm show the maximum wavelength (λ_{max}) for the anthocyanidins. Combinations of acidified ultrapure water (pH 3.0, with anhydrous acetic acid, Merck, Darmstadt, Germany) (A) and acetonitrile (HPLC grade, Tedia, Rio de Janeiro, Brazil) (B) were used as the mobile phase (initially 5% A rising to 95% in 80 min). HPLC column was silica-based C18 (250 mm × 4.6 mm i.d. × 5 μm particle size, ODS Hypersil, Thermo, Waltham, MA, USA). The oven was set at 50 °C and the injection volume was 10 μL for all analyses.

2.5. Preparation of samples of the extracts

A total of 1000 μL of acetonitrile: methanol (both HPLC grade, Tedia, Rio de Janeiro, Brazil) mixture (75:25; v/v) was added to 10 mg of extracts and anthocyanidins-rich fraction, previously weighed in a 4 mL vial. The vial was sealed and the sample was sonicated for 10 min with occasional swirling. The sample was then vortexed to mix thoroughly, followed by filtering through a 0.45 μm PTFE filter (Merck Millipore, Darmstadt, Germany) before further analyses into an HPLC vial.

2.6. Quantification of anthocyanidins, using carajurin as standard

Quantification of anthocyanidins was performed using carajurin as the external standard. Since there are no standards available for all anthocyanidins, their content was expressed in milligrams of carajurin per gram of dry extract. A 200 μg/mL stock solution of the isolated carajurin (98%, chromatographic determined) was prepared in an acetonitrile:methanol (both HPLC grade, Tedia, Rio de Janeiro, Brazil) mixture (75:25; v/v) in a volumetric flask. Six concentrations of solutions (20; 40; 60; 100; 150 and 200 μg/mL) were done on the day, in order to prepare an analytical curve. The solutions were filtered in a 0.45 μm PTFE filter before analyses by HPLC-DAD-UV. Injections of 10 μL were performed in triplicate to obtain the analytical curve from the areas corresponding to the peaks of carajurin. The analytical curve (20–200 μg/mL) of the standard was plotted based on the UV-Vis signal at 480 nm for better selectivity: carajurin content (μg/mL) = (Abs (mAu) + 661228)/48694; R^2 = 0.9993). Carajurin and other anthocyanidin amounts (mg/g of dry extract) were calculated and expressed as carajurin content [23].

2.7. Animals and ethical statements

The study was approved by the local Ethics Committee on Animal Care and Utilization (CEUA-IOC L53/2016), following all procedures described by the Control of Animal Experimentation (CONCEA). Female BALB/c mice aged 4–6 weeks were purchased from the Institute of Science and Technology in Biomodels of the Oswaldo Cruz Foundation.

2.8. Parasites

Leishmania amazonensis strain MHOM/BR/76/MA-76 was maintained in promastigote form by culturing at 26 °C Schneider's Insect Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco (Gaithersburg, MD, USA), 100 U/mL penicillin (Gibco, Gaithersburg, MD, USA), and 100 μg/mL of streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cultures with a maximum of 10 passages *in vitro* were used.

2.9. Peritoneal macrophage isolation and cell culture

Peritoneal macrophages were isolated from BALB/c mice administered with 3 mL thioglycolate 3% intraperitoneal for 72 h. Then, the animals were euthanized with ketamine 10% and xylazine 2% according to the weight of each animal and, after the death and exposure of the peritoneum, 10.0 mL of sterile phosphate buffered saline solution pH 7.2 was inoculated, followed by a light manual massage. Cells were recovered from peritoneum, cultured overnight and maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/ mL), at 37 °C and 5% CO_2.

2.10. Activity against promastigote forms

The exponential phase of L. *amazonensis* promastigote forms were seeded into 96-well flat-bottomed plates at 2×10^6 parasites per well. Then, 100 μL of compounds solution, resuspended in dimethyl sulfoxide (DMSO) 1%, were mixed in the first well, proceeding to serial microdilutions in a 1:1 proportion ratio (compounds solution: Schneider's Insect Medium) until the penultimate cavity. Thus, different concentrations of the ACCE (1000–3.9 μg/mL), ACAF (1000–3.9 μg/mL), or isolated anthocyanidins (100–3.125 μg/mL) were obtained. After diluting the samples, the plates were incubated at 26 °C. Amphotericin B (2.5–0.07 μg/mL) was used as the reference drug, while wells without parasites were used as blanks, and wells with parasites and DMSO 1% only were used as untreated control. After 72 h of treatment, the plates were examined under an inverted microscope to assure the growth of the controls under sterile conditions, and viable promastigotes were counted in a Neubauer chamber [24]. The experiments were conducted in triplicate. The percentage of growth inhibition was calculated from the count of viable parasites relative to the untreated control, and 50% inhibitory concentration (IC$_{50}$) values were determined.

2.11. Cytotoxicity assay

Peritoneal macrophages were cultured in 96-well plates (5×10^5 cells/mL) and tested with different concentrations of ACCE and ACAF (7.81–1000 μg/mL), anthocyanidins (3.9–500 μg/mL) or amphotericin B (0.19–25 μg/mL), in a final volume of 100 μL/well, at 37 °C and 5% of CO_2. Wells without cells were used as blanks and wells with cells and 1% DMSO were used as controls. After 24 and 72 h of treatment, cell viability was evaluated by the modified colorimetric method based on the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, MO, USA). MTT (5 mg/mL) was added to each well in a volume equal to 10% of the total. After 2 h, the supernatant was completely removed and 100 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was read on a spectrophotometer at a wavelength of 570 nm. Data were normalized according to the formula: % survival = (Abs. sample-Abs. blank) / (Abs. control-Abs. blank) × 100 [25].

2.12. Activity against intracellular amastigotes and selectivity index

Peritoneal macrophages of BALB/c mice were cultured in 24-well plates (5×10^5 cells/well) containing round coverslips, at 37 °C and 5% CO_2. The cells were infected with promastigote forms of *L. amazonensis* — in the parasite/cell ratio of 10:1 — for 6 h followed by washing with PBS to remove non-internalized parasites. Infected cells were treated with different concentrations of ACAF (2.5–0.078 μg/mL) or carajurin (25–0.78 μg/mL) for 24 h. The slides of the infected and treated cells were fixed with Bouin, stained with Giemsa, and examined by light microscopy. The IC$_{50}$ was calculated from the intracellular amastigotes count in 200 cells. The percentage of infected cells was

obtained from the number of infected cells divided by two. The average number of amastigotes per cell was obtained from the number of intracellular amastigotes in 200 cells divided by the number of infected cells [25]. Amphotericin B (2.5–0.15 µg/mL) was used as a reference drug. The selectivity index (IS) was obtained from the ratio between the half-maximal cytotoxic concentration (CC_{50}) for BALB/c peritoneal macrophages and the IC_{50} for promastigote or intracellular amastigote.

2.13. Nitrite quantification

BALB/c peritoneal macrophages (5×10^6 cells/mL) were treated with ACAF (2.5 µg/mL) or carajurin (25 µg/mL) and stimulated with *L. amazonensis* (3×10^7 parasites/mL). After 48 h, the supernatant was collected and the nitrite quantification was performed with the Griess reagent. 50 µL of culture supernatant was added to 50 µL of Griess reagent (25 µL of 1% sulfanilamide in 2.5% H_3PO_4 solution and 25 µL of 0.1% N-(1-naphthyl) ethylenediamine solution) in 96-well plates. After 10 min, the plates were read at 570 nm on the spectrophotometer and the nitrite values were obtained from the standard sodium nitrite curve (1.5–100 µM) [26].

2.14. Molecular docking

Molecular docking simulations were performed with GOLD v.2020.2.0 [44] and the ChemPLP [27] scoring function. The receptor (PDB 1DF1, 2.35 Å) was treated as rigid, and the compounds were treated as fully flexible. The preparation of the receptors was made within the GOLD suite. Only was used the chain A of receptor. No crystallographic water molecules were considered. The binding site was defined as all the receptor atoms up to 6 Å of the reference crystallographic inhibitor. At least 10 poses were generated for ligand using the default parameters of the genetic algorithm. For analysis, the top-scoring conformations of the most populated clusters of poses/ligands were selected. Prior to the simulations, the ligands were optimized with a steepest descent algorithm (100 steps. FF: AM1BCC. The atomic charges were assigned with Ammp-Mom) in VEGA ZZ v.3.2 [28]. The propensity maps were generated with SuperStar, a module for knowledge-based pharmacophore generation and prediction of intermolecular interactions available within the GOLD suite. We worked with the default parameters for cavity detection and the PDB data, allowing R-H rotation and [O, N, S]-H bonds. The propensity map figure was generated with Hermes v.1.10.5 (also available within the GOLD suite). The receptor-ligand figures were generated with PyMOL v.1.8.
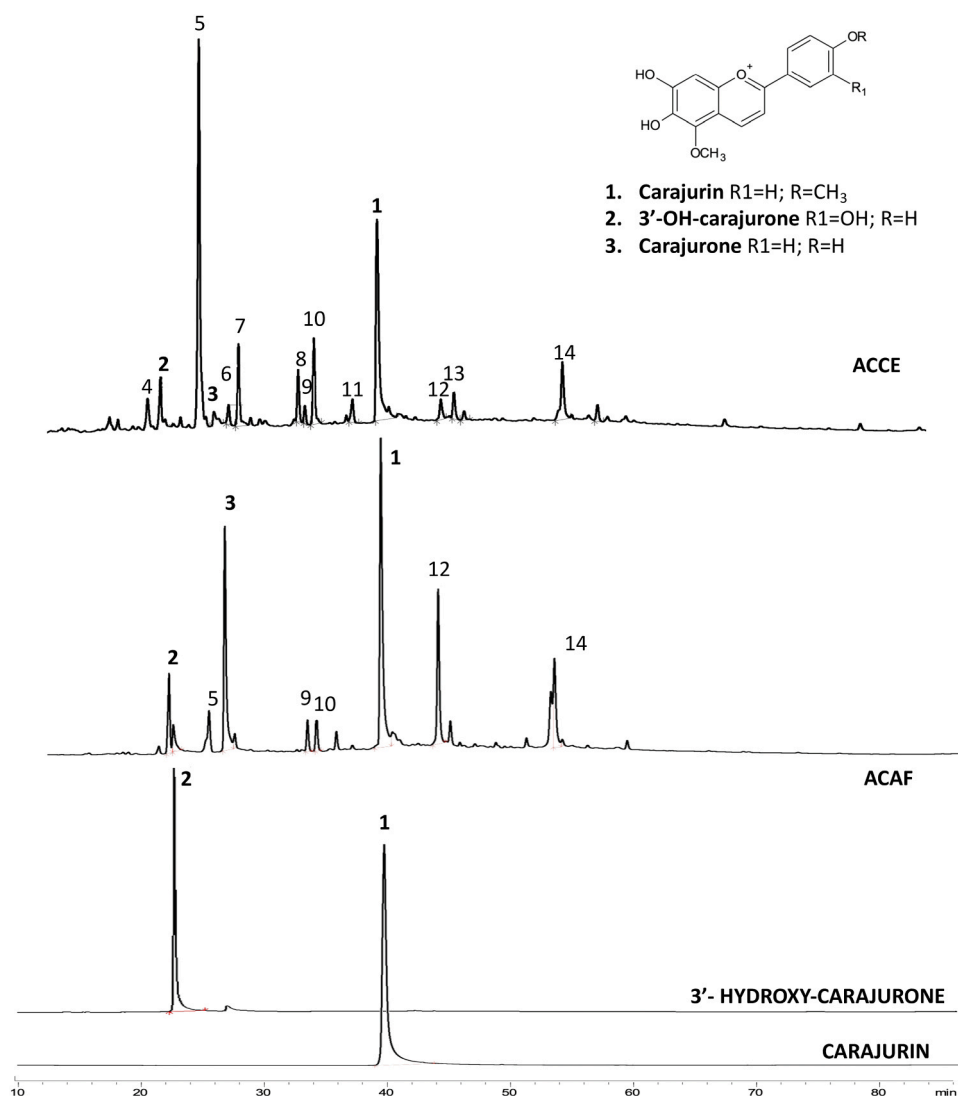
Fig. 1. HPLC-DAD-UV chromatograms of *Arrabidaea chica* crude extract (ACCE), anthocyanidins-rich fraction (ACAF) and isolated compounds carajurin (1, Rt= 38.96 min) and 3'-hydroxy-carajurone (2, Rt= 23.54 min). Note: 3'-OH-carajurone = 3'-hydroxy-carajurone. Rt = retention time.

2.15. Statistical analyses

The statistical analyses were conducted using the statistical software GraphPad Prism® version 7 (GraphPad Software Inc., San Diego, CA, USA). The numerical results were expressed as mean ± standard deviation and differences were considered significant when $p < 0.05$ by one-way analysis of variance (ANOVA) or Mann-Whitney test.

3. Results

3.1. Phytochemical analysis

The bioguided fractionation of *A. chica* crude extract (ACCE) was carried out in this study, leading to an anthocyanidin-rich fraction (ACAF) and the isolation of three known anthocyanidins: carajurin (**1**), 3′-hydroxy-carajurone (**2**), and carajurone (**3**).

HPLC-DAD-UV analyses were performed to determine the ACCE and ACAF profile. The isolated anthocyanidins carajurin (**1**), 3′-hydroxy-carajurone (**2**), and carajurone (**3**) were found in both ACAF and ACCE (Fig. 1). The UV data and retention times (Rt) were important to tentatively identify some other compounds (Table 1).

ACCE chromatogram (Fig. 1) showed 14 peaks, five of them presented UV λ_{max} in the range of 474–485 nm, characteristic of anthocyanidin compounds (peaks 1, 2, 3, 12, and 14). The elution order and the retention times of these compounds followed their structural and polarity characteristics, as had been previously discussed [23]. All five anthocyanidins identified at ACCE were also present at ACAF. Three of them have already been described in the literature: 3′-hydroxy-carajurone (**2**), the first anthocyanidin to be eluted (Rt = 23.54 min) due to its more polar structure, features four hydroxyl groups; carajurone (**3**) (Rt = 27.35 min), which contains three hydroxyl groups; and carajurin (**1**) (Rt = 38.96 min), the least polar of the four known anthocyanidins from *A. chica* (two hydroxyl and two methoxyl groups) [29,30]. The other two anthocyanidins have already been reported by Moragas-Tellis et al. [23] but although they have a proposed structure, they have not yet been confirmed and are still here designated as anthocyanidins A2 (**12**, Rt = 43.51 min) and A3 (**14**, Rt = 52.17 min), in accordance with previous studies [23].

Among the nine non-anthocyanidin compounds found in the ACCE only one — in minor concentration (peak 6) — did not have in its UV spectrum the broad bands of absorption with maximum peaks in the range of 338 and 348 nm (band I) and 256 and 283 nm (band II), which are characteristic of flavonoids [31].

The fractionation steps applied to the ACCE were effective in increasing the content of anthocyanidins (Tables 1 and 2). Luteolin (**7**) and apigenin (**8**) could be identified because they have already been isolated and identified by our group and we could compare their Rt and UV spectral data. Both were present only in the ACCE. According to our analyses, the composition of ACCE and the ACAF showed a flavonoid (**5**) and the anthocyanidin carajurin (**1**), respectively, as the compounds found in major amount.

3.2. Characterization and quantitation of anthocyanidins (mg/g of dry weight) expressed as carajurin content in ACCE and ACAF

As the anthocyanidins of *A. chica*, especially carajurin, had already been pointed out by our group as probably responsible for the leishmanicidal activity of the extracts of *A. chica*, we evaluated the quantitative variations on their content between ACCE and ACAF. The results of the quantitative determination of the 5 anthocyanidins identified (mg/g of dry extract calculated as carajurin content) are presented in Table 2.

Quantitative comparative analyses among anthocyanidins of the ACCE and ACAF showed that all anthocyanidins present in the extract are found in the rich fraction in higher quantities. Thus, the successive steps of fractionation were effective to produce a fraction rich in anthocyanidins.

3.3. Anti-Leishmania activity and cytotoxicity

The effect of the ACCE, the ACAF, and isolated compounds on the promastigote forms of L. *amazonensis* was monitored for 72 h (Table 3). It was possible to observe that the fractionation contributed to enhance the antipromastigote activity, having the ACAF a 20-fold higher level of activity than the ACCE. The evaluation of the antipromastigote activity of the isolated substances showed that 3′-hydroxy-carajurone and carajurone, although being more active than ACCE, were not as effective as the ACAF and the isolated carajurin, which was the most active of all. Additionally, 3′-hydroxy-carajurone exhibited the highest cytotoxicity, being followed by isolated carajurin and carajurone. However, considering the ratio between highest promastigote inhibition and lowest cytotoxicity, ACAF yielded the best results.

Carajurin, which had the highest level of activity against promastigote forms, and the ACAF, which has carajurin as its main component, were selected to further examination of their effects against intracellular amastigote forms. In this evaluation, the rich fraction presented a lower IC_{50} compared to carajurin (Table 4). However, carajurin showed less cytotoxicity, making it possible to obtain greater selectivity (IS 34.8). Such result is similar when compared with the drug used as reference, amphotericin B (SI 32.9). Amphotericin B showed leishmanicidal activity and cytotoxicity as expected. Carajurin was 26.4-fold less cytotoxic than amphotericin B. The SI showed that carajurin was 34.8 times more selective for *L. amazonensis* intracellular amastigote than for BALB/c peritoneal macrophage.

Table 1

Comparative composition in area percentage (area %) of anthocyanidins and other constituents in *Arrabidaea chica* crude extract (ACCE) and the anthocyanidins-rich fraction (ACAF).

Peak	HPLC-DAD-UV		Area %		Tentative Identification
	Rt (min)	UV data (nm)	ACCE	ACAF	
4	22.63	344, 281	3.44	–	flavonoid
2	**23.54**	**484, 295, 275, 243**	**4.48**	**6.73**	**3′-hydroxy-carajurone**
5	26.27	338, 283	33.67	3.15	flavonoid
3	**27.35**	**475, 293, 246**	**1.89**	**21.92**	**carajurone**
6	28.39	262, 234	1.46	–	unknown
7	29.10	347, 292	6.64	–	luteolin
8	33.35	337, 267	3.71	–	apigenin
9	33.82	340, 281	1.21	2.58	flavonoid
10	34.47	345, 274	6.70	3.25	flavonoid
11	37.22	334, 279	1.99	–	flavonoid
1	**38.96**	**475, 294, 245**	**24.19**	**36.80**	**carajurin**
12	43.51	485, 290, 240	1.79	14.86	Anthocyanidin A2
13	44.44	330, 268	2.28	–	flavonoid
14	52.17	480, 292, 240	6.51	10.63	Anthocyanidin A3

Rt = retention time.

Table 2

Quantification of anthocyanidins content (mg/g of dry extract) calculated as carajurin in the crude extract of *Arrabidaea chica* Verlot (ACCE) and the anthocyanidin rich fraction (ACAF).

Anthocyanidin	Rt (min)	Anthocyanidin Content (mg/g of dry weight calculated as carajurin)	
		ACAF	ACCE
3′-hydroxy-carajurone	23.5	4.78 ± 0.028	1.97 ± 0.005
Carajurone	27.5	18.84 ± 0.193	3.54 ± 0.039
Carajurin	39.0	24.96 ± 0.355	17.26 ± 0.011
A2	43.5	7.30 ± 0.029	2.10 ± 0.006
A3	52.1	7.44 ± 0.047	3.89 ± 0.022

Note: Values are expressed as the mean ± SD ($n = 3$). A2 and A3 are unidentified anthocyanidins. Rt = retention time.

Table 3

Activity against promastigote forms of *Leishmania amazonensis* and cytotoxicity in peritoneal macrophages of BALB/c after treatment with compounds of *Arrabidaea chica* for 72 h.

Compounds	Promastigote IC$_{50}$ (µg/mL)	Peritoneal macrophage CC$_{50}$ (µg/mL)
ACCE	101.5 ± 0.06	39.39 ± 1.158
ACAF	4.976 ± 1.09	44.90 ± 1.14
Carajurin (1)	3.662 ± 1.16	16.48 ± 1.10
3′-Hydroxy-carajurone (2)	22.70 ± 1.20	9.640 ± 1.2
Carajurone (3)	28.28 ± 0.07	66.74 ± 0.10
Amphotericin B	0.0345 ± 1.14	9.984 ± 1.12

ACCE: *A. chica* crude extract. ACAF: *A. chica* anthocyanidins-rich fraction. IC$_{50}$: half-maximal inhibitory concentration. CC$_{50}$: half-maximal cytotoxic concentration. Data are presented as the mean ± SD of three independent experiments performed in triplicate.

Table 4

Leishmanicidal activity against intracellular amastigote forms of *Leishmania amazonensis*, cytotoxicity in BALB/c peritoneal macrophages, and selectivity index after 24 h of treatment with *Arrabidaea chica* constituents and amphotericin B.

Compounds	Intracellular amastigote IC$_{50}$ (µg/mL)	Peritoneal macrophage CC$_{50}$ (µg/mL)	SI
ACAF	5.925 ± 1.29	60.60 ± 1.15	10.2
Carajurin	7.065 ± 1.19	264.1 ± 0.32	34.8
Amphotericin B	0.302 ± 1.23	9.984 ± 1.12	32.9

IC$_{50}$: half-maximal inhibitory concentration; CC$_{50}$: half-maximal cytotoxic concentration; SI: selectivity index; ACAF: *A. chica* anthocyanidins-rich fraction. Data are presented as the mean ± SD of three independent experiments performed at least in triplicate.

The analysis of the leishmanicidal activity showed that, in infected cells treated with ACAF, a statistically significant reduction was observed only at the highest concentration evaluated (2.5 µg/mL), according to the following infection parameters: intracellular amastigote number (p = 0.0202, Fig. 2(a)), percentage of infected cells (p = 0.0286, Fig. 2(b)), and the mean of amastigotes per infected cell (p = 0.0285, Fig. 2(c)). The concentrations used were selected from the intracellular amastigote assay, therefore they did not show cytotoxicity against peritoneal macrophages. At 12.5 and 25 µg/mL, carajurin exhibited a statistically significant reduction in the intracellular amastigote number (p = 0.0169 and p = 0.0011, respectively) (Fig. 2(d)), percentage of infected cells (p = 0.0292 and p = 0.0029, respectively) (Fig. 2(e)) and the mean of amastigotes per infected cell (p = 0.0476 and p = 0.0029, respectively, Fig. 2(f)).

3.4. Nitrite quantification in *L. amazonensis*-infected peritoneal macrophages treated with ACAF and carajurin

We analyzed whether the treatment with ACAF and carajurin increased the production of nitric oxide (NO) by measuring the nitrite levels according to the Griess method. We measured nitrite levels in the supernatant of BALB/c peritoneal macrophages and they were higher in cells treated with ACAF (5.02 ± 0.68 µM NaNO$_2$) and carajurin (5.76 ± 0.57 µM NaNO$_2$, p = 0.0476), with an enhancement of 66.22% and 90.72%, respectively, when compared to untreated-unstimulated cells (3.02 ± 0.74 µM NaNO$_2$). However, this difference was statistically significant only for carajurin. The same high nitrite levels pattern was observed in *L. amazonensis* infected macrophages treated with ACAF (5.52 ± 1.93 µM NaNO$_2$) and carajurin (7.10 ± 1.08 µM NaNO$_2$, p = 0.0286), with an increase of 35.62% and 74.44%, respectively, when compared with stimulated-untreated cells (4.07 ± 1.39 µM NaNO$_2$) (Fig. 3).

3.5. Molecular docking

In order to discuss a possible pathway that would lead to an increase in NO production, without disregarding other possibilities due to the complexity of this system, studies of enzyme regulation were developed through molecular docking. Data are presented in Table 5. Of the compounds tested, carajurin had the best score, indicating greater ability to activate the nitric oxide synthase enzyme (NOS). Redocking is reported in Fig. 4. Interactions in the binding mode of carajurin at the activation site of NOS are presented in Fig. 5.

4. Discussion

The crude extract obtained from *A. chica* Verlot (ACCE) is rich in flavonoids [17] including anthocyanidins [29,30,32,33], the chemical markers of the species, as well as flavones [19,34] and flavonols [19]. Previous results of our research network indicated that the leishmanicidal activity of *A. chica* is associated with their anthocyanidin profile, especially the content of carajurin [23]. The extract was therefore fractionated to provide an anthocyanidins-rich fraction, containing the five anthocyanidins with carajurin as the major component. The successive fractionation steps also led to the isolation of carajurin (1), 3′-hydroxy-carajurone (2), and carajurone (3), which allowed the evaluation of the leishmanicidal activity of these isolated anthocyanidins. Some flavones, although it was not isolated in this study, could be identified from the comparison with their authentic samples or isolated compounds, as well as two other anthocyanidins (A2 and A3) for which structures have been presented but remain to be confirmed [23].

The antiparasitic activity of the hydro-alcoholic extract of *A. chica* has been previously reported against *Trypanosoma cruzi* [19,20] and *Leishmania* [13,22]. These previous studies have also shown that the bioguided fractionation process of extracts can promote a progressive increase in activity against *T. cruzi* and *Leishmania*, demonstrating the potential of this plant as a source of biologically active compounds. The evaluation of the effect of the extract, anthocyanidin-rich fraction, and isolated compounds from *A. chica* against the promastigote forms of *L. amazonensis* monitored for 72 h showed that the fractionation also contributed to the improvement of the antipromastigote activity. The increase in leishmanicidal activity of the ACAF may be related to the increase in the concentration of these compounds relative to ACCE or to a possible increase in the synergistic effect between them [35].

Anthocyanidins are active compounds with remarkable biological activities [36]. 3-Deoxyanthocyanidin named carajurin (6,7-dihydroxy-5,4′-dimethoxy-flavylium) [30] is the main constituent of *A. chica* [37]. Thus, in an attempt to identify the component responsible for the antipromastigote activity of the fractions, biological tests were performed with the isolated anthocyanidins. Purified 3′-hydroxy-carajurone and carajurone showed less promising antipromastigote activity than the fraction, but carajurin exhibited an inhibitory capacity greater than 6 times among the tested anthocyanidins. The difference of leishmanicidal activity observed is possibly related to the different patterns of hydroxylation and methoxylation at the flavylium cation (phenyl-2-benzopyrylium) of these substances [38,39].

It has been shown that flavonoids in general are compounds possessing various biological activities. Those characterized by the presence of hydroxyl groups have several biological activities on tumor cells, such as apigenin (4′,5,7-trihydroxyflavone) that induce apoptosis in human hepatoma cell lines, notably Hep G2 [40]. Besides, among the flavonoids active against different species of *Leishmania*, those containing methoxyl groups in one of the rings are referred to as very promising molecules [38,39]. Thus, the antipromastigote results suggest that the presence of a methoxyl group in ring B favors activity expressed by carajurin, since both carajurone and 3′-hydroxy-carajurone, which has one and two hydroxyl groups, respectively, and no methoxyl in ring B, showed lower leishmanicidal activity. 3′-hydroxy-carajurone was the most cytotoxic among the evaluated compounds, suggesting that the presence of the
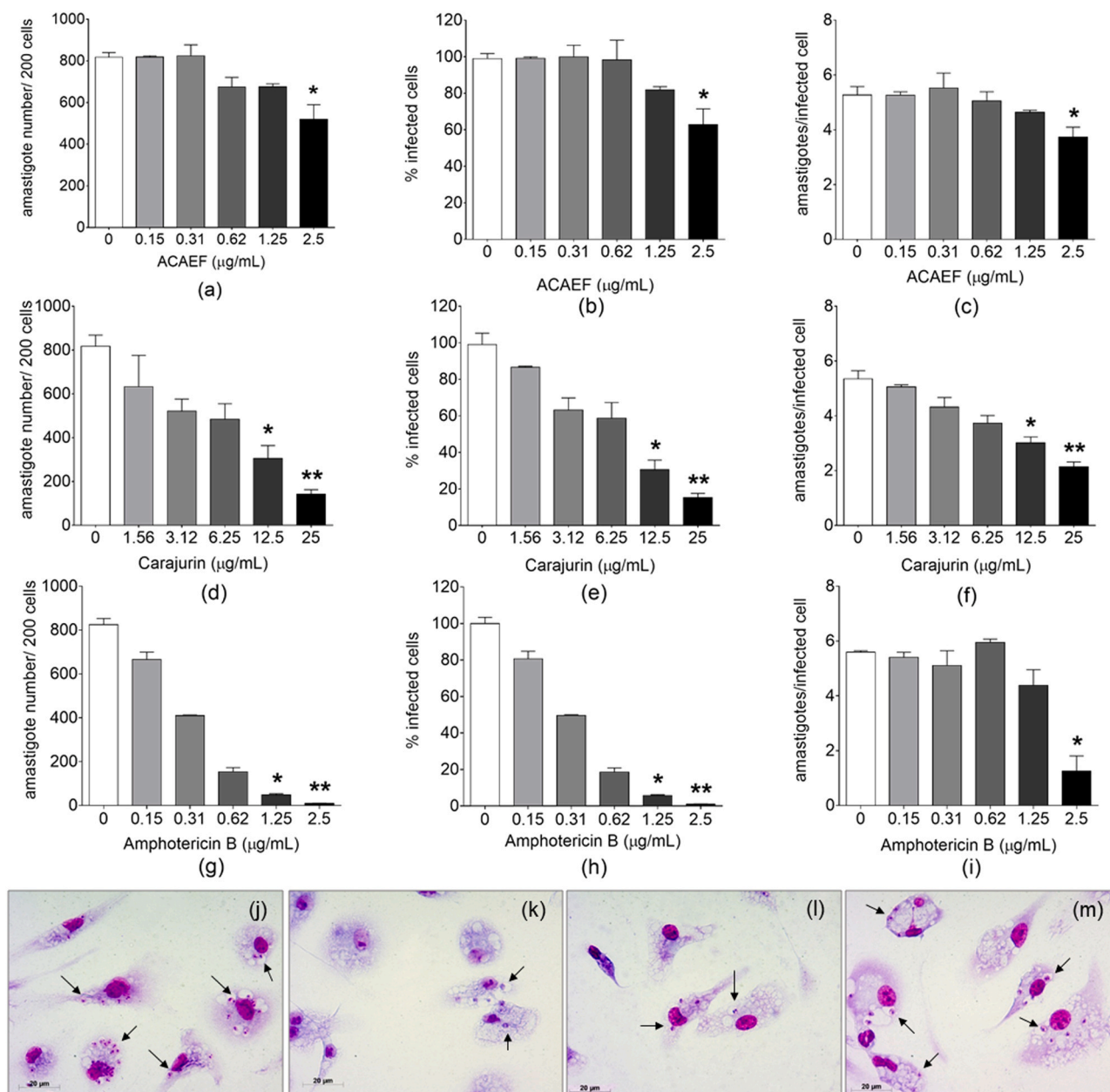
Fig. 2. Infection parameters of BALB/c peritoneal macrophages infected with *Leishmania amazonensis* and treated for 24 h with: anthocyanidins rich fraction of *Arrabidaea chica* (ACAF) (a-c), carajurin (d-f) or amphotericin B (g-i). (j) Untreated infected macrophages; (k) infected macrophages treated with amphotericin B (0.31 μg/mL); (l) infected macrophages treated with ACAF (2.5 μg/mL); (m) infected macrophages treated with carajurin (25 μg/mL). The treatment decreased the number of intracellular amastigotes (arrows). The data represent the mean ± standard deviation. * $p < 0.05$ and ** $p < 0.001$, when compared to the untreated group by the Mann-Whitney test.

hydroxyl group in 3′ increases the harmful effect on the macrophage. This observation is confirmed by the low cytotoxicity presented by carajurone (without hydroxyl group in 3′), but in this case the absence of the methoxy group also leads to lower leishmanicidal activity.

In the activity against promastigote forms, performed at different times of exposure, carajurin showed a time-dependent effect. However, treatment time has also contributed to increased cytotoxicity. Some reports demonstrated that anthocyanins in time-dependent studies markedly induces strong growth inhibitory effects against human hepatoma HepG2 [41], and causes apoptosis in human promyelocytic leukemia cells (HL-60) [42]. Thus, when assessing the selectivity index, we noticed that carajurin has better selectivity at 24 h of treatment.

For *Leishmania*, the intracellular amastigote form of the parasite provides the ideal conditions for carrying out an *in vitro* drug screening

procedure, since it requires conditions that mimic the environment found by the target cell [43]. Therefore, carajurin and ACAF were selected to evaluate the activity against intracellular amastigote forms, for presenting the best activity against promastigote forms of *L. amazonensis* . Moreover, even with the indication of possible synergistic action of the compounds presented in the fraction, it was important to evaluate the activity of the isolated majority compound in an attempt to characterize the possible biological marker of the species *A. chica*. However, there was no significant difference between carajurin and ACAF against intracellular amastigote forms, just a difference in the analysis of cytotoxicity data, resulting in greater selectivity of carajurin. Such results are similar when compared with the drug used as a reference. Furthermore, the infection parameters showed a statistically significant reduction for the treatment with ACAF and carajurin.
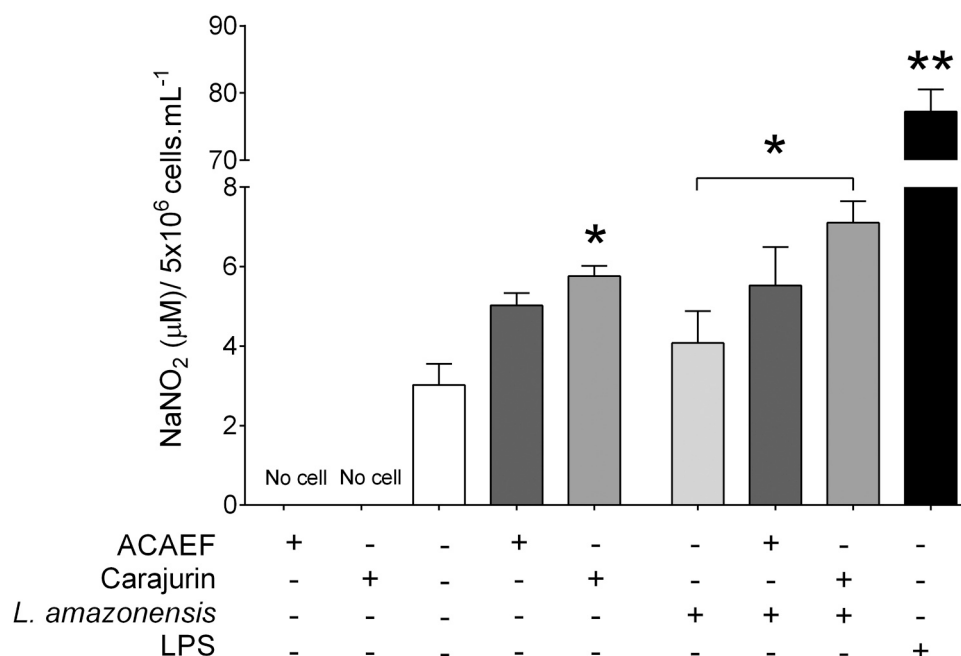
Fig. 3. Nitrite quantification in the supernatant of BALB/c peritoneal macrophages treated with anthocyanidins rich fraction of *Arrabidaea chica* (ACAF) at 2.5 μg/mL or carajurin at 25 μg/mL, stimulated or not with *Leishmania amazonensis*. * $p < 0.05$ when compared with the untreated group by Mann-Whitney test.

Table 5

Scores of the predicted binding modes of anthocyanidins from *Arrabidaea chica* after docking in the murine inosoxy dimer with isothiourea bound in the active site (PDB ID 1DF1).

Compounds	ChemPLP Score
Carajurin	53.800
3′-Hydroxy-carajurone	52.440
Carajurone	50.551
2-amino-6-(1,2-dihydroxypropyl)octahydropteridin-4(1 H)-one[a]	54.126

[a] cocrystallized ligand.

Only a few species from the *Arrabidaea* genus have been investigated for their antiprotozoal activity. Dimeric flavonoids purified from *Arrabidaea brachypoda* presented anti-leishmanial activity [44]. By quantifying the *in vitro* infected macrophages, it was evidenced that the compound brachydin B was the most active against intracellular amastigotes of *L. amazonensis*, without exhibiting host cell toxicity. Brachydin B was possibly more active due to the presence of the methoxyl group, which is necessary to improve membrane penetration [44]. Therefore, this leads us to infer that the presence of two methoxyl groups in the structure of carajurin may possibly be responsible for its higher activity against promastigote forms of *L. amazonensis*.

Some studies have shown that *A. chica* extracts and fractions possess antileishmanial activity against different *Leishmania* species [13,22]. Nevertheless, in the light of our knowledge, our study is the first to show an evaluation of the bioguided antileishmanial activity of ACCE, ACAF, and isolated compounds (carajurin, 3′-hydroxy-carajurone, and carajurone) against forms of *L. amazonensis*. Our findings confirm the results in a previous study [23], pointing out that the variation of the anthocyanidin profile in the extracts of four morphotypes of *A. chica*, especially the content of carajurin, was responsible for the variation of its leishmanicidal activity. In that study, it was also suggested that the presence of anthocyanidin carajurin would enhance this activity. The results of the present study, especially of the intracellular amastigote assays, in addition to the results by Moragas-Tellis et al. [23], allow us to designate carajurin as a biological marker for the leishmanicidal activity of the species.

The differences observed in the leishmanicidal activity of the samples against *Leishmania* promastigotes and intracellular amastigotes may be due to dissimilar biochemical or metabolic characteristics of the two stages of the parasite [45]. Besides a direct effect on intracellular amastigotes, this finding may also be indicative of activation of leishmanicidal macrophage functions, especially inducing NO [46]. Therefore, we evaluated ACAF and carajurin for the production of nitrite, an indirect measure to quantify NO, observing induced NO production by both compounds.

Literature on *A. chica* related to NO and its effects on *Leishmania* confirms our results [22]. After treatment with a fraction hexane:ethyl acetate obtained from *A. chica* hexane extract, the nitrite contents detected in the supernatant of macrophages infected by *L. amazonensis* and *L. infantum* was higher than those found on infected and untreated cell cultures [22]. These results help to contribute to a better understanding of induction of death by macrophage treated with *A. chica* against the intracellular form of *Leishmania*.

The secretion of inflammatory mediators by macrophages, including NO, is important for the success in controlling *Leishmania* multiplication [47]. Thus, the leishmanicidal activity of ACAF and carajurin would be associated with the ability to induce activation of the microbicidal response in macrophages and promote NO production, both of which lead to amastigote death.

NO is produced from L-arginine by a reaction catalyzed by the enzymes constitutive nitric oxide synthase, which is dependent on the interaction with calmodulin and calcium ions, and is involved in cell signaling, and oxide inducible nitric synthase (iNOS), produced by macrophages and other cells activated by cytokines [48].

The NOS functions as a dimer, consisting of two identical monomers, which, in turn, can be divided functionally and structurally into two main domains: a C-terminal reductase domain, homologous to cytochrome P450 and containing binding sites for NADPH, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), and an N-terminal oxidase domain, which abstracts one electron from the substrate L-arginine and has binding sites for heme iron, for the cofactor tetrahydrobiopterin (BH4) and for L-arginine [49]. The catalysis reaction of the constitutive NOS involves two oxidation stages: the hydroxylation of L-arginine to NG-hydroxy-L-arginine, followed by the oxidation of this intermediate using an electron from NADPH, forming
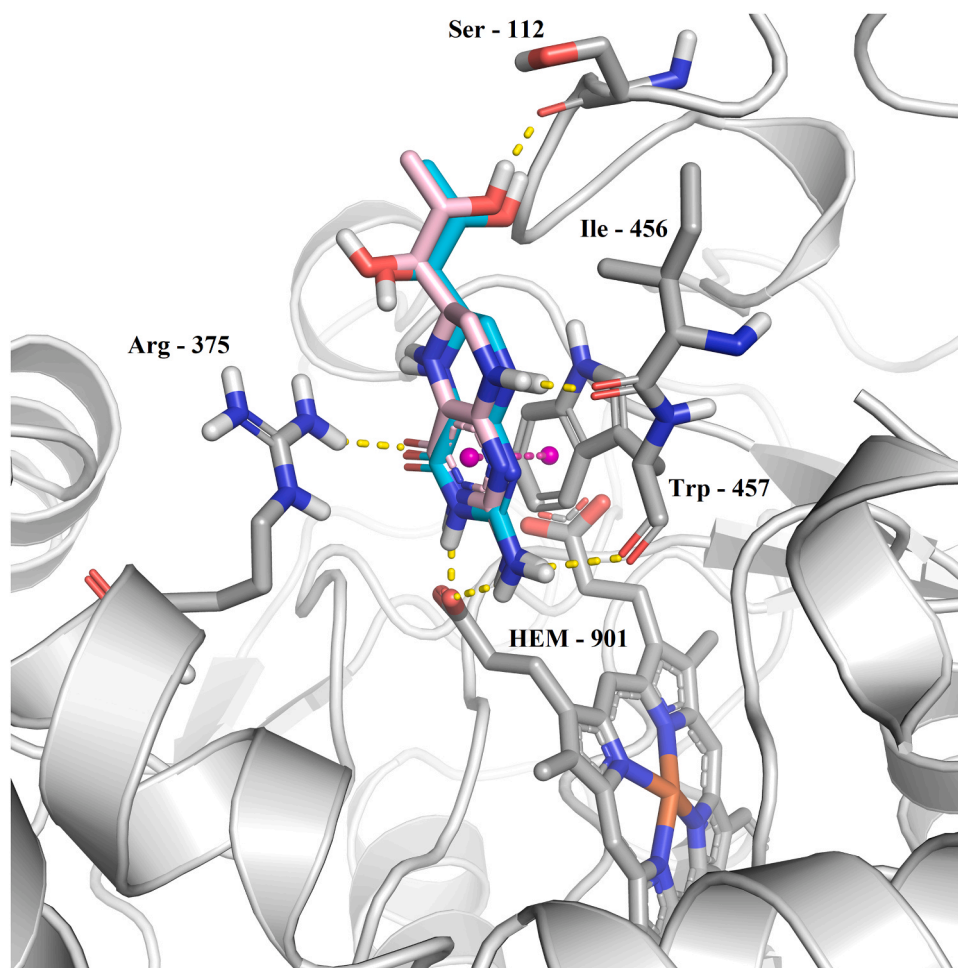
Fig. 4. Redocking image for 2-amino-6- (1,2-dihydroxypropyl) octahydropteridin-4 (1 H) -one (PDB ID 1DF1). The structure of the crystallographic image's binder has a cyan color and the structure resulting from the calculation is represented in pink.

L-citrulline and NO [50]. This reaction consumes 1.5 mol of NADPH and 2 mol of oxygen per mol of L-citrulline formed [51]. Cofactors such as heme iron, BH4 and L-arginine have been particularly studied, and their low bioavailability induces the phenomenon of dysfunctional endothelial NOS (eNOS) [52]. Heme iron is essential for the dimerization of the three isoforms [52], low concentrations or absence of L-arginine catalyze the reduction of oxygen into superoxide (O_2^-), and decreased levels of BH_4 lead to simultaneous production of NO and O_2^-, products that react with each other to form peroxynitrite ($ONOO^-$) [53].

Therefore, and considering that all NOS isoenzymes are hemodimeric, we investigated whether the increase in NO levels investigated by the Griess method, which corresponds to the reported leishmanicidal activity, may correspond to an activation mechanism of one of the isoforms of this enzyme. In this sense, molecular docking studies were directed to the analysis of possible interactions of carajurin with enzyme activation sites, especially in the important region in the dimerization process as reported in the literature.

A more accurate analysis of the interaction shows that carajurin occupies the binding site of 2-amino-6-(1,2-dihydroxypropyl) octahydropteridin-4(1 H)-one (co-crystallized ligand), for which the redocking studies showed an excellent alignment pattern.

In the predicted binding mode on the 1DF1 structure, carajurin interacts with the activation site of the NOS, especially through two non-covalent hydrogen bond-type interactions between the hydrogens of the hydroxyl groups of carujirin and the oxygens of the hydroxyl group and the carbonyl group, present in the serine residue (112). In addition, there is interaction with residue 375 and spatial proximity with the heme group (901), which is of great importance in the process of dimerization and activation of that enzyme. iNOS or isoform II is not constitutively expressed, this means, it is not normally present, being induced in macrophages and other cells by bacterial lipopolysaccharides and/or cytokines. This isoenzyme can also be called macNOS (macrophage NO synthase). Once induced, iNOS is capable of producing NO for a long time, and this characterizes its involvement in various pathological processes.

5. Conclusion and perspective

This is the first study on bioguided assay and characterization of a biological marker for antileishmanial activity of *A. chica*. The compounds were evaluated against *L. amazonensis*, having ACAF and carajurin the highest activity against the promastigote and intracellular amastigote forms, altering all parameters of *in vitro* infection. Both also exhibited a high selectivity index to parasites over cells. Carajurin was able to enhance nitrite levels in the macrophage stimulated or not with *L. amazonensis* , whose docking studies showed a possible interaction of carajurin at the activation site of NOS, the hypothesis that corroborates the *in vitro* results obtained. Underpinned by a set of intertwined pieces of evidence from our experimental results and literature reports, our findings substantiate our proposition that carajurin is a biological marker of the species *A. chica* for antileishmania activity. Nevertheless, further studies are needed to better elucidate the mechanism of action and determine this effect in the experimental murine model of leishmaniasis infection.
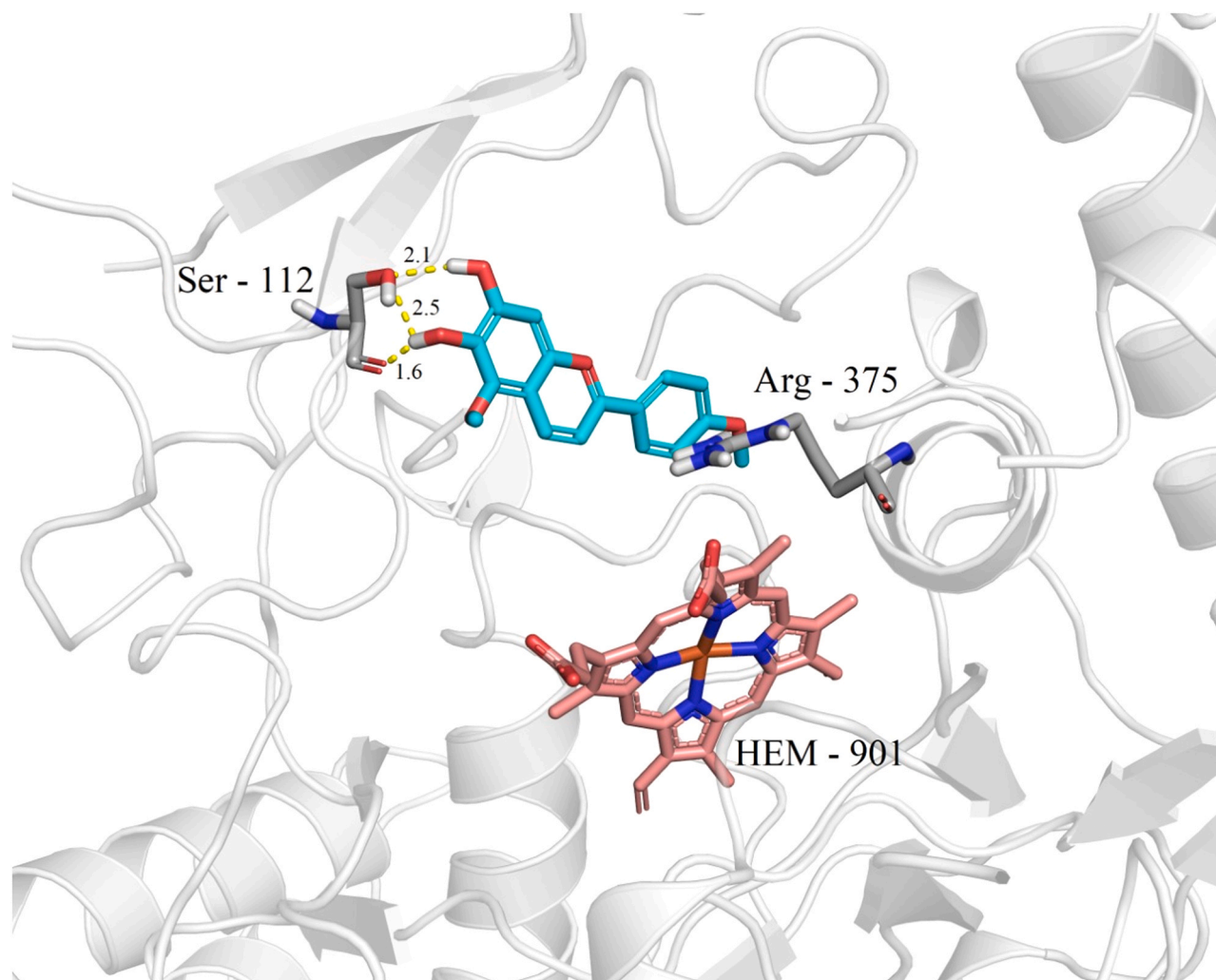
Fig. 5. Noncovalent interactions observed in the predicted binding mode of carajurin at the activation site of nitric oxide synthase. For the sake of clarity, only polar hydrogen atoms are shown.

Funding

CRediT authorship contribution statement

João Victor Silva-Silva: Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Carla J. Moragas-Tellis**: Methodology, Formal analysis, Data curation, Writing – original draft. **Maria S. S. Chagas**: Methodology, Formal analysis. **Paulo Victor R. Souza**: Methodology, Visualization, Formal analysis. **Davyson L. Moreira**: Conceptualization, Data curation, Visualization, Formal analysis. **Celeste S. F. de Souza**: Methodology, Visualization. **Kerolain F. Teixeira**: Methodology, Visualization. **Arthur R. Cenci**: Methodology, Visualization. **Aldo S. de Oliveira**: Conceptualization, Data curation, Formal analysis, Writing – original draft, Visualization, Supervision, Writing – review & editing, Funding acquisition. **Fernando Almeida-Souza**: Conceptualization: Methodology, Formal analysis, Data curation, Writing – original draft, Visualization, Supervision, Writing – review & editing, Funding acquisition. **Maria D. Behrens**: Conceptualization, Formal analysis, Data curation, Supervision, Funding acquisition. **Kátia S. Calabrese**: Conceptualization, Formal analysis, Data curation, Writing – original draft, Visualization, Supervision, Writing – review & editing, Funding acquisition.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

[1] WHO . Leishmaniasis. https://www.who.int/health-topics/leish maniasis#tab=tab_1, 2021 (Accessed 27 May 2021).

[2] S.L. Croft, Neglected tropical diseases in the genomics era: re-evaluating the impact of new drugs and mass drug administration, Genome Biol. 17 (2016) 46, https://doi.org/10.1186/s13059-016-0916-1.

[3] P.J. Hotez, B. Pecoul, S. Rijal, C. Boehme, S. Aksoy, M. Malecela, R. Tapia-Conyer, J.C. Reeder, Eliminating the Neglected Tropical Diseases: Translational Science and New Technologies, PLoS Negl. Trop. Dis. 10 (2016), 0003895, https://doi.org/10.1371/journal.pntd.0003895.

[4] S.H. Carvalho, F. Frézard, N.P. Pereira, A.S. Moura, L.M.Q.C. Ramos, G. B. Carvalho, M.O.C. Rocha, American tegumentary leishmaniasis in Brazil: a critical review of the current therapeutic approach with systemic meglumine

antimoniate and short-term possibilities for an alternative treatment, Trop. Med. Int. Health 24 (2019) 380–391, https://doi.org/10.1111/tmi.13210.

[5] F. Ponte-Sucre, J.C. Gamarro, M.P. Dujardin, R. Barrett, R. López-Vélez, A. W. García-Hernández, R. Pountain, B. Mwenechanya, Drug resistance and treatment failure in leishmaniasis: a 21st century challenge, PLoS Negl. Trop. Dis. 11 (2017), 0006052, https://doi.org/10.1371/journal.pntd.0006052.

[6] A.K. Shakya, Medicinal plants: future source of new drugs, Int. J. Herb. Med. 4 (2016) 59–64.

[7] D.J. Newman, G.M. Cragg, Natural products as sources of new drugs from 1981 to 2014, J. Nat. Prod. 79 (2016) 629–661, https://doi.org/10.1021/acs. jnatprod.5b01055.

[8] L.F.G. de Oliveira, B.A.S. Pereira, B. Gilbert, A.L. Corrêa, L. Rocha, C.R. Alves, Natural products and phytotherapy: an innovative perspective in leishmaniasis treatment, Phytochem. Rev. 16 (2017) 219–233, https://doi.org/10.1007/s11101-016-9471-3.

[9] BRASIL, Ministério da Saúde. RENISUS–Relação Nacional de Plantas Medicinais de Interesse ao SUS. Espécies vegetais. DAF/SCTIE/MS–RENISUS. http://portala rquivos.saude.gov.br/images/pdf/2017/junho/06/renisus.pdf, 2009 (Accessed 27 May 2021).

[10] B.J. Lima de Medeiros, K. Dos Santos Costa, J.F. Alves Ribeiro, J.O. Carrera Silva, W.L. Ramos Barbosa, J.C. Tavares Carvalho, Liver protective activity of a hydroethanolic extract of Arrabidaea chica (Humb. and Bonpl.) B. Verl. (pariri), Pharmacogn. Res. 3 (2011) 79–84, https://doi.org/10.4103/0974-8490.81954.

[11] L. Mafioleti, I.F. da Silva Junior, E.M. Colodel, A. Flach, D.T. Martins, Evaluation of the toxicity and antimicrobial activity of hydroethanolic extract of Arrabidaea chica (Humb. & Bonpl.) B. Verl, J. Ethnopharmacol. 150 (2013) 576–582, https://doi.org/10.1016/j.jep.2013.09.008.

[12] A.F. Michel, M.M. Melo, P.P. Campos, M.S. Oliveira, F.A. Oliveira, G.D. Cassali, V. P. Ferraz, B.B. Cota, S.P. Andrade, E.M. Souza-Fagundes, Evaluation of anti-inflammatory, antiangiogenic and antiproliferative activities of Arrabidaea chica crude extracts, J. Ethnopharmacol. 165 (2015) 29–38, https://doi.org/10.1016/j. jep.2015.02.011.

[13] J. Cortez de Sá, F. Almeida-Souza, R. Mondêgo-Oliveira, IdosS. Oliveira, L. Lamarck, IdeF. Magalhães, A.F. Ataídes-Lima, HdaS. Ferreira, A.L. Abreu-Silva, Leishmanicidal, cytotoxicity and wound healing potential of Arrabidaea chica Verlot, BMC Complement. Altern. Med. 16 (2016) 1, https://doi.org/10.1186/s12906-015-0973-0.

[14] T.F. Gemelli, LdaS. Prado, F.S. Santos, A.P. de Souza, T.N. Guecheva, J. A. Henriques, AdeB. Ferraz, D.S. Corrêa, R.R. Dihl, J.N. Picada, Evaluation of safety of arrabidaea chica verlot (bignoniaceae), a plant with healing properties, J. Toxicol. Environ. Health A 78 (2015) 1170–1180, https://doi.org/10.1080/15287394.2015.1072070.

[15] A.A. Aro, G.F. Simões, M.A. Esquisatto, M.A. Foglio, J.E. Carvalho, A.L. Oliveira, L. Gomes, E.R. Pimentel, Arrabidaea chica extract improves gait recovery and changes collagen content during healing of the Achilles tendon, Injury 44 (2013) 884–892, https://doi.org/10.1016/j.injury.2012.08.055.

[16] F.M. Ribeiro, H. Volpato, D. Lazarin-Bidóia, V.C. Desoti, R.O. de Souza, M.J. V. Fonseca, T. Ueda-Nakamura, C.V. Nakamura, S.O. Silva, The extended production of UV-induced reactive oxygen species in L929 fibroblasts is attenuated by posttreatment with Arrabidaea chica through scavenging mechanisms, J. Photochem. Photobiol. B 178 (2018) 175–181, https://doi.org/10.1016/j. jphotobiol.2017.11.002.

[17] J.T. Siraichi, D.F. Felipe, L.Z. Brambilla, M.J. Gatto, V.A. Terra, A.L. Cecchini, L. E. Cortez, E. Rodrigues-Filho, D.A. Cortez, Antioxidant capacity of the leaf extract obtained from Arrabidaea chica cultivated in Southern Brazil, PLoS One 8 (2013) 72733, https://doi.org/10.1371/journal.pone.0072733.

[18] F.J. Martins, C.A. Caneschi, J.L. Vieira, W. Barbosa, N.R. Raposo, Antioxidant activity and potential photoprotective from amazon native flora extracts, J. Photochem. Photobiol. B 161 (2016) 34–39, https://doi.org/10.1016/j. jphotobiol.2016.05.012.

[19] W.L.R. Barbosa, LdoN. Pinto, E. Quignard, J.MdosS. Vieira, J.O.C. Silva Jr., S. Albuquerque, Arrabidaea chica (HBK) verlot: phytochemical approach, antifungal and trypanocidal activities, Rev. Bras. Farmacogn. 18 (2008) 544–548, https://doi.org/10.1590/S0102-695×2008000400008.

[20] N. Miranda, A.P. Gerola, C.R. Novello, T. Ueda-Nakamura, S. de Oliveira Silva, B. P. Dias-Filho, N. Hioka, J.C.P. de Mello, C.V. Nakamura, Pheophorbide a, a compound isolated from the leaves of Arrabidaea chica, induces photodynamic inactivation of Trypanosoma cruzi, Photodiagnosis Photodyn. Ther. 19 (2017) 256–265, https://doi.org/10.1016/j.pdpdt.2017.05.004.

[21] Y. Estevez, D. Castillo, M.T. Pisango, J. Arevalo, R. Rojas, J. Alban, E. Deharo, G. Bourdy, M. Sauvain, Evaluation of the leishmanicidal activity of plants used by Peruvian Chayahuita ethnic group, J. Ethnopharmacol. 114 (2007) 254–259, https://doi.org/10.1016/j.jep.2007.08.007.

[22] I.A. Rodrigues, M.M. Azevedo, F.C. Chaves, C.S. Alviano, D.S. Alviano, A. B. Vermelho, Arrabidaea chica hexanic extract induces mitochondrion damage and peptidase inhibition on Leishmania spp. Biomed. Res. Int. 2014 (2014), 985171 https://doi.org/10.1155/2014/985171.

[23] C.J. Moragas-Tellis, F. Almeida-Souza, MdoSdoS. Chagas, P.V.R. de Souza, J. V. Silva-Silva, Y.J. Ramos, DdeL. Moreira, KdaS. Calabrese, M.D. Behrens, The influence of anthocyanidin profile on antileishmanial activity of arrabidaea chica morphotypes, Molecules 25 (2020) 3547, https://doi.org/10.3390/molecules25153547.

[24] M.M. Rottini, A.C.F. Amaral, J.L.P. Ferreira, E.S.C. Oliveira, J.RdeA. Silva, N. N. Taniwaki, A.R. Dos Santos, F. Almeida-Souza, CdaS.F. de Souza, KdaS. Calabrese, Endlicheria bracteolata (Meisn.) essential oil as a weapon against

Leishmania amazonensis: In vitro assay, Molecules 24 (2019) 2525, https://doi. org/10.3390/molecules24142525.

[25] IdosSdaS. Oliveira, C.J. Moragas Tellis, MdoSdoS. Chagas, M.D. Behrens, KdaS. Calabrese, A.L. Abreu-Silva, F. Almeida-Souza, Carapa guianensis Aublet (andiroba) seed oil: chemical composition and antileishmanial activity of limonoid-rich fractions, BioMed. Res. Int. 2018 (2018), 5032816, https://doi.org/10.1155/2018/5032816.

[26] F. Almeida-Souza, V.D.D. Silva, G.X. Silva, N.N. Taniwaki, D.J. Hardoim, C. D. Buarque, A.L. Abreu-Silva, KdaS. Calabrese, 1,4-disubstituted-1,2,3-triazole compounds induce ultrastructural alterations in leishmania amazonensis promastigote: an in vitro antileishmanial and in silico pharmacokinetic study, Int. J. Mol. Sci. 21 (2020) 6839, https://doi.org/10.3390/ijms21186839.

[27] J. Cheung, M.J. Rudolph, F. Burshteyn, M.S. Cassidy, E.N. Gary, J. Love, M. C. Franklin, J.J. Height, Structures of human acetylcholinesterase in complex with pharmacologically important ligands, J. Med. Chem. 55 (2012) 10282–10286, https://doi.org/10.1021/jm300871x.

[28] A. Pedretti, L. Villa, G. Vistoli, VEGA - an open platform to develop chemo-bio-informatics applications, using plug-in architecture and script programming, J. Comput. Aided Mol. Des. 18 (2004) 167–173, https://doi.org/10.1023/b: jcam.0000035186.90683.f2.

[29] B. Devia, G. Llabres, J. Wouters, L. Dupont, M.T. Escribano-Bailon, S. de Pascual-Teresa, L. Angenot, M. Tits, New 3-deoxyanthocyanidins from leaves of Arrabidaea chica, Phytochem. Anal. 13 (2002) 114–119, https://doi.org/10.1002/pca.632.

[30] B. Zorn, A.J. García-Piñeres, V. Castro, R. Murillo, G. Mora, I. Merfort, 3-desoxyanthocyanidins from Arrabidaea chica, Phytochemistry 56 (2001) 831–835, https://doi.org/10.1016/s0031-9422(01)00038-3.

[31] T.J. Mabry, K. Markham, M. Thomas, The ultraviolet spectra of flavones and flavonols, in: T.J. Mabry, K.R. Markham, M.B. Thomas (Eds.), The Systematic Identification of Flavonoids, Springer, Berlin, Heidelberg, 1970, pp. 41–164.

[32] E. Chapman, A.G. Perkin, R. Robinson, CCCCII.—the colouring matters of carajura, J. Chem. Soc. 0 (1927) 3015–3041, https://doi.org/10.1039/JR9270003015.

[33] R. Scogin, Anthocyanins of the Bignoniaceae, Biochem. Syst. Ecol. 8 (1980) 273–276, https://doi.org/10.1016/0305-1978(80)90058-7.

[34] O.S. Takemura, M. Iinuma, H. Tosa, O.G. Miguel, E.A. Moreira, Y. Nozawa, A flavone from leaves of Arrabidaea chica f. cúprea, Phytochemistry 38 (1995) 1299–1300.

[35] K.M. Hammi, R. Essid, O. Tabbene, S. Elkahoui, H. Majdoub, R. Ksouri, Antileishmanial activity of Moringa oleifera leaf extracts and potential synergy with amphotericin B, S. Afr. J. Bot. 129 (2020) 67–73, https://doi.org/10.1016/j. sajb.2019.01.008.

[36] E. Kowalczyk, P. Krzesiński, M. Kura, B. Szmigiel, J. Błaszczyk, Anthocyanins in medicine, Pol. J. Pharmacol. 55 (2003) 699–702.

[37] J.T. Paula, L.C. Paviani, M.A. Foglio, I.M.O. Sousa, F.A. Cabral, Extraction of anthocyanins from Arrabidaea chica in fixed bed using CO_2 and CO_2/ethanol/ water mixtures as solvents, J. Supercrit. Fluid. 81 (2013) 33–41, https://doi.org/10.1016/j.supflu.2013.04.009.

[38] E.C. Torres-Santos, D.L. Moreira, M.A. Kaplan, M.N. Meirelles, B. Rossi-Bergmann, Selective effect of 2′,6′-dihydroxy-4′-methoxychalcone isolated from Piper aduncum on Leishmania amazonensis, Antimicrob. Agents Chemother. 43 (1999) 1234–1241, https://doi.org/10.1128/AAC.43.5.1234.

[39] P. Boeck, C.A. Bandeira Falcão, P.C. Leal, R.A. Yunes, V.C. Filho, E.C. Torres-Santos, B. Rossi-Bergmann, Synthesis of chalcone analogues with increased antileishmanial activity, Bioorg. Med. Chem. 14 (2006) 1538–1545, https://doi. org/10.1016/j.bmc.2005.10.005.

[40] L.C. Chiang, L.T. Ng, I.C. Lin, P.L. Kuo, C.C. Lin, Anti-proliferative effect of apigenin and its apoptotic induction in human Hep G2 cells, Cancer Lett. 237 (2006) 207–214, https://doi.org/10.1016/j.canlet.2005.06.002.

[41] C.T. Yeh, G.C. Yen, Induction of apoptosis by the Anthocyanidins through regulation of Bcl-2 gene and activation of c-Jun N-terminal kinase cascade in hepatoma cells, J. Agric. Food Chem. 53 (2005) 1740–1749, https://doi.org/10.1021/jf048955e.

[42] D.X. Hou, T. Ose, S. Lin, K. Harazoro, I. Imamura, M. Kubo, T. Uto, N. Terahara, M. Yoshimoto, M. Fujii, Anthocyanidins induce apoptosis in human promyelocytic leukemia cells: structure-activity relationship and mechanisms involved, Int. J. Oncol. 23 (2003) 705–712, https://doi.org/10.3892/ijo.23.3.705.

[43] S. Gupta, Nishi, , Visceral leishmaniasis: experimental models for drug discovery, Indian, J. Med. Res. 133 (2011) 27–39.

[44] V.P. Rocha, C. Quintino da Rocha, E. Ferreira Queiroz, L. Marcourt, W. Vilegas, G. B. Grimaldi, P. Furrer, É. Allémann, J.-L. Wolfender, M.B. Soares, Antileishmanial activity of dimeric flavonoids isolated from Arrabidaea brachypoda, Molecules 24 (2019) 1, https://doi.org/10.3390/molecules24010001.

[45] H. Kolodziej, A.F. Kiderlen, Antileishmanial activity and immune modulatory effects of tannins and related compounds on Leishmania parasitised RAW 264.7 cells, Phytochemistry 66 (2005) 2056–2071, https://doi.org/10.1016/j. phytochem.2005.01.011.

[46] F. Almeida-Souza, CdaS. de Souza, N.N. Taniwaki, J.J. Silva, R.M. de Oliveira, A. L. Abreu-Silva, KdaS. Calabrese, Morinda citrifolia Linn. fruit juice induces an increase in NO production and death of Leishmania amazonensis amastigotes in peritoneal macrophages from BALB/c, Nitric Oxide 58 (2016) 51–58, https://doi. org/10.1016/j.niox.2016.06.004.

[47] B.J.M. Da Silva, J.R. Souza-Monteiro, H. Rogez, M.E. Crespo-López, J.L.M. Do Nascimento, E.O. Silva, Selective effects of Euterpe oleracea (açaí) on Leishmania (Leishmania) amazonensis and Leishmania infantum, Biomed. Pharmacother. 97 (2018) 1613–1621, https://doi.org/10.1016/j.biopha.2017.11.089.

[48] U. Förstermann, W.C. Sessa, Nitric oxide synthases: regulation and function, Eur. Heart J. 33 (2012) 829–837d, https://doi.org/10.1093/eurheartj/ehr304.

[49] X.L. Wang, J. Wang, Endothelial nitric oxide synthase gene sequence variations and vascular disease, Mol. Genet. Metab. 70 (2000) 241–251, https://doi.org/10.1006/mgme.2000.3033.

[50] E.W. Albrecht, C.A. Stegeman, P. Heeringa, R.H. Henning, H. van Goor, Protective role of endothelial nitric oxide synthase, J. Pathol. 199 (2003) 8–17, https://doi.org/10.1002/path.1250.

[51] O.W. Griffith, D.J. Stuehr, Nitric oxide synthases: properties and catalytic mechanism, Annu. Ver. Physiol. 57 (1995) 707–736, https://doi.org/10.1146/annurev.ph.57.030195.003423.

[52] H. Cai, D.G. Harrison, Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress, Circ. Res. 87 (2000) 840–844, https://doi.org/10.1161/01.res.87.10.840.

[53] J.S. Beckman, W.H. Koppenol, Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly, Am. J. Physiol. 271 (5 Pt 1) (1996) C1424–C1437, https://doi.org/10.1152/ajpcell.1996.271.5.C1424.