Journal of Ethnopharmacology 155 (2014) 387–395

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Research Paper

Evaluation of anti-inflammatory activity of hydroethanolic extract of *Dilodendron bipinnatum* Radlk

Ruberlei Godinho de Oliveira [a], Clarisse Pinto Azevedo Neto Mahon [a], Poliana Guerino Marson Ascêncio [b], Sérgio Donizeti Ascêncio [b], Sikiru Olaitan Balogun [a], Domingos Tabajara de Oliveira Martins [a,*]

[a] Department of Basic Health Sciences, Faculty of Medicine, Federal University of Mato Grosso (UFMT), Av. Fernando Correa da Costa, no. 2367, Coxipó, Boa Esperança, Cuiabá 78060-900, Mato Grosso, Brazil

[b] Natural Products Research Laboratory, Faculty of Medicine, Federal University of Tocantins (UFT), Av. NS15, Palmas 77020-210, Tocantins, Brazil

ARTICLE INFO

Article history:
Received 27 February 2014
Received in revised form
17 May 2014
Accepted 23 May 2014
Available online 12 June 2014

Keywords:
Dilodendron bipinnatum
Secondary metabolites
HPLC fingerprint
Mechanism of action
Inflammation

Chemical compounds studied in this article:
Gallic acid (PubChem CID: 370)
(+) catechin (PubChem CID: 9064)
(−)-epigallocatechin gallate
(PubChem CID: 65064)
(−) gallocatechin (PubChem CID: 65084)
lambda Carrageenan
(PubChem CID: 11966249)
Dexamethasone acetate
(PubChem CID: 5702036)
N-ω-Nitro-L-arginine methyl ester
hydrochloride (PubChem CID: 135193)
Doxorubicin (PubChem CID: 31703)
indomethacin (PubChem CID: 3715)
Alamar Blue (PubChem CID: 11077)
lipopolysaccharide PubChem CID:
53481793)

ABSTRACT

Ethnopharmacological relevance: *Dilodendron bipinnatum* Radlk. (Sapindaceae), popularly known as "mulher-pobre", is a native tree of the Pantanal of Mato Grosso, Brazil. The stem bark of *Dilodendron bipinnatum* is used by the population, in the forms of decoction and maceration in the treatment of inflammatory conditions. There is no information in the literature demonstrating the anti-inflammatory activity of *Dilodendron bipinnatum* and its respective mechanism of action. This study aimed to evaluate the anti-inflammatory activity and mechanism of action of the hydroethanolic extract of the stem bark of *Dilodendron bipinnatum* (HEDb) using in vivo and in vitro experimental models.

Materials and methods: The stem bark of *Dilodendron bipinnatum* was macerated in 70% hydroethanolic solution (1:3, w/v) for 7 days, filtered, concentrated on a rotary evaporator and the residual solvent removed in oven at 40 °C, thus obtaining HEDb. Cytotoxicity of HEDb in RAW 264.7 was assessed by the Alamar blue assay. in vivo anti-inflammatory activity of HEDb was evaluated with carrageenan and dextran-induced paw edemas and lipopolysaccharide (LPS)-induced peritonitis in mice. Effects of HEDb on the inflammatory cytokines (TNF-α, IL-1β and IL-10) concentrations in the peritoneal fluid were evaluated using commercial ELISA kits. The in vitro anti-inflammatory activity was evaluated using RAW 264.7 cells stimulated with LPS and/or INF-γ, while a Griess method was employed to determine nitric oxide (NO) concentrations in the peritoneal lavage and in the supernatants of RAW 264.7 cells. Preliminary phytochemical analysis was carried out using classical methods and secondary metabolites detected on HEDb were analyzed and confirmed by high performance liquid chromatography (HPLC).

Results: HEDb showed very low cytotoxicity with $IC_{50} > 200 \pm 0.38$ μg/mL. HEDb effectively inhibited paw edema by carrageenan in the 2nd hour at 20 mg/kg (36%, $p < 0.001$), and by dextran in the 1st hour at 100 mg/kg (46%, $p < 0.01$), after induction with the phlogistic agents. Furthermore, HEDb reduced total leukocytes and neutrophils migration at all doses tested producing maximum effect at 20 mg/kg (45% and 64%, $p < 0.001$ respectively). HEDb also attenuated increases in the concentrations of the pro-inflammatory cytokines (IL-1β and TNF-α) and increased the level of the anti-inflammatory cytokine IL-10 in the peritonitis model. However, it had no effect on NO production in activated RAW 264.7 cells. Preliminary phytochemical analysis revealed the presence of phenolic compounds, chalcones, flavones, flavonones, flavonoids, saponins and coumarins. HPLC analyses identified some tannins, with epigallocatechin gallate being the major compound.

Conclusions: Our findings provide evidence for the popular use of the stem bark of *Dilodendrum bipinnatum* in inflammation. Its anti-inflammatory action was due, at least in part, to the inhibition of cell migration, of the inflammatory mediators and Th1 cytokines and an increase in Th2 cytokines, without affecting NO pathway. It can be suggested that tannins account at least in part for the anti-inflammatory activity of HEDb.

* Corresponding author. Tel.: +55 6536158852; fax: +55 6536158862.
E-mail address: taba@terra.com.br (D.T.d.O. Martins).

http://dx.doi.org/10.1016/j.jep.2014.05.041
0378-8741/

1. Introduction

Inflammation is a response to tissue injury or infection, and it is characterized in its acute phase by an increase in vascular permeability and plasma extravasation, resulting in accumulation of fluid, leukocytes and mediators to the inflamed site (Guo et al., 2012).

A variety of soluble mediators is involved in the recruitment of circulating leukocytes and in the regulation of the activation process of resident cells in the early stages of inflammation (Morais-Lima et al., 2011). These soluble mediators involve lipid metabolites such as: platelet-activating factor (PAF) and arachidonic acid derivatives (eicosanoids), proteases/substrates related to coagulation and complement system cascade, kinins, nitric oxide and a group of polypeptide derived cells called cytokines (Kim et al., 2010).

In addition, an inflammatory response is related, in part, to reactive oxygen species (ROS) released by neutrophils and activated macrophages (Conforti et al., 2008).

In order to aleviate this situation, anti-inflammatory drugs are used, represented by steroidal agent (SAs) and non-steroidal drugs (NSAIDs), on symptomatic effects (Gautam and Jachak, 2009). However, a prolonged use of these agents is followed by severe side effects such as gastro-duodenal and kidney damage, bone marrow depression, retention of salts and water, among others (Qandil, 2012).

There is a clinical need to identify new compounds that are safe, for the prevention and treatment of inflammatory diseases (Hur et al., 2012). Medicinal plants are viable alternative to the discovery of new safer bioactive compounds (Gautam and Jachak, 2009).

In fact, there are evidences that drugs derived from natural products modulate various inflammatory mediators, including their effects on the expression of pro-inflammatory molecules that are key to inflammation, such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), IL-1β, TNF-α and IL-10 cytokines (Bellik et al., 2013).

Dilodendron bipinnatum (Sapindaceae), commonly known as "mulher pobre" is a native tree from Pantanal in Mato Grosso, Brazil, where the habitat is generally semi-deciduous forest. It occurs in savannah, flooded gallery forests, and develops in sandy or clay fertile soils (Lorenzi, 2000).

The inner stem bark of *Dilodendron bipinnatum* is used by the population, in the form of decoction and macerate, for the treatments of uterine inflammation, bone fractures, general pain and dermatitis because of its diuretic, stimulant, expectorant, sedative and anthelmintic properties (Bieski et al., 2012).

In a study involving the pharmacological evaluation of *Dilodendron bipinnatum*, Santos et al. (2010) reported that the ethanolic extract of the leaves, branches and stem bark were inactive against Gram-positive and Gram-negative bacteria and to *Candida albicans*. The same authors described that the leaf and stem bark extracts present in their composition a mixture of steroids such as β-sitosterol, stigmasterol, campesterol, 3-O-β-D-sitostenone; as well as triterpenos: cicloeucalenol and 24-methylene cicloartanol.

As a result of the widespread use of *Dilodendron bipinnatum* inner stem bark in popular medicine for inflammatory processes combined with a lack of studies proving the popular beliefs, this study was carried out aiming to evaluate the anti-inflammatory activity of hydroethanolic extract of *Dilodendron bipinnatum*, using in vivo and in vitro experimental models.

2. Material and methods

2.1. Botanic material

Inner stem bark of *Dilodendron bipinnatum* Radlk. [Family: Sapindaceae]– Sitzungsber. Math.-Phys. Cl. Königl. Bayer. Akad. Wiss. München viii. (1878) 357. (IK) (www.ipni.org) used in this study was harvested from Poconé, Mato Grosso, Brazil, coordinates S 15°56′528 and W 05°70′567. The plant collection was authorized by the Chico Mendes Institute of Biodiversity Conservation (Instituto Chico Mendes de Conservação da Biodiversidade - ICMbio), registry number 14360, while access to the associated traditional knowledge and to genetic patrimony for the purpose of research was authorized by the Council on Genetic Patrimony of the Ministry of Environment (CGEN/MMA) under registry number 045/2009. Botanical identification was done at the Herbarium of Federal University of Mato Grosso and voucher specimen (No. 20,529) was deposited at the same Herbarium. Since *Dilodendron bipinnatum* is not included in the list of endangered Brazilian plants, as such, its collection for the purpose of scientific studies does not require prior authorization by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA/MMA).

2.2. Animals

Male albino Wistar rats (180–200 g) and male Swiss mice (25–30 g) were used for the studies. Animals were maintained in propylene cages at 26 ± 1 °C in a 12 h dark/12 h light cycle, with free access to standard laboratory chow and water. Groups of six to eight animals were used for each experiment. The experimental protocol followed the International Principles for the Biomedical Research Involving Animal (CIOMS/OMS, 1985) and was approved by the Committee on the Use of Animal for experimentation (CEUA/UFMT) with Protocol no. 23108.015729/13-0.

2.3. Cell culture

Murine macrophage-like RAW 264.7 cell lines were obtained from the Cell Bank of Rio de Janeiro. The cells were maintained in DMEM (Dulbecco's modified Eagle's Medium plus 10% fetal bovine serum), supplemented by penicillin (100 U/mL) and streptomycin (100 μg/mL), under a temperature of 37 °C, and atmosphere of 5% of CO_2 and 90% humidity.

2.4. Drugs and reagents

Carrageenan, dextran, *Escherichia coli* lipopolysaccharide (serotypes 055:B5 and 055:B8), dexamethasone acetate, indomethacin, ethylenediaminetetraacetic acid (sodium-EDTA), N-ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), cyproheptadine, Griess reagent and sodium nitrite were obtained from Sigma (USA). Doxorubicin was acquired from Fluka (USA), Alamar Blue from Invitrogen and interferon-γ from Preprotec (Brazil). HPLC standards used were gallic acid (Vetec® 444), (−)-gallocatechin (Sigma® G6657), (+)−catechin (Sigma® C1251) and (−)-epigallocatechin-gallate. All reagents and drugs used were of analytical grade.

2.5. Extract preparation

The inner stem bark of *Dilodendron bipinnatum* was cleaned, dried at room temperature, and milled in an electric mill (model TE-625 TECNAL, São Paulo, Brazil) coupled with mesh sieve size 40. The dried powder (200 g) obtained was macerated in 70% hydroethanolic solution (1:3, w/v) for 7 days, filtered and concentrated in a rotary evaporator (Marconi MA 120, São Paulo, Brazil) under reduced pressure of 600 mm Hg at 40 °C. The residual solvent was eliminated in an oven at 45 °C, for 24 h, obtaining hydroethanolic extract of *Dilodendron bipinnatum* (HEDb) (with a yield of 16.02% w/w), which was stored in amber bottle and kept at 4 °C. At the time of use, HEDb was dissolved in distilled water to obtain the desired concentration.

2.6. Phytochemical analysis

2.6.1. Preliminary phytochemical screening

Preliminary phytochemical tests for secondary metabolites present in HEDb was performed according to the methods described by Matos (2009), which rely on chemical reactions of coloration, precipitation and foam formation.

2.6.2. Fingerprint HPLC analysis

The experiment was performed using a high performance liquid chromatography (HPLC-Shimadzu® chromatograph - LC-10 Avp series, Japan) equipped with a pump (LC-10AD), degasser (DGU-14A), UV–vis detector (SPD-10A), oven column (CTO-10A), rheodyne manual injector (loop 20 μL) and integrating CLASS (LC-10A). The extract was dissolved in the eluting solvent and the standard in methanol. All solutions were filtered with Millipore membrane (0.45 mM pore diameter). The samples were eluted using a Phenomenex Luna reverse phase column C18 5 μm (2) (250 × 4.6 mm) and Phenomenex C18 pre-column (4 × 3.0 mm) filled with similar material to the main column. The chromatographic separation of the compounds was carried out in isocratic elution using methanol/water Milli-Q/methanol (1:18:1), at 40 °C and flow of 1 mL min^{1}. The quantification was performed and expressed in micrograms per milligram of extract (μg/ mg), correlating the area of the analyte with the calibration curve of standards built in concentrations of 125–1000 μg/mL. The extract solutions and standards were prepared with methanol and filtered through a Millipore® (0.45 mM pore size) membrane as previously described by Tamashiro-Filho et al. (2012).

2.7. Cell viability assay

RAW 264.7 cells, of density 2×10^4 cells per dish were plated on a 96-well microplate containing medium (growth control) with/without HEDb with concentration range from 3.125 to 200 μg/mL. Doxorubicin (0.0058–58 μg/mL) was used as a positive control. After incubation for 24 h at 37 °C and 5% CO_2, the treatments were removed and 200 μL of 10% Alamar Blue solution was added (Nakayama et al., 1997). After 5 h, absorbance plate at 540 nm for oxidized state and 620 nm for reduced state, through ELISA reader (Bio-Tek, Elx800), and then cell viability was calculated. Drugs that presented IC_{50} < 50 μg/mL were considered cytotoxic (Fröelich et al., 2007).

2.8. in vivo anti-inflammatory assay

2.8.1. Paw edema induced by carrageenan and dextran

The animals were orally treated with vehicle (distilled water), HEDb (20, 100 and 500 mg/kg) and indomethacin (5 mg/kg) dissolved in 2% (w/v) sodium bicarbonate. After 1 h, 0.1 mL of 1% carrageenan was injected in posterior left paw of each animal and the same amount of 0.9% sterile sodium chloride solution was injected in the contralateral paw. The volume of each paw was measured using digital plethysmometer (Model 7140, Ugo Basile, Italy) 0, 1, 2, 3 and 4 h after the phlogistic stimulus injection. The measurement variation (mL) between right and left paws represented edema volume (Winter et al., 1962).

In the case of dextran-induced paw edema, the procedure was the same as used for carrageenan, except that cyproheptadine (5 mg/kg) was used as positive control and the edema measurements were carried out at 0, 15, 30, 60 and 120 min after the injection.

2.8.2. Peritonitis induced by lipopolysaccharide

In order to evaluate the effect of HEDb on leukocyte recruitment into the peritoneal cavity, the mice were orally pre-treated, with vehicle (0.9% saline solution), HEDb (20, 100 and 500 mg/kg) or dexamethasone (0.5 mg/kg). After 1 h, LPS (250 ng/cavity/ 0.2 mL), of *Escherichia coli* dissolved in sterile saline solution was administered intraperitoneally. Six hours after the intraperitoneal injection of LPS, mice were anesthetized with 180 mg/kg ketamine and 30 mg/kg xylazin by intraperitoneal (ip.) and the cells in the peritoneal cavity were collected through injection of 3 mL saline solution containing EDTA. The abdomen was slightly massaged and the cell suspension was aspirated using a syringe. The peritoneal lavage collected was used for cellular counting in Neubauer chamber, while an aliquot of the lavage was used to make smear for differential counting. Aliquots of peritoneal washing were stored in a freezer at −80 °C for posterior dosage of cytokines (Cunha et al., 1989; Orlandi et al., 2011).

2.8.3. Cytokine quantification in the peritoneal lavage

The levels of cytokines (pg/mL) TNF-α, IL-1β and IL-10 were determined using ELISA kit (eBioscience, USA), in accordance with manufacturer's instructions. Microplate reader Multiskan® (Thermo Scientific, EUA) was used for reading the absorbance.

2.9. in vitro anti-inflammatory assay

2.9.1. Nitrite dosage

Nitrite (NO_2^-), a stable product from nitric oxide (NO), was used as an indicator of NO production in the culture medium. Nitrite released in the culture medium was measured according to the Griess reaction (Minghetti et al., 1997). In summary, RAW cells 264.7 (1.0×10^6 cells/dish) were plated in a 24-well plate overnight. Cells were pre-treated with HEDb at concentrations of 1, 5 and 20 μg/mL for 1 h, and incubated at 37 °C and 5% CO_2. Next, the cells were stimulated with LPS (0.5 μg/mL) and/or IFN-γ (0.5 ng/mL) for 24 h, in the presence or absence of HEDb (1, 5 and 20 μg/mL) under the same condition. L-NAME (2.69 μg/mL), a specific inhibitor of iNOS, was used as a positive control. For negative control, the same amount of medium was used in the microplate well. Supernatant from cell culture was measured for nitrite concentration and 100 μL of it was mixed with the same volume of Griess reagent for 10 min at room temperature. Absorbance (540 nm) was measured using a microplate reader and nitrite concentration was determined using a standard curve of sodium nitrite prepared in RPMI-1640 exempt of phenol red.

2.10. Data analysis

The results were expressed as mean ± standard error of mean ($\bar{x} \pm$ SEM). Comparisons between means were analyzed by one-way analysis of variance (Anova). When significant, it was followed by Student-Newman–Keuls test for multiple comparisons. P values < 0.05 were considered significant. In vitro assays were performed in triplicate. All values were analyzed with GraphPad Prism® software version 5.01 GraphPad Software, Inc. La Jolla, CA 92037 USA www.graphpad.com.

The IC_{50} was determination from a linear regression relating the percentage of inhibition versus the logarithm of the concentrations tested and assuming a confidence level of 99% ($p < 0.01$) for the straight obtained. For in vitro assays that do not involve statistical analysis, we used the mean ± SEM three independent experiments performed in duplicate.

3. Results

3.1. Preliminary phytochemical screening

Preliminary phytochemical screening of HEDb revealed the presence of phenols, chalcones, flavonoids, saponins and coumarins. The phenols identified were confirmed qualitatively and quantitatively by HPLC.

3.2. Fingerprint HPLC analysis

Analysis by HPLC confirmed the presence of phenolic compounds detected in the preliminary analysis (Fig. 1). These compounds in HEDb were analyzed and revealed the presence of gallic acid (GA, retention time 4.87 min at a concentration of 0.30 mg of GA/g of HEDb, representing 0.03% of the extract), (−)-gallocatechin (retention time 5.99 min, qualitatively analyzed), (+)−catechin (CAT, retention time 13.97 min) at concentration of 7.74 mg of CAT/g of HEDb (0.77% of extract) and gallate of (−)-epigallocatechin (EGTC) (retention time 27.62 min) at concentration of 10.98 mg of EGTC/g of HEDb (1.10% of extract).

3.3. Cell viability assay

Fig. 2 shows the cell viability curve of RAW cells 264.7 treated with decreasing concentrations of HEDb and doxorubicin. The extract demonstrated to be non-cytotoxic with $IC_{50} > 200 \pm 0.38$ μg/mL, while doxorubicin, the positive control in this assay, was highly cytotoxic with IC_{50} of 4.8 ± 2.56 μg/mL.

3.4. in vivo anti-inflammatory assay

3.4.1. Paw edema induced by carrageenan and dextran

For the group treated with vehicle, a slow and progressive volume increase of the posterior intraplantar left paw injected with 0.1 mL of 1.0% carrageenan was observed, reaching the peak of edema at the 3rd hour (0.61 ± 0.05 mL). The effect of HEDb at 20, 100 and 500 mg/kg caused a non-dose-dependent reduction of the edema until the 3rd hour, in all doses, reaching a maximum effect (36%, $p < 0.01$) at the dose of 20 mg/kg. Indomethacin (5 mg/kg) also reduced significant edema until the 3rd hour, producing the greatest effect at the second hour (32%, $p < 0.01$), as shown in Fig. 3.

In the group of rats orally treated with the vehicle, the intraplantar injection of 0.1 mL of 1.5% dextran promoted an edema characterized by sudden onset and reaching the peak at 30 min (0.72 ± 0.05 mL), as shown in Fig. 4. HEDb was active only in the first hour after induction at the dose of 100 mg/kg, reducing the paw edema by 46% ($p < 0.01$). Cyproheptadine (5 mg/kg) caused an intense inhibition of paw edema at all times, with the effect starting from 15 min (42.8%, $p < 0.01$) after induction and reaching the peak (91.5%, $p < 0.001$) at 30 min.

3.4.2. Peritonitis induced by lipopolysaccharide

3.4.2.1. Total leukocytes. In LPS-induced peritonitis, the sham group (distilled water p.o. and 0.9% sterile saline solution, ip.)

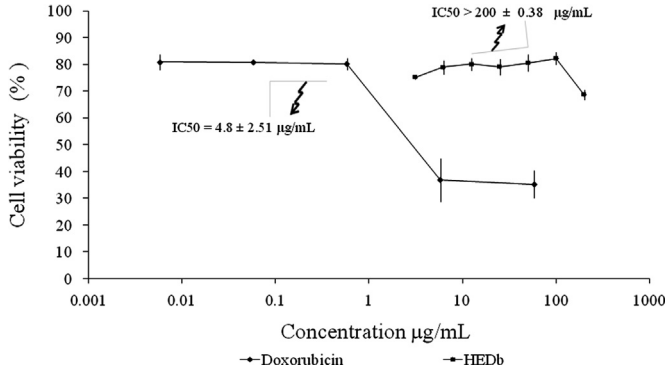

Fig. 2. Cell viability curve of RAW 264.7 cells exposed to varying concentrations of hydroethanolic extract of *Dilodendron bipinnatum* inner stem bark (HEDb) and doxorubicin for 24 h. Expressed as minimum inhibitory concentration 50% (IC_{50}).

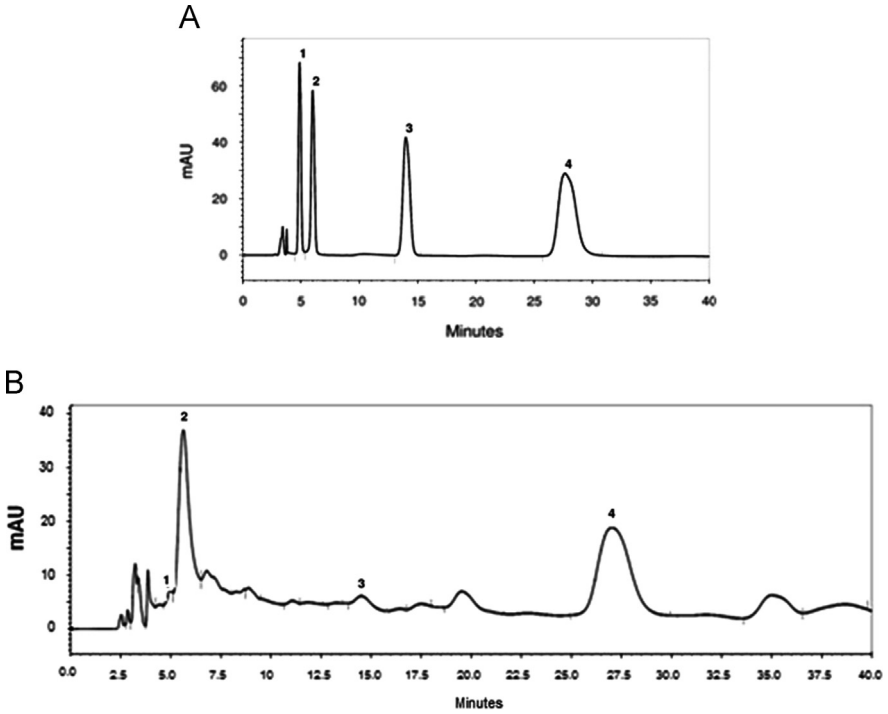

Fig. 1. HPLC chromatogram of authentic standards tested (A) and HPLC fingerprint of hydroethanolic extract of Dilodendron bipinnatum (B), detected at 280 nm. Peak 1: gallic acid (time 4.87 min.) 2: (−) gallocatechin (time 5.99 min.); 3: (+)-catechin (time 13.97 min.); 4: gallate of (−)-epigallocatechin (time 27.62 min).

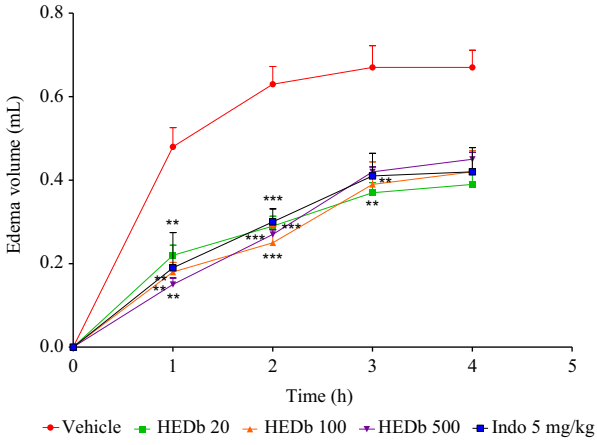

Fig. 3. Effect of oral administration of vehicle (1 mL of distilled water/100 g), 70% hydroethanolic extract of *Dilodendron bipinnatum* inner stem bark (HEDb −20, 100, and 500 mg/kg), and 5 mg/kg indomethacin on paw edema induced by 1% carrageenan in rats. Each point represents a mean of 6 animals. The vertical lines represent S.E.M. One-way analysis of variance, followed by Student-Newman–Keuls test. ** $p < 0.01$ and *** $p < 0.001$ vs. vehicle.

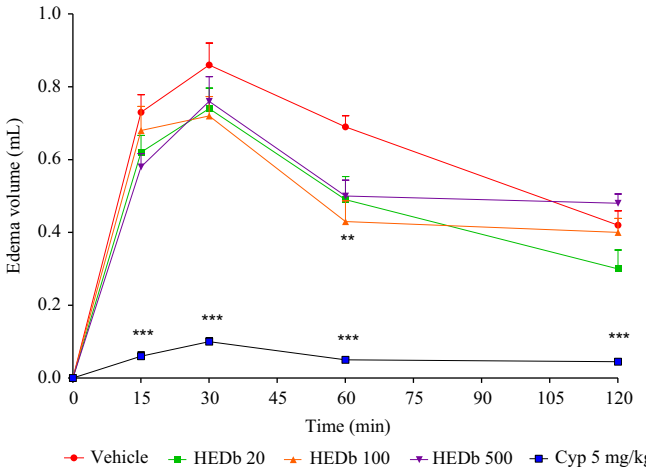

Fig. 4. Effect of oral administration of vehicle (1 mL of distilled water/100 g), 70% hydroethanolic extract of *Dilodendron bipinnatum* inner stem bark (HEDb −20, 100, and 500 mg/kg), and cyproheptadin (Cyp – 5 mg/kg) on paw edema induced by 1.5% dextran in rats. Each point represents a mean of 6 animals. The vertical lines represent S.E.M. One-way analysis of variance, followed by Student-Newman–Keuls test. ** $p < 0.01$ and *** $p < 0.001$ vs. vehicle.

presented $7.9 \pm 0.47 \times 10^6$ total leukocytes in the peritoneal cavity. The intraperitoneal injection of 250 ng of LPS (0.2 mL/cavity) in mice of the vehicle group caused a significant increase (77.0%; $p < 0.001$) in leukocyte migration to the peritoneal cavity compared to the sham group. Pre-treatment with HEDb (20, 100 and 500 mg/kg p.o.) caused reduction in leukocyte migration and attaining maximum effect at the dose of 20 mg/kg (45.4%, $p < 0.001$) compared to vehicle group. Pre-treatment with dexamethasone at 0.5 mg/kg, inhibited leukocytes influxes by 50.8% ($p < 0.001$) when compared to the vehicle group (Fig. 5).

3.4.2.2. Differential cell counting. In the sham group, the number of neutrophils present in the peritoneal cavity was $4.7 \pm 0.48 \times 10^6$. In the vehicle group, LPS injection caused an increase of 79.1% ($p < 0.001$) in the number of neutrophils that migrated to the peritoneal cavity, compared to the sham group.

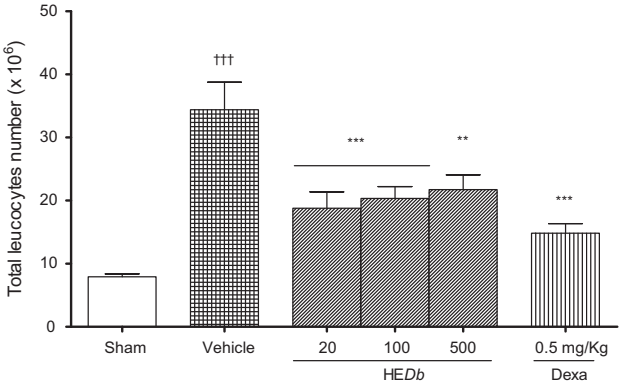

Fig. 5. Effect of oral administration of vehicle (0.1 mL/10 g), 70% hydroethanolic extract of *Dilodendron bipinnatum* inner stem bark (HEDb-20, 100, and 500 mg/kg), and dexamethasone (Dexa-0.5 mg/kg) on total number of leukocytes present in the peritoneal lavage of male mice with LPS-induced peritonitis (250 ng LPS/0.2 mL/cavity). The sham group received vehicle (1 mL water/10 g, p.o) and intraperitoneal injection of 0.9% sterile saline solution (0.1 mL/10 g). Each point represents a mean of 8 animals. The vertical lines represent S.E.M. One-way analysis of variance, followed by Student-Newman–Keuls test. ††† $p < 0.001$ vs. sham; ** $p < 0.01$ and *** $p < 0.001$ vs. vehicle.

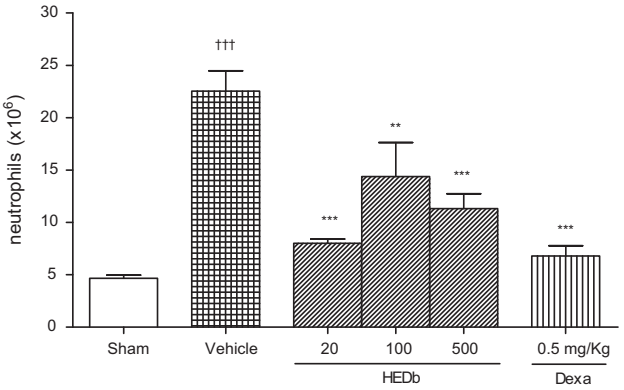

Fig. 6. Effect of oral administration of vehicle (0.1 mL/10 g), hydroethanolic extract 70% of *Dilodendron bipinnatum* inner stem bark (HEDb-20, 100, and 500 mg/kg), and dexamethasone (Dexa-0.5 mg/kg) on number of neutrophils present during peritoneal lavage of male mice with peritonitis LPS-induced (250 ng LPS/0.2 mL/cavity). The sham group received vehicle (water, 1 mL/10 g, v.o) and an intraperitoneal injection of 0.9% saline solution sterile (0.1 mL/10 g). Each point represents a mean of 8 animals. The vertical lines represent S.E.M. One-way analysis of variance, followed by Student-Newman–Keuls test. ††† $p < 0.001$ vs. sham; ** $p < 0.01$ and *** $p < 0.001$ vs. vehicle.

HEDb reduced neutrophil migration at all doses tested, in a non dose-dependent manner and producing greater effect at the dose of 20 mg/kg (64.0%, $p < 0.001$), in comparison to the vehicle group, while in dexamethasone treated group, the reduction was 69.8% ($p < 0.001$) as shown in Fig. 6.

3.4.2.3. Cytokines quantification in the peritoneal lavage. As shown in Fig. 7, the concentration of TNF-α in peritoneal lavage of animals from sham group was 8.6 ± 1.95 pg/mL. In the vehicle group, which received an intraperitoneal injection of LPS, presented a significant increase of 94.8% ($p < 0.001$) of this cytokine, compared to sham group.

HEDb reduced TNF-α concentration in the peritoneal lavage, at all doses tested (in non dose-dependent manner), with 100 mg/kg having the maximum effect (65.8%, $p < 0.001$), while with dexamethasone, the reduction was 78.6% ($p < 0.001$), in comparison to vehicle group.

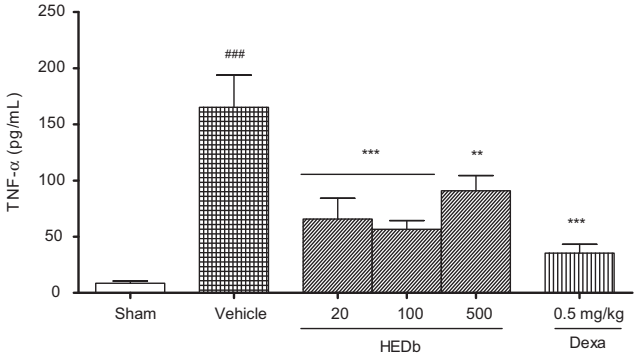

Fig. 7. Effect of oral administration of vehicle (0.1 mL/10 g), 70% hydroethanolic extract of *Dilodendron bipinnatum* inner stem bark (HEDb-20, 100, and 500 mg/kg), and dexamethasone (Dexa-0.5 mg/kg) on the concentration of TNF-α, on peritoneal lavage of male mice with peritonitis LPS-induced (250 ng LPS/0.2 mL/cavity). The sham group received vehicle (water, 1 mL/10 g, v.o) and intraperitoneal injection of 0.9% sterile saline solution (0.1 mL/10 g). Each point represents a mean of 8 animals. The vertical lines represent S.E.M. One-way analysis of variance, followed by Student-Newman–Keuls test. ††† $p < 0.001$ vs. sham; ** $p < 0.01$ and *** $p < 0.001$ vs. vehicle.

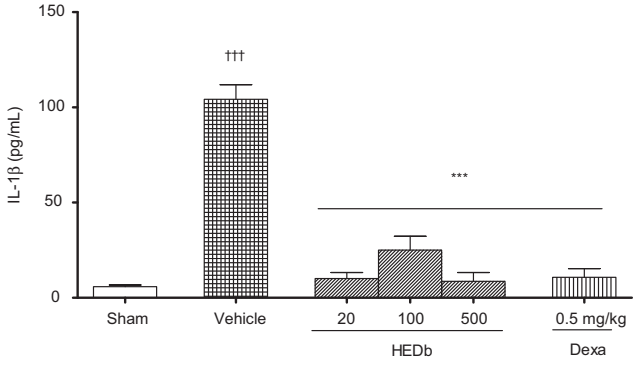

Fig. 8. Effect of oral administration of vehicle (0.1 mL/10 g), 70% hydroethanolic extract of *Dilodendron bipinnatum* inner stem bark (HEDb-20, 100, and 500 mg/kg), and dexamethasone (Dexa-0.5 mg/kg) on the concentration of Interleukin-1β (IL-1β), on peritoneal lavage of male mice with peritonitis LPS-induced (250 ng LPS /0.2 mL/cavity). The sham group received vehicle (water, 1 mL/10 g, v.o) and intraperitoneal injection of 0.9% sterile saline solution (0.1 mL/10 g). Each point represents a mean of 8 animals. The vertical lines represent S.E.M. One-way analysis of variance, followed by Student-Newman–Keuls test. ††† $p < 0.001$ vs. sham; *** $p < 0.001$ vs vehicle.

As shown in Fig. 8, the concentration of IL-1β determined in the peritoneal lavage of animals from the sham group was 5.9 ± 0.84 pg/mL. Vehicle group presented an increase of 94.4% ($p < 0.001$), of the concentration of this cytokine, in comparison to sham group.

HEDb treatment attenuated the increase in the concentration of IL-1β at all dose tested, in a non-dose-dependent manner, with maximum effect being at the dose of 500 mg/kg (91.7%, $p < 0.001$), whereas with dexamethasone, the inhibition was 89.4% ($p < 0.001$) when compared to the vehicle group.

As shown in Fig. 9, the concentration of IL-10 determined in the peritoneal lavage of animals from the sham group was 279.5 ± 19 pg/mL. The intraperitoneal injection of LPS in the vehicle group did not significantly alter the concentration of IL-10 (188.2 ± 10.9 pg/mL) in comparison to the sham group ($p > 0.05$).

HEDb increased the concentration of IL-10 in the peritoneal lavage at all doses tested, in a non-dose-dependent manner, with

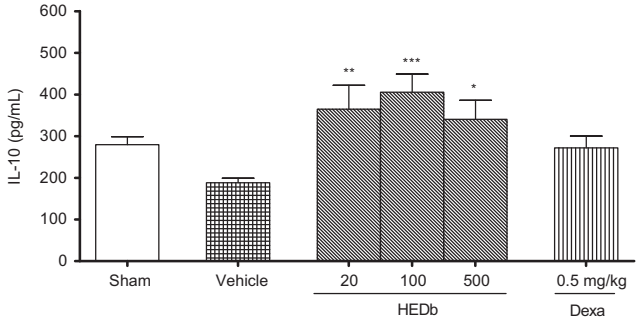

Fig. 9. Effect of oral administration of the vehicle (0.1 mL/10 g), 70% hydroethanolic extract of *Dilodendron bipinnatum* inner stem bark (HEDb-20, 100, and 500 mg/kg), and dexamethasone (Dexa-0.5 mg/kg) on the concentration of Interleukin 10 (IL-10), on peritoneal lavage of male mice with peritonitis LPS-induced (250 ng LPS/0.2 mL/cavity). The sham group received vehicle (water, 1 mL/10 g, v.o) and intraperitoneal injection of (0.1 mL/10 g). Each point represents a mean of 8 animals. The vertical lines represent S.E.M. One-way analysis of variance, followed by Student-Newman–Keuls test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. vehicle.

the peak effect at the dose of 100 mg/kg (53.6%, $p < 0.001$), while dexamethasone did not alter at the concentration of this cytokine (271.8 ± 28.4 pg/mL, $p > 0.05$) in comparison to the vehicle group.

3.5. Evaluation of in vitro anti-inflammatory activity

3.5.1. Nitrite dosage

The basal nitrite concentration in non-stimulated RAW cells 264.7 was 0.1 ± 0.23 μM. The vehicle group, which was stimulated with LPS 0.5 μg/mL or co-stimulated with LPS 0.5 μg/mL and IFN-γ 0.5 ng/mL presented, increases of 95.7% and 94.9% ($p < 0.001$) respectively, in the nitrite concentration, compared to the sham group.

Pre-treatment with HEDb (1, 5 and 20 μg/mL) did not alter ($p > 0.05$) nitrite levels in the cellular supernatant stimulated by LPS or LPS with IFN-γ, in comparison to vehicle group. L-NAME (2.69 μg/mL), the standard used in the assay, inhibited nitrite production by 79.4% and 57.9% in the LPS and LPS with IFN-γ respectively, compared to the vehicle group ($p < 0.001$).

4. Discussion

The scientific evidence of the popular use of HEDb in inflammatory processes has been demonstrated by Mahon (2012). In order to evaluate possible mechanisms of action involved in the anti-inflammatory action of HEDb, in vivo and in vitro inflammation models were employed in this present study.

Towards this end, carrageenan induced paw edema was used. In this inflammatory model, oral treatment of HEDb present antiedemal activity at all the three doses tested, which suggests that the extract acts by the inhibition of mediators related to arachidonic acid, prostaglandin and cyclooxygenase pathways (Silva et al., 2005). However, further studies are needed to elucidate this mechanism of action.

Due to the promising result obtained in the preliminary screening with carrageenan-induced paw edema, we sought to investigate the effects of HEDb in other animal models of acute inflammation.

Edema produced by subplantar injection of dextran in animals is characterized by a rapid increase in the paw edema and spontaneous decrease after 30 min, with histamine and serotonin being the main mediators (Lo et al., 1982; Calixto, 2005).

HEDb at the dose of 100 mg/kg presented a reduction in dextran-induced paw edema in the first hour only, indicating that the anti-edematogenic activity observed in this model could be related to the inhibition of histamine and serotonin releases (Stucki and Thompson, 1958). It is interesting to note that only the intermediate dose was effective, the exact mechanism responsible for this kind of effect is not known.

Although experimental models of carrageenan-induced and dextran-induced paw edema are important to evaluate the potential of anti-inflammatory drugs, they are not suitable for quantifying the cellular component of an acute inflammatory response, where neutrophils are particularly relevant (Vinegar et al., 1987). In this way, HEDb was evaluated in an LPS-induced leukocyte migration model (Orlandi et al., 2011).

During the course of inflammation induced by LPS, the neutrophils and the macrophages are the principal cells involved (Pinho et al., 2011). A reduction in the leukocyte migration by HEDb was accompanied by inhibition in the accumulation of the neutrophils to the site of inflammation, indicating that inhibition of this polymorphonuclear cell is involved in the anti-inflammatory action of the extract.

In peritonitis caused by LPS, phagocytes synthesize and release several mediators, including pro-inflammatory cytokines such as TNF-α and IL-1β and anti-inflammatory IL-10. Pre-treatment of animals with HEDb resulted in reductions of TNF-α and IL-1β comparable to dexamethasone and, surprisingly, increased the levels of IL-10, unlike the standard drug which, in this model did not cause increase in the production of this cytokine.

IL-1β exerts a strong pro-inflammatory activity and it is produced mainly by macrophages and to a lesser extent by neutrophils, lymphocytes, endothelial cells and other cell types (Gabay et al., 2010). IL-1β is important for initiation and increased inflammatory response to the proliferation of certain microorganisms (Pinho et al., 2011). It plays a key role in acute and chronic inflammatory and autoimmune diseases (Dinarello, 2010). IL-1β promotes the recruitment of inflammatory cells to the site of inflammation by inducing the expression of adhesion molecules on endothelial cells and release of chemokines by stromal cells (Gabay et al., 2010).

TNF-α, primarily produced by macrophages and to a lesser extent by other cell types (Chu, 2013) is the prototype of pro-inflammatory cytokines, because it not only induces its own secretion, but also stimulates the production of other inflammatory cytokines and chemokines (McDermott, 2001). This cytokine has been directly implicated as a mediator of septic shock, inflammation and cytotoxicity (Aggarwal and Natarajan, 1996).

Like IL-1β, TNF-α plays an important role in the control of leukocyte migration, especially of neutrophils, by increasing the expression of adhesion molecules on the surface of endothelial cells and chemokines such as MCP-1 which contribute to the recruitment of circulating monocytes into the tissues (Cavaillon, 1994).

Studies have shown that IL-10 controls the degree and duration of the inflammatory response, acting as a potent anti-inflammatory cytokine, affecting both Th1 and Th2 responses. Its main effect is due to the selective blockade of expression of genes encoding cytokines (TNF-α, IL-1β and IL-6 mRNA) and pro-inflammatory CXR chemokines (MCP-1, IL-8, IP-10 and MIP -2) in myeloid cells activated by PRR ligands such as LPS (Moore et al., 2001). Accordingly, IL-10 causes blockade of cellular migration, especially the initial influx of neutrophils to the site of the injury (Bazzoni et al., 2010).

Based on these results, a model for signaling induced by EHDb in peritonitis induced by LPS is proposed here. First, HEDb may be acting directly or indirectly, by inhibiting the activation of TLRs by LPS and thereby leading to reduced cell migration, and in turn, reducing the release of pro-inflammatory cytokines in the peritoneal cavity. Second, HEDb may be acting directly through the inhibition of the production or release of pro-inflammatory cytokines such as TNF-α and IL-1β, and by reducing the release of other inflammatory mediators which are important in the induction and maintenance of inflammatory response caused by LPS. Third, HEDb may be acting directly or indirectly (via IL-10) by increasing the expression of anti-inflammatory molecules. Fourth, HEDb may be acting directly or indirectly by the activation of IL-10R receptor, and ultimately inducing expression of genes that inhibit the synthesis of new proteins by neutrophils and macrophages, including pro-inflammatory cytokines (IL-1β, TNF and IL-8) (Bazzoni et al., 2010).

The increasingly restricted use of tests with laboratory animals has led to the development of alternative in vitro methods. Compared to the in vivo methods, in vitro methods offer the following advantages: limited number of experimental variables; simpler acquisition of meaningful data, and short test period (Rogero et al., 2003).

In the present study, we evaluated the viability of RAW 264.7 cells with different concentrations of HEDb cells using the Alamar Blue method (Nakayama et al., 1997). HEDb presented $IC_{50} > 200$ μg/mL, indicating its very low cell toxicity according to criteria established by Fröelich et al. (2007).

Analysis of cell viability does not only enable evaluation of cytotoxicity superficially, but also in the selection of the concentrations of HEDb to be used for the in vitro anti-inflammatory assays.

RAW 264.7, a murine macrophage cell line was selected for the in vitro studies of mechanisms of action, and has been shown to be an excellent model for screening anti-inflammatory drugs and subsequent evaluation of inhibitors of the pathways that lead to induction of pro-inflammatory enzymes and cytokines (Yang et al., 2012).

in vitro studies to elucidate the possible mechanisms involved in the anti-inflammatory action HEDb were performed in RAW 264.7 cells activated by LPS and/or IFN-γ, since among immune systems that participate in host defense, macrophages are the main cells targeted by this bacterial endotoxin (Lai et al., 2013).

NO, a reactive nitrogen species is produced from iNOS and has important biological functions including vasodilation, immunoregulation, inflammation, and neurotransmitter. This small gas molecule modulates the synthesis of prostaglandins, thromboxanes and other inflammatory molecules (Moncada et al., 1991). The increase of NO in RAW 264.7 activated with LPS and IFN-γ was not affected by pre-treatment with HEDb, indicating that the anti-inflammatory activity of the extract is independent of the modulation of the NO.

Chemical analysis revealed the presence of gallic acid, gallocatechin, catechin and epigallocatechin gallate in HEDb, the latter being the major compound.

Activities and possible mechanisms of anti-inflammatory action of these four tannins are well documented in the literature and involve actions on different targets of the inflammatory process (Kroes et al., 1992; Delporte et al., 2003; Geraets et al., 2009; Singh et al., 2010; Hirao et al., 2010; Muthuraman et al., 2011).

Based on preliminary phytochemical analysis, it can be stated that the anti-inflammatory action of HEDb, depends at least in part, on the synergistic interaction of the four tannins identified by the HPLC technique. One cannot rule out the possibility of other compounds belonging to different metabolic classes contributing to the anti-inflammatory action of HEDb, since in the preliminary phytochemical analysis of the extract chalcones, flavonoids, saponins and coumarins were found in addition to the phenols. Santos et al. (2010) showed that the ethanol extract of leaves,

branches and stem of the *Dilodendron bipinnatum* present steroids (β-sitosterol, stigmasterol, campesterol and 3-O-β-D-sitostenone) and triterpenes (cicloeucalenol and) all of proven anti-inflammatory activities (Safayhi and Sailer 1997; Gabay et al., 2010; Loizou et al., 2010; Kaur et al., 2011).

As can be seen in most of the experiments, a non-dose dependent effect of HEDb was observed. The exact mechanism by which the extract produced a non-dose dependent effect is unknown. However, there are plausible hypotheses to explain this phenomenon. The crude extract is composed of complex of phytochemical compounds whose diverse biological and pharmacological effects are well documented. Many of these compounds are known to be pleiotropic, producing multiple effects by acting on several cellular and or molecular targets (Vattem and Shetty, 2005). In this manner, it is possible that a single molecular entity may produce different functional outputs. Likewise, from the perspective of ligand-receptor interactions, there could be an agonism, partial agonism or even antagonism effects due to the combined actions of the various phytoconstituents (Vattem and Shetty, 2005; Spedding, 2011). In addition, various factors such as onsets and offsets kinetics, functional selectivity (ligand induced differential signaling) and a host of others determine the ultimate effect of even a single compound, and therefore by extension may be the case with a crude extract. As noted in the review of Vattem and Shetty (2005), some phytochemicals have been shown to interact with proteins and to alter their configurations, and can directly interact with the cell surface receptors and ion pumps, thus directly activating signaling cascades. These signaling cascades can result in the changes in many physiological pathways.

The in vivo and in vitro results provided evidence for the popular use of the stem bark of *Dilodendron bipinnatum* in inflammation. HEDb presented innovative multitargeted anti-inflammatory action mechanisms and was non-cytotoxic. Its anti-inflammatory action was due, at least in part, to the inhibition of cell migration and mediators of the inflammatory response, by inhibiting Th1 cytokines and stimulating Th2 cytokine without affecting the NO pathway. It can be suggested that the tannins are responsible, at least in part for the anti-inflammatory activity of HEDb.

Acknowledgments

We thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (process no. 551737/2010-7), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (23038.000731/2013-56) and Instituto Nacional de Ciência e Tecnologia em Áreas Umidas (INAU) (04.2/2012) for financial assistance. We are grateful to Dr. Germano Guarin Neto of UFMT Herbarium for technical assistance with plant identification.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2014.05.041.

References

Aggarwal, B.B., Natarajan, K., 1996. Tumor necrosis factors: developments during the last decade. European Cytokine Network 7, 93–124.
Bazzoni, F., Tamassia, N., Rossato, M., Cassatella, M.A., 2010. Understanding the molecular mechanisms of the multifaceted IL-10-mediated anti-inflammatory response: lessons from neutrophils. European Journal Immunology 40, 2360–2368.
Bellik, Y., Boukraâ, L., Alzahrani, H.A., Bakhotmah, B.A., Abdellah, F., Hammoudi, S.M., Iguer-Ouada, M., 2013. Molecular mechanism underlying anti-Inflammatory and anti-allergic activities of phytochemicals: an update. Molecules 18, 322–353.
Bieski, I.G.C., Santos, F.R., Oliveira, R.M., Espinosa, M.M., Macedo, M., Albuquerque, U.P., Martins, D.T.O., 2012. Ethnopharmacology of medicinal plants of the Pantanal Region (Mato Grosso, Brazil). Evidence-based complementary and alternative medicine. 2012, 1–36.
Calixto, J.B., 2005. Twenty-five years of research on medicinal plants in Latin America a personal review. Journal of Ethnopharmacology 100, 131–134.
Cavaillon, J.M., 1994. Cytokines and macrophages. Biomedecine pharmacotherapie 48, 445–453.
Chu, W.M., 2013. Tumor necrosis factor. Cancer Letters. 328, 222–225.
Conforti, F., Sosa, S., Marrelli, M., Menichini, F., Statti, G., Uzunov, D., Tubaro, A., Menichini, F., Loggia, R.D., 2008. in vivo anti-inflammatory and in vitro antioxidant activities of Mediterranean dietary plants. Journal of Ethnopharmacology 116, 144–151.
Cunha, F.Q., Souza, G.E., Souza, C.A., Cerqueira, B.C., Ferreira, S.H., 1989. in vivo blockage of neutrophil migration by LPS is mimicked by a factor released from LPS-stimulated macrophages. British Journal of Experimental Biology 70, 1–8.
Delporte, C., Backhouse, N., Salinas, P., San-Martín, A., Bórquez, J., Loyola, A., 2003. Pharmaco-toxicological study of diterpenoids. Bioorganic and Medicinal Chemistry. 11, 1187–1190.
Dinarello, C.A., 2010. Anti-inflammatory agents: present and future. Cell 140, 935–950.
Fröelich, B.O., Kakooko, A., Siems, K., Schubert, C., Jenett-Siems, K., 2007. Plants traditionally used against malaria: phytochemical and pharmacological investigation of Momordica foetida. Brazilian Journal of Pharmacognosy 17, 1–7.
Gabay, C., Lamacchia, C., Palmer, G., 2010. IL-1 pathways in inflammation and human diseases. Nature Review Rheumatology 6, 232–241.
Gautam, R., Jachak, S.M., 2009. Recent developments in anti-inflammatory natural products. Medicinal Research Reviews. 29, 767–820.
Geraets, L., Haegens, A., Brauers, K., Haydock, J.A., Vernooy, J.H., Wouters, E.F., Bast, A., Hageman, G.J., 2009. Inhibition of LPS-induced pulmonary inflammation by specific flavonoids. Biochemical and Biophysical Research Communications 382, 598–603.
Guo, W., Sun, J., Jiang, L., Duan, L., Huo, M., Chen, N., Zhong, W., Wassy, L., Yang, Z., Feng, H., 2012. Imperatorin attenuates LPS-induced inflammation by suppressing NF-κB and MAPKs activation in RAW 264.7 macrophages. Inflammation 35, 1764–1772.
Hirao, K., Yumoto, H., Nakanishi, T., Mukai, K., Takahashi, K., Takegawa, K., Matsuo, T., 2010. Tea catechins reduce inflammatory reactions via mitogen-activated protein kinase pathways in toll-like receptor 2 ligand-stimulated dental pulp cells. Life Science 86, 654–660.
Hur, S.J., Kang, S.H., Jung, H.S., Kimb, S.C., Jeon, H.S., Kim, I.H., Lee, J.D., 2012. Review of natural products actions on cytokines in inflammatory bowel disease. Nutrition Research 32, 801–816.
Kaur, N., Chaudhary, J., Jain, A., Kishore, L., 2011. Stigmasterol: a comprehensive review. International Journal of Pharmaceutical Sciences and Research 2, 2259–2265.
Kim, J.M., Jung, H.A., Choib, J.S., Lee, N.G., 2010. Identification of anti-inflammatory target genes of Rhizoma coptidis extract in lipopolysaccharide-stimulated RAW 264.7 murine macrophage-like cells. Journal of Ethnopharmacology 130, 354–362.
Kroes, B.H., Van Den Berg, A.J., Quarles van Ufford, H.C., van Dijk, H., Labadie, R.P., 1992. Anti-inflammatory activity of gallic acid. Planta Medica 58, 499–504.
Lai, X., Ye, Y., Sun, C., Huang, X., Tang, X., Zeng, X., Yin, P., Zeng, Y., 2013. Icaritin exhibits anti-inflammatory effects in the mouse peritoneal macrophages and peritonitis model. International Immunopharmacology. 16, 41–49.
Lo, T.N., Almeida, A.P., Beaven, M.A., 1982. Dextran and carrageenan evoke different inflammatory responses in rat with respect to composition of infiltrates and effect of indomethacin. Journal of Pharmacology and Experimental Therapeutics 221, 261–267.
Loizou, S., Lekakis, I., Chrousos, G.P., Moutsatsou, P., 2010. β-Sitosterol exhibits anti-inflammatory activity in human aortic endothelial cells. Molecular Nutrition & Food Research 54, 551–558.
Lorenzi, H., 2000. 3rd ed.Árvores Brasileiras – Manual de Identificação e Cultivo de Plantas Arbóreas Nativas do Brasil, 1. Editora Plantarum. Nova Odessa, São Paulo.
Mahon, C.P.A.N.M., 2012. Avaliação da atividade anti-inflamatória de Dilodendron bipinnatum Radlk. (Dissertação de Mestrado) – Faculdade de Medicina, Universidade Federal de Mato Grosso, Cuiabá, Mato Grosso, Brasil.
Matos, F.J.A., 2009. third. ed.Introdução à Fitoquímica Experimental, 3. UFC, Fortaleza.
McDermott, M.F., 2001. TNF and TNFr biology in health and disease. Cellular Molecular Biology 47, 619–635.
Minghetti, L., Polazzi, E., Nicolini, A., Creminon, C., Levi, G., 1997. Up-regulation of cyclooxygenase-2 expression in cultured microglia by prostaglandin E2, cyclic AMP and non-steroidal anti-inflammatory drugs. European Journal Neuroscience 9, 934–940.
Moncada, S., Palmer, R.M., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacological Review 43, 109–142.
Moore, K.W., Waal Malefyt, R., Coffman, R.L., O'Garra, A., 2001. Interleukin-10 and the interleukin-10 receptor. Annual Review Immunology 19, 683–765.
Morais-Lima, G.R., Montenegro, C.A., Almeida, C.L., Athayde-Filho, P.F., Barbosa-Filho, J.M., Batista, L.M., 2011. Database survey of anti-inflammatory plants in South America: a review. International Journal Molecular Science 12, 2692–2749.
Muthuraman, A., Sood, S., Singla, S.K., 2011. The anti-inflammatory potential of phenolic compounds from Emblica officinalis L. in rat. Inflammopharmacoly 19, 327–334.

Nakayama, G.R., Caton, M.C., Nova, M.P., Parandoosh, Z., 1997. Assessment of the Alamar Blue assay for cellular growth and viability in vitro. Journal of Immunological Methods 204, 205–208.
Orlandi, L., Vilela, F.C., Santa-Cecília, F.V., Dias, D.F., Alves-da-Silva, G., Giusti-Paiva, A., 2011. Anti-inflammatory and antinociceptive effects of the stem bark of Byrsonima intermedia A. Juss. Journal of Ethnopharmacology 137, 1469–1476.
Pinho, B.R., Sousa, C., Valentão, P., Andrade, P.B., 2011. Is nitric oxide decrease observed with naphthoquinones in LPS stimulated RAW 264.7 macrophages a beneficial property? PLoS One 6, 1–8.
Qandil, A.M., 2012. Prodrugs of nonsteroidal anti-inflammatory drugs (NSAIDs), more than meets the eye: a critical review. International Journal Molecular Sciences 13, 17244–17274.
Rogero, S.O., Lugao, A.B., Ikeda, T.I., Cruz, A.S., 2003. Teste in vitro de citotoxicidade: estudo comparativo entre duas metodologias. Materials Research 6, 317–320.
Safayhi, H., Sailer, E.R., 1997. Anti-inflammatory actions of pentacyclic triterpenes. Planta Medica 63, 487–493.
Santos, J.C., Oliveira, C.A.N., Varella, L., Matos, A.P., Terezan, A.P., Leite, A.C., Fernandes, J.B., Vieira, P.C., Silva, M.F.G.F., Pirani, J.R., 2010. Constituintes químicos e atividade antimicrobiana dos extratos de *Dilodendron bipinnatum* (Sapindaceae). Química Nova 33, 2080–2082.
Silva, M.G., Oliveira, F.S., Quintans-Junior, L.J., Oliveira, T.M.L., Diniz, M.F.F.M., 2005. Investigação do efeito analgésico central e antiinflamatório de *Conocliniopsis prasiifolia* (DC) R.M. King & H. Robinson em roedores. Acta Farmacêutica Bonaerense 24, 533–537.
Singh, R., Akhtar, N., Haqqi, T.M., 2010. Green tea polyphenol epigallocatechi3-gallate: inflammation and arthritis. Life Sciences 86, 907–918.
Spedding, M., 2011. Resolution of controversies in drug/receptor interactions by protein structure. Limitations and pharmacological solutions. Neuropharmacology 60, 3–6.
Stucki, J.C., Thompson, C.R.A., 1958. Screening procedure for substances which inhibit dextran edema in the rat. American Journal Physiology 193, 275–282.
Tamashiro-Filho, P., Sikiru Olaitan, B., Tavares de Almeida, D.A., Lima, J.C., Marson-Ascêncio, P.G., Donizeti Ascêncio, S., Rios-Santos, F., Martins, D.T.O., 2012. Evaluation of antiulcer activity and mechanism of action of methanol stem bark extract of *Lafoensia pacari* A. St.-Hil. (Lytraceae) in experimental animal. Journal of Ethnopharmacology 144, 497–505.
Vattem, D.A., Shetty, K., 2005. Biological functionality of ellagic acid: a review. Journal of Food Biochemistry 29, 234–266.
Vinegar, R., Truax, J.F., Selph, J.L., Johnston, P.R., Venable, A.L., McKenzie, K.K., 1987. Pathway to carrageenan-induced inflammation in the hind limb of the rat. Federation Proceedings 46, 118–126.
Winter, C.A., Risley, E.A., Nuss, G.W., 1962. Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. Experimental Biology and Medicine 111, 544–547.
Yang, Y.I., Shin, H.C., Kim, S.H., Park, W.Y., Lee, K.T., Choi, J.H., 2012. 6,6′ Bieckol, isolated from marine alga *Ecklonia cava*, suppressed LPS-induced nitric oxide and PGE2 production and inflammatory cytokine expression in macrophages: The inhibition of NFκB. International Immunopharmacology. 12, 510–517.