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Leishmanicidal activity of *Cecropia pachystachya* flavonoids: Arginase inhibition and altered mitochondrial DNA arrangement

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ABSTRACT

The plant *Cecropia pachystachya* Trécul is widely used in Brazilian ethnomedicine to treat hypertension, asthma, and diabetes. Arginase is an enzyme with levels that are elevated in these disorders, and it is central to *Leishmania* polyamine biosynthesis. The aims of this study were to evaluate antileishmanial activity and inhibition of the arginase enzyme by *C. pachystachya* extracts, and to study changes in cellular organization using electron microscopy. The ethanol extract of *C. pachystachya* was tested on *Leishmania* (*Leishmania*) *amazonensis* promastigote survival/proliferation and arginase activity *in vitro*. Qualitative ultrastructural analysis was also used to observe changes in cell organization. The major bioactive molecules of the ethanol extract were characterized using liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS). The ethyl acetate fraction of the ethanol extract diminished promastigote axenic growth/survival, inhibited arginase activity, and altered a mitochondrial kinetoplast DNA (K-DNA) array. The bioactive compounds of *C. pachystachya* were characterized as glucoside flavonoids. Orientin (**9**) (luteolin-8-C-glucoside) was the main component of the methanol-soluble ethyl acetate fraction obtained from the ethanol extract and is an arginase inhibitor (IC_{50} 15.9 μM). The ethyl acetate fraction was not cytotoxic to splenocytes at a concentration of 200 μg/mL. In conclusion, *C. pachystachya* contains bioactive compounds that reduce the growth of *L.* (*L.*) *amazonensis* promastigotes, altering mitochondrial K-DNA arrangement and inhibiting arginase.

1. Introduction

Leishmaniasis is a debilitating and potentially fatal parasitic disease that currently affects at least 12 million people, with an estimated incidence of 2 million new annual cases (ca. 1.5 million cases of cutaneous leishmaniasis and 500,000 of visceral form) in 88 countries (WHO, 2011). The current chemotherapeutic treatment is often associated with severe side-effects and refractory cases (Astelbauer and Walochnik, 2011). The search for new therapeutic targets led to characterization of arginase from *Leishmania* (*Leishmania*) *mexicana* (Roberts et al., 2004), *Leishmania* (*Leishmania*) *amazonensis* (da Silva et al., 2008) and *Leishmania* (*Leishmania*) *major* (Reguera et al., 2009). Arginase is a manganese metalloenzyme that catalyzes the hydrolysis of L-arginine to L-ornithine and urea. Ornithine is decarboxylated into putrescine, the diamine precursor of polyamines (PA) that is involved in many different mechanisms required for cell proliferation, and is thus a valuable target for antiparasitic chemotherapy (Birkholtz et al., 2011). *Leishmania* arginase knockouts have shown that the enzyme plays a central role in PA biosynthesis (Reguera et al., 2009; Roberts et al., 2004). The PA spermidine is required for trypanothione (N1,N8-bis(glutathionyl)spermidine) synthesis in trypanosomatid

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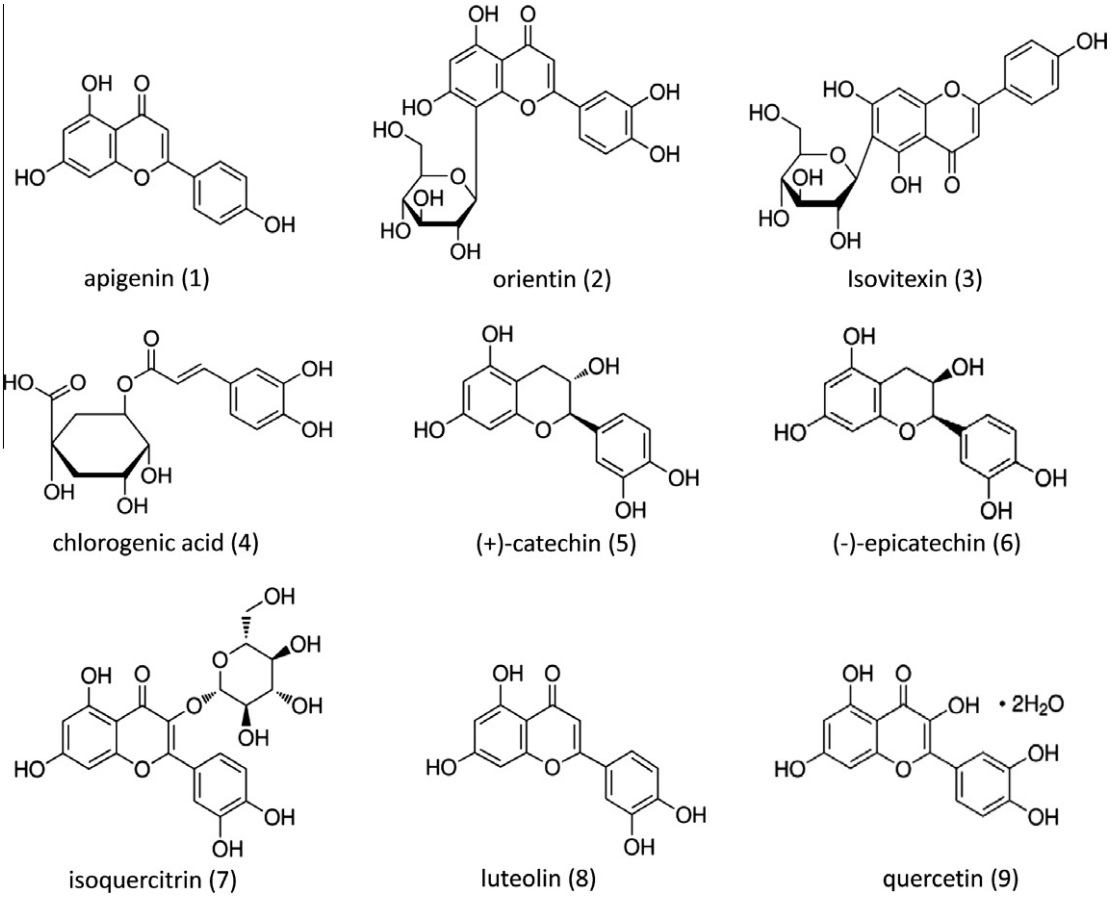

Fig. 1. Compounds 1–9.

parasites such as *Leishmania*. This molecule scavenges reactive oxygen (Fairlamb and Cerami, 1992; Fonseca-Silva et al., 2011) and nitrogen species (Bocedi et al., 2010), protecting protozoa from endogenous and immune-response-derived oxidative stress.

Reduced PA synthesis and transport is implicated in mitochondrial damage and k-DNA disorganization in *Trypanosoma cruzi* (Menezes et al., 2006) and *L.* (*L.*) *amazonensis* (Vannier-Santos et al., 2008). To survive within macrophages, *Leishmania* uses arginase to drive the conversion of L-arginine to ornithine and to down-regulate the production of nitric oxide by inducible NO synthase (iNOS) (Gaur et al., 2007). TH1 cytokine-activated macrophages trigger iNOS to convert L-arginine to NO and citrulline, killing the parasites, whereas TH2 cytokines mediate macrophage arginase activation, providing ornithine to polyamine biosynthesis and enabling parasite survival and proliferation (Iniesta et al., 2002; Wanderley and Barcinski, 2010).

The use of N^{ω}-hydroxy-L-arginine (NOHA), a physiologic arginase inhibitor, can control *Leishmania* infection in a susceptible mice strain (Iniesta et al., 2001). In fact, NOHA can inhibit both host and *Leishmania* arginase, providing a rational target for the design of drugs to treat leishmaniasis (Riley et al., 2011).

Several Brazilian plants show ethnopharmacological activity against *L.* (*L.*) *amazonensis* promastigotes (de Toledo et al., 2011; Muzitano et al., 2009). The *Cecropia* genus is used in folk medicine to treat asthma and hypertension (Delarcina et al., 2007; Lima-Landman et al., 2007; Tanae et al., 2007), diseases that are correlated with increased arginase activity and decreased production of NO, a potent bronchodilator and vasodilator (Bergeron et al., 2007; Demougeot et al., 2007). Previous work has shown that *Cecropia pachystachya* produces chlorogenic acid (**4**), (+)-catechin (**5**), (−)-epicatechin (**6**), isoquercitrin (**7**), isovitexin (**3**), isoorientin, orientin (**2**) and procyanidin B2 (Lacaille-Dubois et al., 2001) (Fig. 1). Both (+)-catechin (**5**) and (−)-epicatechin (**6**) were previously tested and shown to be microbicidal against *Leishmania* (*Leishmania*) *donovani* amastigotes (Tasdemir et al., 2006). In this work, the *C. pachystachya* extract was studied as well as its fractions and isolated compounds, for activity against *L.* (*L.*) *amazonensis* arginase. It is demonstrated here that the compound orientin (**2**) in the ethyl acetate fraction of the *C. pachystachya* ethanolic extract is leishmanicidal, inhibiting arginase and altering parasite mitochondrial K-DNA organization.

2. Results

2.1. Activity of the C. pachystachya extract on arginase activity

At a concentration of 10 μg/mL, the ethanol extract (EE) inhibited 90% of *L.* (*L.*) *amazonensis* arginase activity. DFMO (α-difluormethylornithine), a selective irreversible ornithine decarboxylase inhibitor, was then used for a comparison with other polyamine pathway antagonists; DFMO caused 30% inhibition of arginase activity at 218.6 μg/mL (1 mM). The IC_{50} values for the ethyl acetate fraction (AF), which was divided into methanol-soluble (AF–MS), methanol-insoluble (AF–MI), *n*-butanol (BUF) and dichloromethane fractions (DFs), are presented in Table 1.

2.2. Characterization of the AF–MS

Analysis of AF–MS by LC–ESI-MS showed four predominant symmetrical peaks using UV detection and +MS (Fig. 2). The retention times (RT) observed (280 nm) were 13.2, 15.0, 16.1 and

Table 1
Inhibition of arginase and promastigotes growth by extract fractions derived from the EE through fractionation of the organic phase.

Fraction[a]	IC_{50} ± SE (μg/mL)	
	Arginase inhibition	Promastigotes growth
AF	17 ± 2	53.3 ± 6.4
AF–MS	48 ± 4	nd[b]
AF–MI	64 ± 7	nd
BUF	90 ± 11	24.8 ± 1.7
DF	200 ± 25	5.6 ± 0.5

[a] AF, ethyl acetate fraction; MS, methanol-soluble; MI, methanol-insoluble; BUF, *n*-butanol fraction; DF, dichloromethane fraction.
[b] nd, not determined.

16.8 min using an Ascentis RP–Amide column, and the $[M+H]^+$ values for these compounds were *m/z* 581, 449, 595 and 565, respectively. The relative abundance for these major four peaks, obtained by integration of peaks and assessed by UV detection, were 13.8%, 23.6%, 13.5% and 16.6% for *m/z* 581, 449, 595 and 565, respectively.

The UV/Vis spectrum shows that the four compounds had broad bands of absorption, with maximum peaks at λ = 350 nm (peak 2, 3 and 4) and 338 nm (peak 5) that are characteristic of flavonoids (Fig. 3). The second narrow absorption band at 270 nm was characteristic of apigenin (**1**) (Fig. 1) and can be observed in peak 5 of the chromatogram (Fig. 2). Peak 3 with *m/z* 449 (RT 14.9 min) was identified as orientin (**2**) by comparison with RT, UV and mass spectra of an authentic sample. Peak 6 (lower intensity) with *m/z* 433 (RT 19.35) was identified as isovitexin (**3**) (apigenin-6-C-glucoside) by comparison with its RT, UV and mass spectra to an authentic sample. Data for the authentic samples of orientin (**2**) and isovitexin (**3**) were collected separately and compared to data collected for the extract (Table 2).

2.3. Arginase inhibition by natural compounds

Chlorogenic acid (**4**), (+)-catechin (**5**), (−)-epicatechin (**6**), and isoquercitrin (**7**) showed inhibitions above 50% at 20 μM (Table 3). Orientin (**2**) exhibited an IC_{50} of 7 μg/mL (16 μM), while the IC_{50} for the AF–MS, which contained approximately 24% orientin (**2**), was 48 μg/mL. Thus, orientin (**2**) contributes significantly to arginase inhibition by AF–MS. All inhibition experiments were conducted at pH 9.5 because arginase activity drops off steeply at pH 7.5 (Riley et al., 2011).

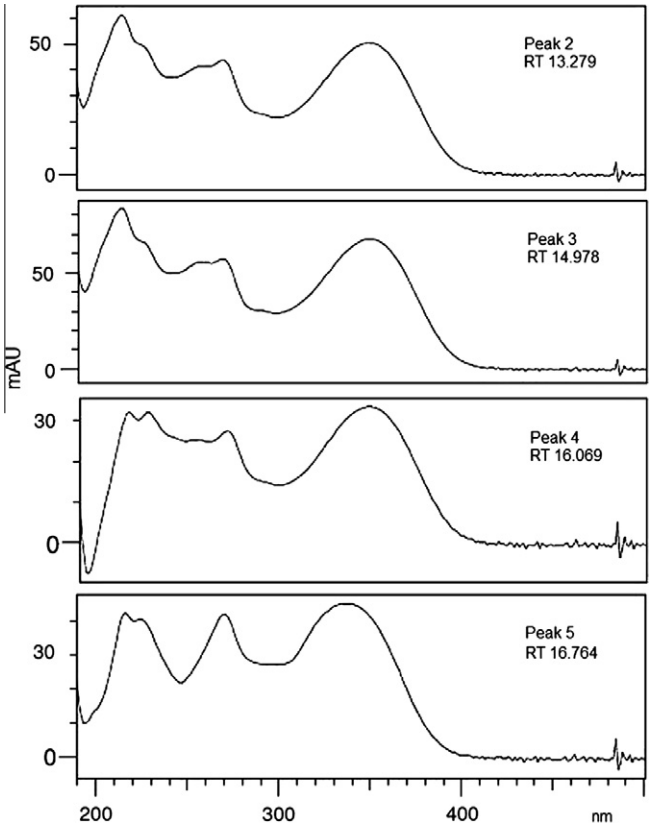

Fig. 3. UV–Vis spectra of the major constituents of AF–MS. (RT = retention time in minutes).

2.4. Ultrastructural changes in promastigote cells of L. (L.) amazonensis caused by the AF–MS fraction of C. pachystachya extract

The AF–MS was tested in a 100 μg/mL culture of promastigote cells of *L.* (*L.*) *amazonensis* and inhibited 70% of parasite growth in 96 h. An incubation time of 72 h was therefore used to better assess early changes in the parasite. In ultrastructural qualitative evaluation of *L.* (*L.*) *amazonensis* promastigotes, it was observed that control cells remained intact, with characteristic organelles including the single mitochondrion that spans the entire cell and contains the kinetoplast (Fig. 4A). In the analysis of cells treated

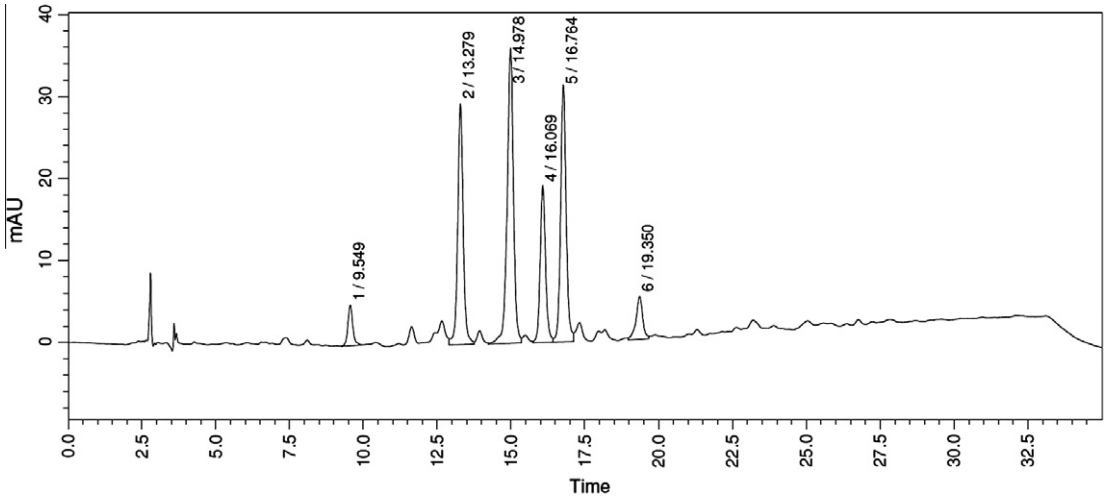

Fig. 2. Analysis of the AF–MS by LC–ESI-MS using an RP-amide column. The chromatogram shows absorbance at 280 nm.

Table 2
Active compounds identified from the AF–MS.

m/z $[M-H]^+$	Peak/RT (min)	UV peaks (nm)	Compound
449	3/14.978	257,270,350	Orientin (**2**) (luteolin-8-C-glucoside)
433	6/19.350	270,338	Isovitexin (**3**) (apigenin-6-C-glucoside)

Table 3
Inhibition of arginase by natural compounds at 20 μM.

Compound	Inhibition ± SE (%)
Chlorogenic acid (**4**)	67 ± 5
(+)-Catechin (**5**)	66 ± 8
(−)-Epicatechin (**6**)	62 ± 7
Isoquercitrin (**7**)	54 ± 6
Isovitexin (**3**)	14 ± 3

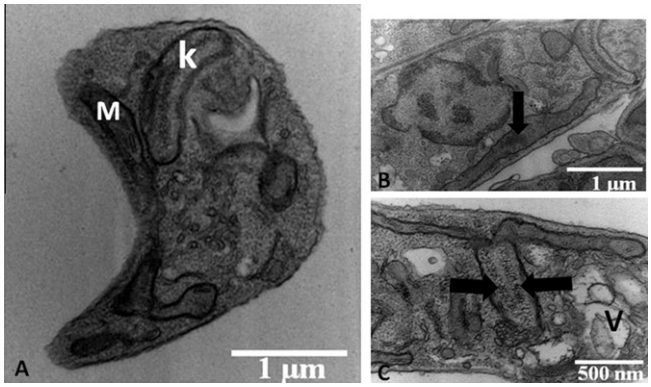

Fig. 4. Transmission electron microscopy of *L.* (*L.*) *amazonensis* promastigotes after treatment with 100 μg/mL ethyl acetate extract for 72 h. (A) Untreated control displaying a single mitochondrion (M) with kinetoplast (K). (B) Treated cells showing swollen mitochondria with electrodense matrix deposits (arrow). (C) Treated cells showing kinetoplasts with two k-DNA masses in a parallel arrangement (arrows). Treated parasites also presented cytoplasmic vacuolization (V). (N = nucleus).

with 100 μg/mL AF–MS, swollen mitochondria were observed displaying electrondense concretions in the matrix (Fig. 4B) and changes in the arrangement of k-DNA in the kinetoplast (Fig. 4C).

2.5. Antileishmanial susceptibility

Antileishmanial activity was evaluated for the three fractions derived from EE (Table 1). The lowest IC_{50} was obtained with the dichloromethane following *n*-butanol and ethyl acetate fractions. The differences of the IC_{50} between fractions was significant ($p < 0.05$).

2.6. Toxicity to splenocytes

The toxicity of the AF was evaluated in splenocyte cultures at 200, 100, 50 and 25 μg/mL (Fig. 5). There were no significant changes in cell viability at the concentrations used ($p > 0.05$).

3. Discussion

Polyamine metabolic pathways furnish promising drug targets and strategies for parasitic diseases caused by protozoa such as *Leishmania* spp. (Birkholtz et al., 2011). The arginase knockouts of *L.* (*L.*) *mexicana* (Roberts et al., 2004), *L.* (*L.*) *major* (Reguera et al., 2009) and *L.* (*L.*) *amazonensis* (da Silva et al., 2012b) demonstrated the importance of the enzyme for the growth of the parasite. This

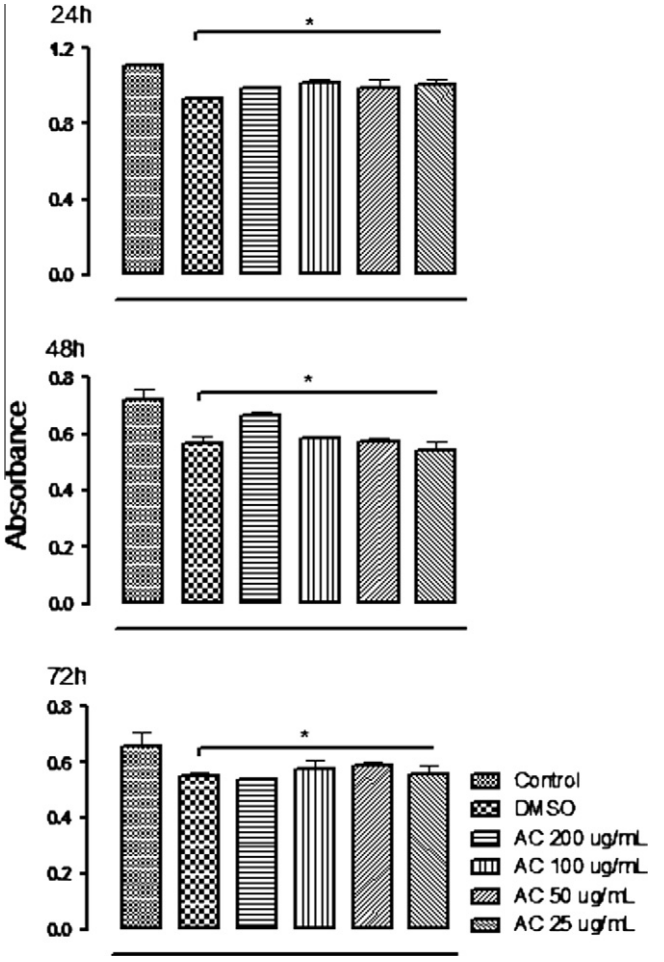

Fig. 5. Assessment of toxicity to splenocytes using the MTT method in the presence of different ethyl acetate extracts concentrations for 24, 48 and 72 h. AC = acetate fraction*$p > 0.05$.

enzyme plays a pivotal role in PA biosynthesis and pathogenesis (Balaña-Fouce et al., 2012). Promastigotes display arginine transporters that sense the amino acid pools (Castilho-Martins et al., 2011).

Comparative molecular modeling showed differences in the vicinity of the active site between the human and *L.* (*L.*) *amazonensis* enzymes (da Silva et al., 2002). Synthetic inhibitors not only are not selective for *L.* (*L.*) *mexicana* arginase, but also inhibit the parasite growth with a potency lower than the inhibition of the parasite enzyme (Riley et al., 2011).

Cecropia extracts constitute a valuable tool in the search for antiparasitic drugs, as species of this genus have been reported in ethnopharmacological studies to be cardiotonic, diuretic, hypotensive, anti-inflammatory, hypoglycemic and anti-asthmatic (Aragão et al., 2010; Consolini et al., 2006; Schinella et al., 2008). These extracts have been particularly of interest for treatment of leishmaniasis (Odonne et al., 2011). All fractions derived from *C. pachystachya* extract showed IC_{50} lower than pentostam ($IC_{50} > 64$ μg Sb^V/mL) against promastigotes, but showed lower potency than amphotericin (IC_{50} 0.65 μg/mL) and miltefosine (IC_{50} 0.6 μg/mL) (Vermeersch et al., 2009). The fraction AF showed an IC_{50} against promastigotes of the 53 μg/mL, while IC_{50} for arginase inhibition was 17 μg/mL. The difference between the inhibitory action of AF on the arginase and the parasite may be due to the access of drugs to the glycosome, the organelle where arginase is located in the parasite (da Silva et al., 2012b). No genotoxic effects were associated with *Cecropia obtusifolia* (Toledo et al., 2008), which suggests the possibility of developing innovative non-toxic drugs.

Here, the action of a plant extract rich in flavonoids is described that inhibits arginase and is active against *L.* (*L.*) *amazonensis* promastigotes (Table 1). In studies of the quantitative structure–activity relationship, flavonoids were tested on cultures of *L.* (*L.*) *donovani*, *Trypanosoma brucei rhodesiense* and *T. cruzi* (Tasdemir et al., 2006); most compounds tested had greater activity against *L.* (*L.*) *donovani* and *T. brucei rhodesiense*. Species of the genus *Leishmania* (Camargo et al., 1978) and *T. brucei rhodesiense* (Heby et al., 2007), but not *T. cruzi*, express arginase (Camargo et al., 1978). Flavonoids such as luteolin (**8**) and quercetin (**9**) cause apoptosis by inducing cleavage of kinetoplast DNA mediated by topoisomerase II, as previously reported (Mittra et al., 2000). Quercetin (**9**) has been characterized as an inhibitor of arginase (da Silva et al., 2012a) and ribonucleotide reductase (Sen et al., 2008). Quercetin (**9**) is also able to chelate Mn^{2+} (cofactor of arginase), inducing cleavage of plasmid DNA (Jun et al., 2007) and Fe^{+2} and causing a decrease in the supply of iron needed for the development of *L.* (*L.*) *donovani* (Sen et al., 2008). Inhibition of arginase blocks the first step of the synthesis of polyamines that are essential for the multiplication and maintenance of the antioxidant mechanism through synthesis of trypanothione (Colotti and Ilari, 2011). Here, it was shown that an ethanol extract fractionated with ethyl acetate resulted in an enriched flavonoid fraction soluble in methanol, which largely shows the presence of six natural products possibly derived from luteolin (**8**) and apigenin (**1**). One of the four major components was identified as orientin (**2**); the other related compounds were the product of conjugations of apigenin (**1**) or luteolin (**8**) with glucose, xylose and arabinose. Apigenin-7-O-glucoside, luteolin-5-O-glucoside, and luteolin-7-O-glucoside have shown high activity against amastigotes from *L.* (*L.*) *donovani* (Tasdemir et al., 2006).

The toxicity of the ethyl acetate fraction was tested in splenocytes and showed no significant change to cell cultures. In this work, it was shown that arginase from *Leishmania* is a target for *C. pachystachya* flavonoids. The lack of cell toxicity in splenocytes and selectivity for parasite enzymes are important parameters for the development of selective inhibitors. Thus, the components of the ethyl acetate fraction of the ethanolic extract of *C. pachysyachya* did not affect the host, while showing good activity against promastigotes of *Leishmania* at 100 μg/mL, the concentration that reduced the growth of parasites by 70% in 96 h. Flavonoids can contribute to reduced PA synthesis and consequently down-modulate the parasite antioxidant system, triggering oxidative stress and apoptosis. However, the activity of arginase at near-neutral pH drops steeply (Riley et al., 2011), and the activity and inhibition of arginase is not detected *in vitro*. At pH 9.5, the flavonoids did not inhibit rat liver arginase, used as a mammalian enzyme model, but instead showed selective inhibition of protozoan enzymes.

Electron microscopy approaches have provided valuable data in the elucidation of mechanisms of action of antiparasitic compounds (Vannier-Santos and De Castro, 2009; Vannier-Santos and Lins, 2001; Vannier-Santos et al., 2008). Kinetoplast segregation precedes the onset of nuclear division, and the elongated kDNA is usually slightly rotated or tilted during division (Ambit et al., 2011), but not arranged in a parallel array as reported here. This configuration presumably precludes the segregation driven by side-by-side basal bodies. It is therefore noteworthy that protozoa cell-cycle machinery may be a target in antiparasitic chemotherapy (Hammarton et al., 2003). Here, it was shown mitochondrial damage and kDNA disorganization by transmission electron microscopy. These alterations maybe due to PA deficiency, as these polycations are antioxidant (Maia et al., 2008; Menezes et al., 2006; Tkachenko et al., 2011; Vannier-Santos et al., 2008) and can stabilize and regulate the function of nucleic acids (Igarashi and Kashiwagi, 2010). Such an inference is in agreement with that previously demonstrated by the effect of reduced PA synthesis in *Trypanosoma cruzi* (Menezes et al., 2006) and *L.* (*L.*) *amazonensis* (Vannier-Santos et al., 2008). Polyamine depletion also leads to the destruction of *Tritrichomonas foetus* hydrogenosomes (Reis et al., 1999), the redox organelles inside anaerobic trichomonad parasites. Parasite cytoplasmic vacuolation may be caused by autophagic triggering due to oxidative stress (Menezes et al., 2006; Vannier-Santos et al., 2008).

Pleiotropic drugs against cancer, cardiovascular and parasitic diseases have been considered by several researchers (Cavalli et al., 2010; Frantz, 2005; Hampton, 2004). Considered as multitarget compounds against *Leishmania* and *Trypanosoma* (Cavalli et al., 2010), the flavonoids can comprise multifunctional drugs or can be used as lead compounds in multifunctional drug design schemes. The biodiversity of flavonoids can contribute to research based on the mechanism of action of isolated or mixed flavonoids on multiple targets related to leishmaniasis.

4. Conclusion

Analysis by transmission electron microscopy showed that the *C. pachystachya* extract rearranged mitochondrial DNA, which can lead to interference with the negative modulation of polyamines and trypanothione biosynthesis. This effect is relevant because *Leishmania* arginase has been implicated in the maintenance of infection in mammalian hosts, and flavonoids presents in the ethyl acetate fraction, orientin (**2**) and isovitexin (**3**), might be used as prototypes for rational drug design or as part of a multidrug treatment for leishmaniasis.

5. Experimental

5.1. Extract and fractions

Leaves of *C. pachystachya* were collected in February 2008 on the Ondina Campus of the Federal University of Bahia – UFBA, Salvador, Bahia, Brazil. Taxonomic identification was made by Dr. Maria L.S. Guedes, Biology Institute – UFBA. The voucher specimen is deposited in the Herbarium Alexandre Leal Costa – Biology Institute – UFBA as n° 82550.

Leaves were washed in H_2O, superficially dried with absorbent paper, dried for 3 weeks at 25 °C and ground in a mill (Thomas Wiley Laboratory Mill – Model 4). The extraction process was performed by exhaustive maceration of dry powdered leaves (50 g) in EtOH (1 L) with three changes of solvent, once every 48 h at 25 °C. The filtrate was concentrated in a rotary evaporator (BUCHI Model R-3000) under reduced pressure at 45 °C, to yield the crude ethanol extract (EE). Aliquots of the EE were resolubilized in EtOH, with subsequent addition of H_2O–EtOH (1:4, v/v) to perform the partition. The soluble fraction in EtOH:H_2O (4:1) was subjected to extraction with *n*-hexane to yield the hexane fraction (HF). The remaining phase in EtOH:H_2O (4:1) was extracted with CH_2Cl_2, to yield a CH_2Cl_2 soluble fraction (DF). After extraction with CH_2Cl_2, the remaining phase in EtOH:H_2O (4:1) was subjected to extraction with EtOAc, resulting in the EtOAc soluble fraction (AF). Finally, the remaining fraction was extracted with *n*-BuOH (BUF). Three partitions with each solvent were used at each stage, each with one-third of the total volume of the hydro-alcoholic phase. After evaporation of solvent under reduced pressure, each fraction was resuspended in H_2O, and the resulting solutions were used to determine the IC_{50}.

5.2. Characterization of the active constituents from C. pachystachya

Characterization of the most active constituents from *C. pachystachya* was performed using liquid chromatography–mass spectra (LC–MS) using an Esquire 3000 Plus (Bruker Daltonics). Separation

was performed using an RP-amide (Ascentis-Supelco, Sigma–Aldrich) column (25 cm × 4.6 mm; 5 μM). Samples (5 mg/mL) were prepared in 0.1% HCOOH, and aliquots (10 μL) were injected onto the column at a flow rate of 1 mL/min. The constituents were eluted using a linear gradient from 10% to 35% CH_3CN–H_2O containing 0.1% HCOOH in 30 min. Detection was carried out at 280 nm using diode array detection (DAD) and mass detection. The actives constituents were characterized by comparison with an authentic sample by the retention time (RT), mass spectra and UV–Vis spectra.

5.3. Parasites

The MHOM/Br/75/Josefa strain of *L.* (*L.*) *amazonensis* isolated from a human case of LCD in Brazil by Dr. CA Cuba-Cuba (University of Brasilia, Brazil) was maintained in axenic culture as promastigotes at 27 °C in Warren medium (broth infusion of bovine brain and heart, folic acid 20 mg/L and hemin 20 mg/L) supplemented with 10% fetal bovine serum. To verify the leishmanicidal activity of the fractions, 5 mL cultures of stationary-phase promastigotes were centrifuged at 1000*g* for 10 min at 27 °C. The pellet was resuspended in the medium (1 mL) and used to inoculate 5.0×10^5 parasites/mL in 10 mL tubes with medium (3 mL). The growth of parasites in the absence or presence of inhibitors was assessed by direct counting using a Neubauer chamber under phase microscopy.

5.4. Assessment of toxicity to splenocytes

For experiments with splenocytes, BALB/c mice spleens were obtained to assess mitochondrial redox activity using methylthiazolyldiphenyl-tetrazolium bromide (MTT) test. Inocula of 1.0×10^7 splenocytes were incubated in 96-well plates with 200 μL of RPMI (supplemented with 10% fetal bovine serum, 80 μg/mL gentamycin, 10 IU/mL penicillin and 10 μg/mL streptomycin) and either the extract or fractions. Cultures were incubated for 24, 48 and 72 h at 37 °C in a humidified atmosphere containing 5% CO_2 (Mosmann, 1983). After incubation with the extract or fractions, 20 μL of MTT at 5 mg/mL were added. After 3 h of incubation, DMSO (100 μL) was added to each well. The plates were centrifuged for 10 min at 550*g*, and the supernatants were read in a Versamax® microplate reader at 570 nm. A positive control was used to verify the MTT test.

5.5. Transmission electron microscopy

L. (*L.*) *amazonensis* promastigotes were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in sodium cacodylate buffer 0.1 M, pH 7.2 for 60 min at room temperature. Samples were post-fixed in a solution containing 1% OsO_4, 0.8% $K_3[Fe(CN)_6]$ and 5 mM $CaCl_2$ in the same buffer for 40 min at room temperature and protected from light (Vannier-Santos and Lins, 2001). After washing with cacodylate buffer, the samples were dehydrated in increasing concentrations of acetone and embedded in epoxy resin (Polybed 820, PolySciences). The ultrathin sections were contrasted with 7% uranyl acetate in MeOH and aqueous 1% lead citrate for viewing under a transmission electron microscope (Zeiss EM109).

5.6. IC_{50} determination and inhibition kinetics

L. (*L.*) *amazonensis* arginase activity was determined as previously described (da Silva et al., 2008; Silva and Floeter-Winter, 2010). To evaluate the inhibitory activity of EE and derived fractions, the tests were performed in a reaction mixture containing recombinant arginase (10 μL) from *L.* (*L.*) *amazonensis* (equivalent to 30 μg/mL protein) 100 mM L-arginine at pH 9.5 and 70 μL of the previously obtained aqueous solution fraction (HF, DF AF–MS and BUF) or H_2O (positive control). The reaction mixture was incubated at 37 °C for 15 min.

Arginase activity was determined using the Berthelot enzymatic-colorimetric assay method (Fawcett and scott, 1960), which detects urea production. Briefly, the reaction mixture (10 μL) was transferred to R1 (20 mM phosphate buffer, 750 μL), pH 7.0, containing 60 mM salicylate, 1 mM sodium nitroprusside and >500 IU urease). The R1 mixture was incubated at 37 °C for 10 min, and R2 (750 μL, 10 mM NaOCl and 150 mM NaOH) was added and incubated at 37 °C for 10 min. Spectrophotometric measurements were performed at 600 nm using a Hitachi 2810U spectrophotometer. Control experiments were performed under the same conditions, but in the absence of the inhibitor. IC_{50} measurements were performed with inhibitor concentrations obtained by the following two serial dilutions: the first set of dilutions contained 1250, 125, 12.5 and 1.25 μM inhibitor, and the second dilution 250, 25, 2.5, 0.25 μM inhibitor. The reaction was performed with 50 mM L-arginine in 50 mM CHES buffer at pH 9.5. All assays were performed in duplicate on three independent measurements.

5.7. Antileishmanial susceptibility test

Promastigote forms of *L.* (*L.*) *amazonensis* MHOM/BR/75/Josefa strain were cultured at 26 °C in Warren's medium (brain heart infusion plus haemin and folic acid) pH 7.2, supplemented with 10% heat-inactivated fetal bovine serum. Promastigotes from a 48-h-old logarithmic-phase culture were suspended to yield 10^5 -cells/ml, and treated with fractions obtained from crude extracts of *C. pachystachya*. The fraction concentrations used were 6.25, 12.5, 25, 50 and 100 μg/ml. Cultures were incubated at 26 °C for 72 h. The activity of the fraction was evaluated by cell counting using a Neubauer chamber. The antileishmanial activity was expressed as the IC_{50} (50% inhibitory concentration) after a 72 h incubation period. The control experiment was made with solvent used to solubilize the fractions.

5.8. Data analysis

For all tests, differences greater than $p < 0.05$ were considered significant. Analysis was performed using an ANOVA and a posteriori Tukey's tests by use of Prism 5.0 software (GraphPad). All tests were performed in triplicate with at least three independent repetitions. Inhibition data were analyzed also using the GraphPad Prism 5.0 software.

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