Title

Anti-inflammatory and antinociceptive activities of Croton urucurana Baillon bark

Authors

Cordeiro, Kátia Wolff

Felipe, Josyelen Lousada

Malange, Kauê Franco

et al.

Publication Date

2016-05-01

DOI

10.1016/j.jep.2016.02.051

Journal of Ethnopharmacology 183 (2016) 128–135

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Anti-inflammatory and antinociceptive activities of *Croton urucurana* Baillon bark

Kátia Wolff Cordeiro [a], Josyelen Lousada Felipe [b], Kauê Franco Malange [b], Pâmela Rafaela do Prado [c], Patrícia de Oliveira Figueiredo [c], Fernanda Rodrigues Garcez [c], Karine de Cássia Freitas [b], Walmir Silva Garcez [c], Mônica Cristina Toffoli-Kadri [b,*]

[a] Faculdade de Medicina, Universidade Federal do Mato Grosso do Sul, Campo Grande, MS, Brazil
[b] Centro de Ciências Biológicas e da Saúde, Universidade Federal do Mato Grosso do Sul, Campo Grande, MS, Brazil
[c] Instituto de Química, Universidade Federal do Mato Grosso do Sul, Campo Grande, MS, Brazil

ARTICLE INFO

Article history:
Received 18 November 2015
Received in revised form
24 January 2016
Accepted 28 February 2016
Available online 2 March 2016

Keywords:
Euphorbiaceae
Medicinal plants
Sangra-d'água
Pain
Inflammation

Chemical compounds studied in this article:
(+)-gallocatechin (PubChem CID: 65084)
procyanidin B3 (PubChem CID: 146798)
(+)-Catechin (PubChem CID: 9064)
(−)-epicatechin (PubChem CID: 72276)
tembetarine (PubChem CID: 167718)
magnoflorine (PubChem CID: 73337)
taspine (PubChem CID: 215159)
Methyl-3-oxo-12-*epi*-barbascoate (8) methyl-12-*epi*-barbascoate (9)
hardwickiic acid (PubChem CID: 161454)

ABSTRACT

Ethnopharmacological relevance: *Croton urucurana* (Euphorbiaceae) is popularly used in Brazil to treat inflammatory processes, pain, and gastric ulcers.
Aim of study: To evaluate the anti-inflammatory and antinociceptive properties of the methanol extract from the bark of *C. urucurana* (MECu) in mice and identify its chemical constituents.
Materials and methods: The extract was characterized by UHPLC-DAD-ESI-Q-TOF-MS/MS. Extract doses of 25, 100, and 400 mg/kg were employed in the biological assays. Evaluation of anti-inflammatory activity was based on paw edema and leukocyte recruitment into the peritoneal cavity of mice, both induced by carrageenan. Abdominal writhing caused by acetic acid and duration of formalin-induced paw-licking were the models employed to evaluate antinociceptive activity.
Results: Ten compounds were identified in the extract: (+)-gallocatechin (**1**), procyanidin B3 (**2**), (+)-catechin (**3**), (−)-epicatechin (**4**), tembetarine (**5**), magnoflorine (**6**), taspine (**7**), methyl-3-oxo-12-*epi*-barbascoate (**8**), methyl-12-*epi*-barbascoate (**9**), and hardwickiic acid (**10**). This is the first report of compounds **2**, **4**, **6**, **7**, and **10** in *C. urucurana* and compound **5** in the genus *Croton*. In addition to inhibiting paw edema and leukocyte recruitment (particularly of polymorphonuclear cells) into the peritoneal cavity of mice, MECu reduced the number of abdominal writhings induced by acetic acid and the duration of formalin-induced paw licking.
Conclusions: The methanol extract of *C. urucurana* bark exhibited anti-inflammatory and antinociceptive properties, corroborating its use in folk medicine. These effects may be related to the presence of diterpenes, alkaloids, and flavonoids.

1. Introduction

Globally, non-steroidal anti-inflammatory drugs (NSAIDs) are currently the most frequently used compounds for medication, owing to their wide range of therapeutic indications, including pain, edema, inflammation, osteoarthritis, rheumatoid arthritis, and skeletal muscle disorders (Al-Saeed, 2011). Gastrointestinal lesions, however, are the most common, and severe, side effects of NSAIDs, implicating these drugs as the leading cause of gastroduodenal ulcers (Yuan et al., 2006).

Native to Paraguay, Uruguay, Argentina, and Brazil (Rao et al., 2007), *Croton urucurana* Baillon, popularly known as "sangra-d'água" in Portuguese, is an arboreal species popularly employed for its putative anti-inflammatory, analgesic (Peres et al., 1998a) and wound-healing (Peres et al., 1998b), properties to treat gastritis, ulcers, and back pain (Alves et al., 2008).

The anti-ulcer activity of the methanol extract of *C. urucurana* bark has been previously demonstrated *in vivo* (Wolff Cordeiro et al., 2012). Antimicrobial (Peres et al., 1997), antidiarrheal (Gurgel et al., 2001), and wound-healing properties (Esmeraldino et al., 2005) have also been observed for this species. Nonetheless, the effectiveness of the popular use of *C. urucurana* bark as an anti-inflammatory and analgesic has not been corroborated by published investigations.

This study evaluated the anti-inflammatory and antinociceptive activities of the methanol extract of *C. urucurana* bark in mice. Although the antinociceptive activity of *C. urucurana* latex had

* Correspondence to: Centro de Ciências Biológicas e da Saúde, Universidade Federal do Mato Grosso do Sul, Cidade Universitária, Caixa postal 549, Campo Grande, MS 79070-900, Brazil.
E-mail address: monica.kadri@ufms.br (M.C. Toffoli-Kadri).

http://dx.doi.org/10.1016/j.jep.2016.02.051
0378-8741/

preliminarily been investigated by Rao et al. (2007), studies conducted by our group revealed differences in latex chemical composition, compared with the bark methanol extract. Although Peres et al. (1998a) evaluated the antinociceptive activity of *C. urucurana* bark, we consider their data inconsistent, since the doses employed in their assay were not fully reported and a detailed description of the procedure used in the antinociceptive test was not provided. The present paper therefore reports a novel investigation of the activities referred above, employing UHPLC-DAD-ESI-Q-TOF-MS/MS to establish the extract's phytochemical profile.

2. Materials and methods

2.1. Plant material and extract preparation

Bark material from *C. urucurana* was collected in Dourados county, Mato Grosso do Sul state, Brazil (22°20′013″S; 54°84′014″W; 388 m mean altitude) in January 2013. Species identification was performed by Prof. Zefa Valdivina Pereira, of the Universidade Federal da Grande Dourados (voucher specimen 4869, deposited at the DDMS Herbarium, in Dourados).

The material was dried in a circulating air oven at 40 °C, powdered in a four-blade mill, extracted with methanol (99.5%, Synth, São Paulo, Brazil) by maceration at a ratio of 1 kg of bark powder to 4 L of solvent for seven days, and filtered. The residue was re-extracted with methanol three more times following the same procedure. The resultant methanol extract of *C. urucurana* bark (MECu) was concentrated under reduced pressure at 37 °C and lyophilized, with a 15.4% yield (686.0 g). Before administration to the animals, the extract was solubilized in distilled water, which also served as a vehicle (negative control) in all treatments.

2.2. Extract preparation for phytochemical screening

Type I water (Milli-Q Synthesis, Millipore, Bedford, MA, USA), acetonitrile (ACN), and HPLC-grade methanol (Tedia, Rio de Janeiro, Brazil) were employed to determine the chemical profile of MECu by UHPLC-DAD-ESI-Q-TOF-MS/MS. Authentic samples (standards) of (+)-gallocatechin, (+)-catechin, (−)-epicatechin, procyanidin B3, methyl-3-oxo-12-*epi*-barbascoate, methyl-12-*epi*-barbascoate, and (-)-hardwickiic acid, obtained from the Laboratory of Research of Natural Bioactive Products (PRONABIO) of the Universidade Federal de Mato Grosso do Sul were used to interpret the chromatograms and the high-resolution mass spectra of secondary metabolites present in the extract.

Five milligrams of MECu were solubilized in 2.5 mL of MeOH-H_2O (85:15 v:v), placed in an extraction cartridge for solid-phase extraction (SPE; Waters Sep-Pak Classic, C18), and eluted with further 2.5 mL of MeOH-H_2O (85:15 v:v) to remove lipophilic components. To this end, the SPE cartridge was preconditioned by elution of 5 mL of MeOH, followed by 5 mL of MeOH-H_2O (85:15 v:v). The eluted materials were dissolved at a concentration of 1 mg/mL in MeOH-H_2O (85:15 v:v) and filtered through a 0.22 μm PVDF membrane (Allcrom, São Paulo, Brazil). For later analysis by UHPLC-DAD-ESI-Q-TOF-MS/MS, the standards were solubilized in MeOH-H_2O (85:15 v:v) at a concentration of 300 μg/mL and filtered through 0.22 μm PVDF membranes (Allcrom, São Paulo, Brazil).

2.3. UHPLC-DAD-ESI-Q-TOF-MS/MS analysis

Phytochemical screening involved reversed-phase column separation using an Ultra Fast Liquid Chromatograph (UFLC) system, a Prominence SIL-20A autosampler, and LC-20AT pumps, coupled to an M20A diode array detector (DAD) (all Shimadzu, Kyoto, Japan) and a micrOTOF Q-II high-resolution time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Germany) with an electrospray ionization (ESI) source. The samples were detected online using the DAD and then directed to the mass spectrometer. The micrOTOF II-Q device was operated in positive and negative modes. Positive mode parameters: spray voltage, 4.5 kV; nebulizer pressure (N_2), 4 bar; drying gas (N_2) 9.0 L/min; drying gas temperature, 200 °C; collision cell energy, 10 eV; collision gas, N_2, 45%. Negative mode parameters: spray voltage, 3.5 kV; nebulizer pressure, 4 bar (N_2); drying gas (N_2) 9.0 L/min; drying gas temperature, 200 °C; collision cell energy, −10 eV; collision gas, N_2, 45%. The mass-to-charge ratio was calibrated using TFA adducts, both in positive and negative modes. Mass spectra were acquired in the 120–1300 range of mass-to-charge ratios (*m/z*).

For UHPLC-DAD-ESI-Q-TOF-MS/MS analysis, 5 μL aliquots of MECu and standards were separately injected into an Kinetex RP-18 column (2.6 μm, 150 × 2.1 mm, Phenomenex, USA) coupled to a sub-2 SecurityGuard Ultra cartridge for C18 UHPLC and core-shell column with an internal diameter of 2.1 mm (Phenomenex, USA). Column temperature was maintained at 50 °C and the samples were eluted at a flow of 0.3 mL/min. The mobile phase was prepared using two solvents: 1% (v:v) acetic acid in Milli-Q water (solvent A) and 1% (v:v) acetic acid in acetonitrile (solvent B). Separation of analytes entailed isocratic elution in 3% B (0–2 min), followed by a 3–25% linear gradient in B (2–25 min) and a 25–80% linear gradient in B (25–40 min). Solvent composition remained unaltered until elution was complete (43 min). To condition the column for a new injection, the solvent was linearly modified to 3% B until 44 min and maintained as such until 48 min.

2.4. Animals

Eight weeks old male albino Swiss mice (*Mus musculus*), weighing 25–30 g, supplied by the UFMS Central Animal Facility, were kept in an alternating 12 h light/dark cycle in a temperature-controlled room (22 °C ± 2 °C) and given standard chow and water *ad libitum*. Food and water were withdrawn 6 h and 1 h before the experiments, respectively. During fasting, the animals were kept in cages equipped with raised floors to prevent coprophagy. A CO_2 chamber was used for euthanasia. The experiments, approved by the UFMS Animal Ethics Committee (protocol number 563/2013), complied with National Institutes of Health regulations on the use and care of animals for scientific purposes.

2.5. Carrageenan-induced paw edema

The method employed to induce paw edema by carrageenan was adapted from Winter et al. (1962). After fasting, the animals were assigned to one of five groups ($n=7$) and pretreated orally (gavage) with water (10 mL/kg), indomethacin (15 mg/kg; Sigma Aldrich, St. Louis, USA), or MECu (25, 100, or 400 mg/kg). Sixty minutes later, edema was induced by an intraplantar injection of 40 μL of carrageenan (1%, w:v; Sigma Aldrich, St. Louis, USA) in the right hind paw. As a control, 40 μL of 0.9% saline solution (vehicle) was injected into the contralateral paw. Edema, measured with a digital plethysmometer (Insight®) 30, 60, 120, and 240 min after injection, was defined as the difference between the volumes (in millimeters) of treated and control paws.

2.6. Leukocyte recruitment into the peritoneal cavity

The method employed to induce leukocyte recruitment into the peritoneal cavity was adapted from Souza and Ferreira (1985). The animals were distributed into five groups ($n=8$) and pretreated orally (gavage) with water (10 mL/kg), indomethacin (15 mg/kg),

or MECu (25, 100, or 400 mg/kg). After 60 min, the animals were intraperitoneally injected with 0.5 mL of 1% carrageenan suspended in saline solution and sham group (n=4, injected with 0.5 mL saline/cavity). The animals were euthanized in a CO_2 chamber 4 h later. For cell collection, a 3 mL volume of phosphate-buffered saline (PBS) solution containing 10 U/mL of heparin (Basel, Switzerland) and 0.03% albumin was injected into the peritoneal cavity, followed by massaging and, finally, collection of cell suspension with a disposable syringe. Total leukocyte count was performed in a Neubauer chamber after diluting the exudate to 1:20 (v:v) with Türk's solution (0.2% crystal violet in 30% acetic acid). For differential analysis and determination of cell number, the exudate was centrifuged at 1000 rpm for 5 min, the sediment was resuspended in 200 μL of 3% bovine serum albumin, smeared onto slides, and subsequently stained with Hema3 (Instant-Prov, NewProv). The cells were categorized as polymorphonuclear and mononuclear, based on conventional morphological criteria. The results were expressed as cells per cubic millimeter.

2.7. Acetic acid-induced writhing response

The method for inducing abdominal writhing with acetic acid was adapted from Koster et al. (1959). After fasting, the animals were assigned to one of five groups (n=7) and pretreated orally (gavage) with water (10 mL/kg), indomethacin (15 mg/kg), or MECu (25, 100, or 400 mg/kg). Sixty minutes later, the animals were injected intraperitoneally with 10 mL/kg of 0.6% acetic acid diluted in distilled water. The results were expressed as number of abdominal writhings within 30 min of injection.

2.8. Formalin test

The method employed in this test was adapted from Hunskaar et al. (1985). After fasting, the animals were assigned to one of six groups (n=8) and pretreated orally (gavage) with vehicle (10 mL/kg), indomethacin (15 mg/kg), morphine (5 mg/kg), or MECu (25, 100, or 400 mg/kg). Sixty minutes later, the animals were given a 40 μL intraplantar injection of 1.2% formalin diluted in saline solution into the right hind paw. Duration of paw licking was evaluated during two intervals: from 0 to 5 min (first phase) and 15 to 30 min (second phase). The results were expressed in seconds.

2.9. Statistical analysis

Data were expressed as means ± standard error of the mean (SEM). Statistical differences were determined by analysis of variance followed by Tukey's post-test (for one-way ANOVA) or Bonferroni's post-test (for two-way ANOVA) using GraphPad Prism 5 software (GraphPad, USA). Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Phytochemical screening

From the chromatogram obtained by UHPLC-DAD-ESI-Q-TOF-MS/MS (in positive and negative modes) (Fig. 1), ten compounds were identified (Fig. 2) based on retention times, high-resolution mass spectrum data (MS and MS/MS), UV data, and comparisons with authentic samples and published data. These ten compounds were categorized into three groups, according to metabolite class.

The first group (compounds **1**–**4**) comprised flavonoids, that exhibited UV bands with absorption maxima in the 270–280 nm range and were identified by analyzing their deprotonated $[M–H]^-$ peaks and MS and MS/MS fragments (Table 1). The mass spectra of compounds **3** and **4** showed peaks at *m/z* 289.0711 $[M–H]^-$ and 289.0704 $[M–H]^-$, consistent with the molecular formula $C_{15}H_{13}O_6$ (Δ 2.4 and 4.7 ppm, respectively), identifying these substances as catechin (**3**, *Rt*=7.2 min) and epicatechin (**4**, *Rt*=11.1 min), while the $[M–H]^-$ ion of **1**, at *m/z* 305.0630, was consistent with the molecular formula $C_{15}H_{13}O_7$ (Δ 1.9 ppm), identifying the compound as (+)-gallocatechin (**1**, *Rt*=3.2 min). Substance **2** (*m/z* 577.1331, $[M–H]^-$), with a molecular formula defined as $C_{30}H_{25}O_{12}$

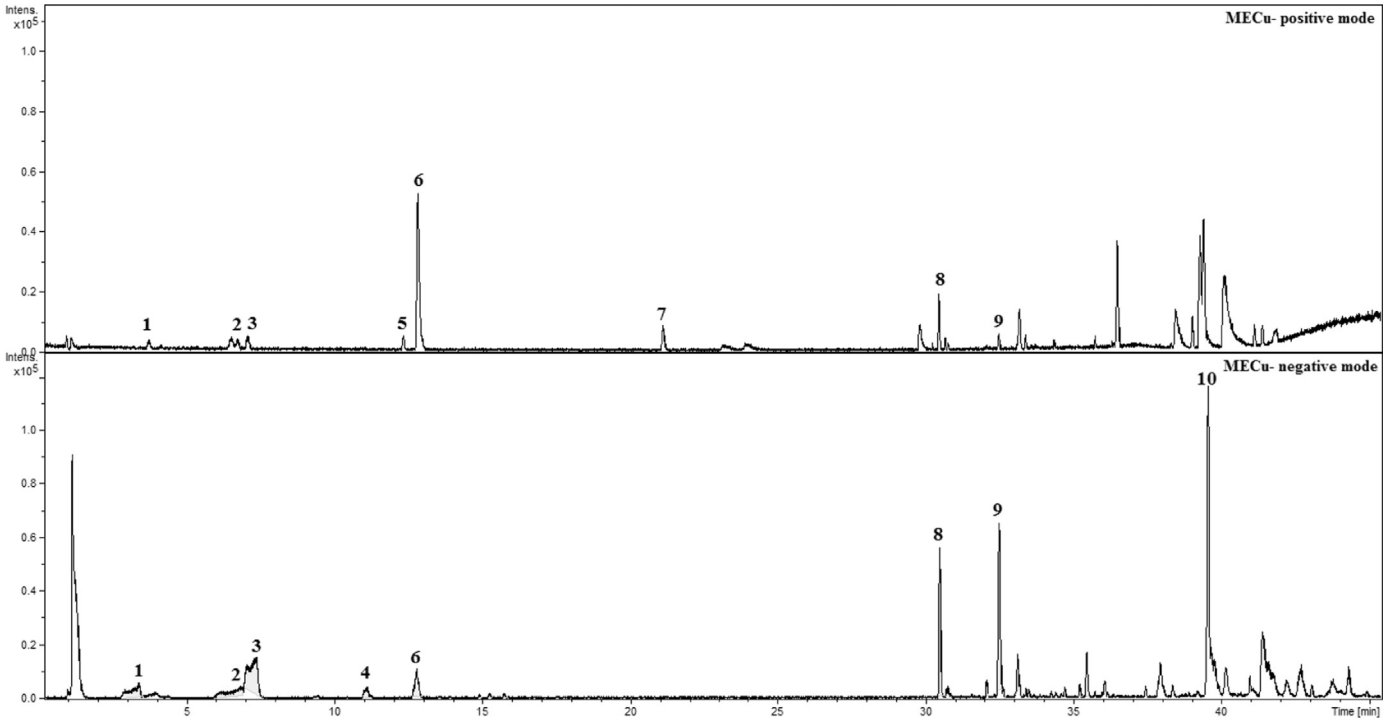

Fig. 1. Analytical UFLC-DAD-ESI-TOF-MS chromatogram of the methanol extract of *Croton urucurana* bark. Injections were performed in positive (A) and negative (B) modes.

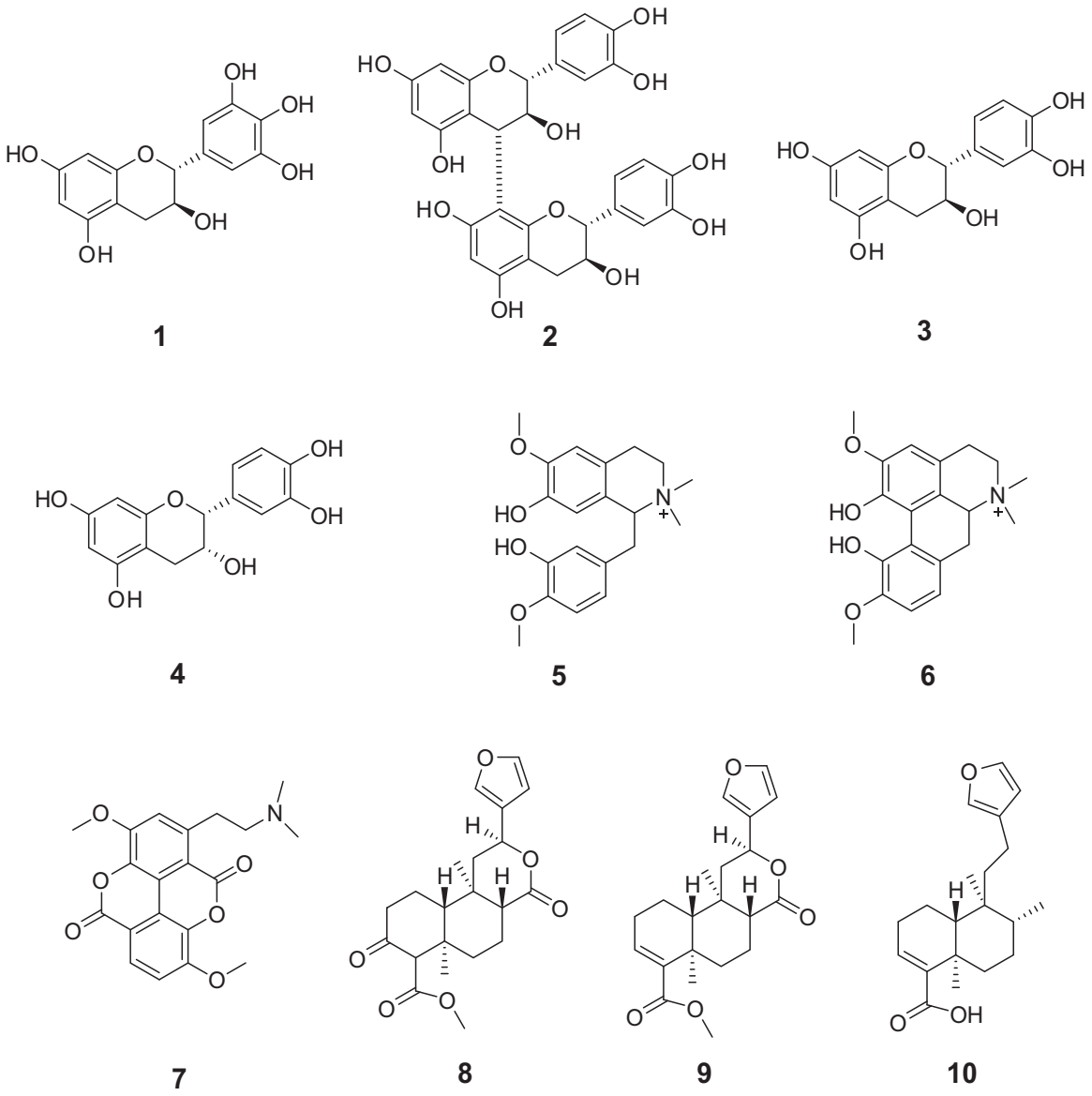

Fig. 2. Compounds identified in the methanol extract of *Croton urucurana* bark, as determined from UV and HR-ES-MS/MS data.

(Δ 3.6 ppm), was identified as procyanidin B3 [(+)-catechin-(4–8)-(+)-catechin]. Major fragments (*m/z*) observed in the ESI-MS and ESI-MS/MS spectra of **1**–**4** were consistent with fragmentation patterns reported for these compounds (Callemien and Collin, 2008; Jaiswal et al., 2012) and with high-resolution ESI-MS and ESI-MS/MS spectra of the authentic samples.

The second group (**5**–**7**) exhibited bands with absorption maxima in the 275–278 nm range in the UV spectra. The ions observed in the HR-ESI-MS spectra of **5**–**7** at *m/z* 344.1854 $[M]^+$, *m/z* 342.1695 $[M]^+$, and *m/z* 370.1279 $[M+H]^+$, respectively, were consistent with the molecular formulas $C_{20}H_{26}NO_4$ (**5**, Δ 0.6 ppm), $C_{20}H_{24}NO_4$ (**6**, Δ 1.4 ppm), and $C_{20}H_{20}NO_6$ (**7**, Δ 1.7 ppm), suggesting these compounds were alkaloids (Table 1). Based on UV and HR-ESI-MS data and their respective fragments observed in the HR-ESI-MS/MS experiments, supplemented by comparisons against published data for alkaloids with the same molecular formulas established for **5**–**7**, these compounds were putatively identified as the aporphinic alkaloids magnoflorine (**6**) (Yan et al., 2013) and taspine (**7**) (Pieters et al., 1993) and the alkaloid tembetarine (**5**) (Pieters et al., 1993).

In the third group, the UV spectra of compound **8** (*m/z* 373.2386 $[M-H]^-$) exhibited a band with an absorbance maximum at 275 nm, while compounds **9** (*m/z* 359.1863 $[M+H]^+$) and **10** (*m/z* 315.1977 $[M-H]^-$) showed no absorbance bands in the range investigated (240–800 nm). The *m/z* values for these ions were consistent with the molecular formulas $C_{21}H_{25}O_6$ (**8**, Δ 3.9 ppm), $C_{21}H_{27}O_5$ (**9**, Δ 2.8 ppm), and $C_{20}H_{27}O_3$ (**10**, Δ 3.5 ppm). Based on the molecular formulas, together with analysis of HR-ESI-MS and HR-ESI-MS/MS spectra of authentic samples, the following skeletons were proposed: furane clerodane diterpene methyl-3-oxo-12-*epi*-barbascoate (**8**), methyl-12-*epi*-barbascoate (**9**), and hardwickiic acid (**10**).

3.2. Effect of extract on carrageenan-induced paw edema

Treatment with MECu inhibited paw edema (Table 2). Inflammatory response peaked 30 min after stimulus. Indomethacin inhibited paw edema in all the periods investigated. Administered at 25 and 400 mg/kg, the extract significantly inhibited paw edema at the peak of inflammatory response, while at 25 and 100 mg/kg this effect was sustained beyond peak response.

Table 1
Chromatographic, UV, MS, and MS/MS data of compounds identified in the methanol extract of *Croton urucurana* bark.

No.	Rt (min)	Compound	Product ions	UV (nm)
1	3.2	(+)-gallocatechin	MS: 305.0630 $[M-H]^-$, 261.0756, 221.0378, 179.0332. MS/MS (305.0630, 30.1 eV): 167.0328, 165.0171, 164.0086	271
2	6.7	procyanidin B3	MS: 577.1331 $[M-H]^-$, 461.1583, 291.9886, 217.0004. MS/MS (577.1331, 45.8 eV): 407.0736, 339.0825, 289.0700, 245.0719, 203.0596, 179.0389, 161.0194.	278
3	7.2	(+)-catechin	MS: 289.0711 $[M-H]^-$, 245.0799, 221.0779, 217.0019, 179.0323. MS/MS (289.0711, 29.7 eV): 221.0757, 203.0669, 188.0442, 161.0536	278
4	11.1	(−)-epicatechin	MS: 289.0704 $[M-H]^-$, 245.0815, 201.0345, 179.0351. MS/MS (289.0704, 29.7 eV): 221.0671	278
5	12.3	tembetarine	MS: 344.1854 $[M]^+$, 289.0662, 269.0676, 233.9823, 205.0291, 191.9723, 173.9627, 163.0181, 155.9728, 149.0168, 145.0074. MS/MS (344.1854, 35.9 eV): 175.0746.	278
6	12.8	magnoflorine	MS: 342.1695 $[M]^+$, 327.2036, 297.1035, 285.2757, 233.9818, 219.0230, 214.9906, 205.0286, 191.9689, 173.9594, 158.9955. MS/MS (342.1695, 35.8 eV) 297.1123, 282.0881, 265.0861, 250.0594, 237.0913, 222.0684, 191.0834	278
7	21.1	taspine	MS: 370.1279 $[M+H]^+$, 285.2741, 214.9826, 191.9721, 173.9600, 158.9954, 145.0024. MS/MS (370.1299, 36.6 eV) 325.0723, 310.0508	275
8	32.5	methyl-3-oxo-12-*epi*-barbascoate	MS: 409.1434 $[M+Cl]^-$, 373.1671 $[M-H]^-$, 377.2386, 305.0243, 232.9773, 217.0024, 201.0399. MS/MS (.1671, 31.8 eV): 341.1427, 297.1563, 273.1205	275
9	36.5	methyl-12-*epi*-barbascoate	MS: 717.3637 $[2M+H]^+$, 359.1863 $[M+H]^+$, 265.1452, 233.1181. MS/MS (359.1863, 36.3 eV): 233.1184, 219.1403, 187.1119, 159.1171	–
10	39.5	hardwickiic acid	MS: 315.1977 $[M-H]^-$, 311.1696. MS/MS (315.1977, 35.8 eV): 185.0097.	–

3.3. Effect of extract on carrageenan-induced leukocyte recruitment into the peritoneal cavity

Fig. 3 depicts the effect of MECu on carrageenan-induced recruitment of total leukocytes (*A*), mononuclear cells (*B*), and polymorphonuclear cells (*C*) into the peritoneal cavity.

Indomethacin (the positive control) reduced the concentration of total leukocytes by 54.0% (2044.0 ± 273.0 cells/mm^3). At 25 mg/kg, MECu inhibited leukocyte recruitment by 41.9% (2581.0 ± 387.4 cells/mm^3), and by 49.2% (2256.0 ± 272.8 cells/mm^3) at 100 mg/kg. At 400 mg/kg, however, the effect exhibited (5475.0 ± 460.9 cells/mm^3) did not differ significantly from that elicited by the vehicle (4444.0 ± 169.4 cells/mm^3) (Fig. 3 A).

Neither indomethacin nor MECu significantly inhibited recruitment of mononuclear cells (Fig. 3B). For polymorphonuclear cells (Fig. 3 C), indomethacin inhibited recruitment by 76.2% (485.7 ± 106.3 cells/mm^3), while treatment with MECu at 25 and 100 mg/kg inhibited by 62.6% (764.1 ± 139.7 cells/mm^3) and 58.7% (844.6 ± 116.3 cells/mm^3), respectively. These values differed significantly from those obtained for vehicle (2043.0 ± 140.4 cells/mm^3) and MECu at 400 mg/kg (2043.0 ± 194.8 cells/mm^3).

3.4. Effect of extract on acetic acid-induced writhing response

The number of abdominal writhings induced by acetic acid was 60.3 ± 5.9 in the first 30 min (Fig. 4). Pretreatment with indomethacin decreased this effect by 60.2% (24.0 ± 5.1). Administered at 400 mg/kg, the extract reduced writhing by 42.2% (34.8 ± 3.5), while doses of 25 and 100 mg/kg failed to significantly decrease writhing frequency.

3.5. Formalin test

In the first phase of the formalin test (neurogenic nociception), MECu reduced paw licking duration when administered at 100 mg/kg (29.6%; 69.7 ± 3.3 s) and 400 mg/kg (54.6%; 45.0 ± 5.6 s). With morphine (the positive control), licking response was reduced by 57.8% (41.9 ± 5.4 s), while the effects of extract at 25 mg/kg (93.5 ± 5.9 s) and indomethacin (79.6 ± 4.6 s) did not differ significantly from that obtained with vehicle (99.1 ± 7.7 s) (Fig. 5 A).

In the second phase (inflammatory nociception), only MECu at 400 mg/kg (64.9 ± 5.5; 63.8%) and indomethacin (54.2 ± 6.5; 69.8%) significantly reduced paw licking duration. Morphine (145.2 ± 15.7 s) and extract at 25 mg/kg (165.6 ± 35.4 s) and 100 mg/kg (128.2 ± 14.8 s) failed to change licking response (Fig. 5B).

4. Discussion

In the present investigation, the methanol extract of *C. urucurana* bark exhibited anti-inflammatory and antinociceptive properties in the models evaluated, corroborating ethnomedicinal data available for this species (Alves et al., 2008; Peres et al., 1998a, 1998b).

The phytochemical profile of MECu revealed the presence of flavonoids, alkaloids and terpenes, of which gallocatechin (**1**) and catechin (**3**) had been previously identified in *C. urucurana* extracts (Peres et al., 1998a, 1998b). The alkaloids identified were magnoflorine (**6**), previously described for other *Croton* species, such as *C. xalapensis* (Arevalo et al., 2009) and *C. cumingi* (Tomita et al., 1965); taspine (**7**), previously described for *C. lechleri* (Vaisberg et al., 1989), *C. draco*, *C. palanostigma* (Salatino et al., 2007), and *C. heliotropiifolius* (Queiroz et al., 2014); and tembetarine (**5**), a benzyl tetrahydroisoquinolinic alkaloid, that, although unreported in the genus *Croton*, is probably the precursor of alkaloids **6**

Table 2
Effects of the methanol extract of *Croton urucurana* bark (MECu) on carrageenan-induced mouse paw edema.

Group	Dose (mg/kg)	30 min		60 min		120 min		240 min	
		Paw edema (mL)	% Inhibition	Paw edema (mL)	% Inhibition	Paw edema (mL)	% Inhibition	Paw edema (mL)	% Inhibition
Water (vehicle)	–	0.043 ± 0.004^{a}	–	0.033 ± 0.003^{a}	–	0.020 ± 0.002^{a}	–	0.021 ± 0.004^{a}	–
Indomethacin	15	0.023 ± 0.002^{c}	46.7	0.016 ± 0.002^{bc}	52.2	0.010 ± 0.003^{a}	50.0	0.009 ± 0.003^{bc}	60.0
MECu	25	0.024 ± 0.005^{bc}	43.3	0.019 ± 0.003^{b}	43.5	0.013 ± 0.005^{a}	35.7	0.010 ± 0.004^{b}	53.3
	100	0.034 ± 0.002^{ab}	20.0	0.021 ± 0.001^{b}	34.8	0.011 ± 0.003^{a}	42.9	0.009 ± 0.003^{b}	60.0
	400	0.016 ± 0.005^{c}	63.3	0.023 ± 0.002^{ab}	30.4	0.017 ± 0.003^{a}	14.3	0.013 ± 0.003^{ab}	40.0

Results expressed as means ± SEM ($n=7$). Data were subjected to two-way ANOVA followed by Bonferroni's post-test. Different letters on the same column indicate significant difference ($p < 0.05$).

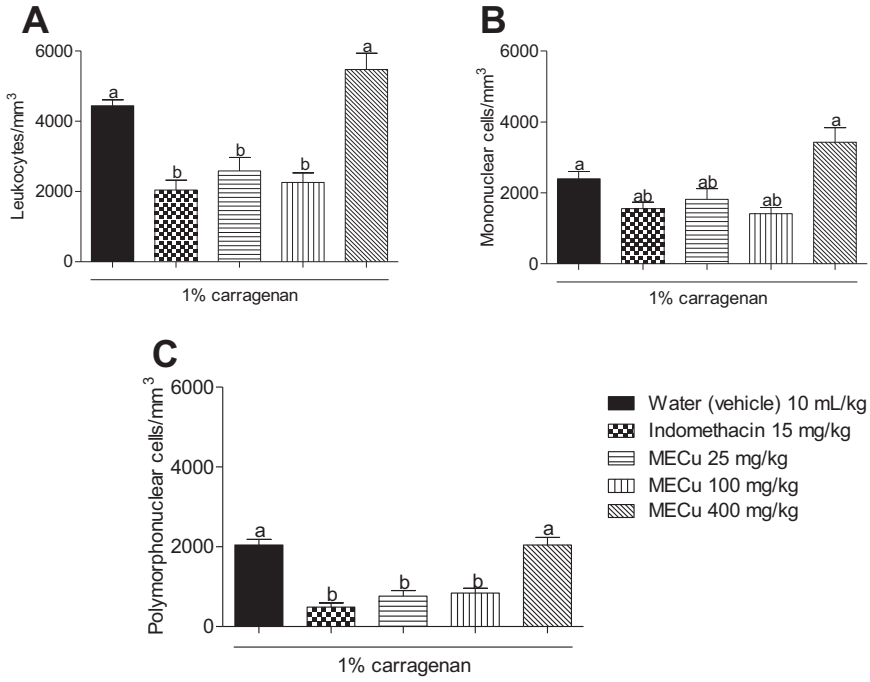

Fig. 3. Effect of methanol extract of *Croton urucurana* bark (MECu) on total leukocytic (A), mononuclear (B), and polymorphonuclear (C) cell recruitment into the peritoneal cavity of mice. Animals were pre-treated 1 h before injection of carrageenan (1%, 0.5 mL, i.p.). Four hours later, exudates were collected for cell counting. Columns represent means ± SEM ($n=4$–8) of cell numbers. Data were subjected to one-way ANOVA followed by Tukey's post-test. Different letters indicate significant differences ($p < 0.01$).

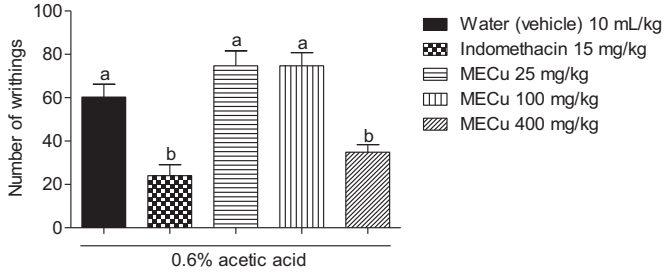

Fig. 4. Effect of methanol extract of *Croton urucurana* bark (MECu) on acetic-acid–induced writhing in mice. One hour after pre-treatment, acetic acid (0.6%, 10 mL/kg, i.p.) was injected and the writhings induced were counted for the first 30 min. Columns represent means ± SEM ($n=7$) of number of writhings. Data were subjected to one-way ANOVA followed by Tukey's post-test. Different letters indicate significant differences ($p < 0.05$).

(aporphinic) and **7** (*seco*-aporphinic).

Identification of the diterpenes methyl-3-oxo-12-*epi*-barbascoate (**8**), methyl-12-*epi*-barbascoate (**9**), and hardwickiic acid (**10**) drew on chromatographic comparisons, as well as on HR-ESI-MS and HR-ESI- MS/MS spectra of authentic samples.

This is the first report of procyanidin B3 (**2**), epicatechin (**4**), tembetarine (**5**), magnoflorine (**6**), taspine (**7**), and hardwickiic acid (**10**) for *C. urucurana* and the first for the alkaloid tembetarine (**5**) in the genus *Croton*.

Induction of paw edema by carrageenan is a standard model for screening novel anti-inflammatory agents (Di Rosa et al., 1971). In mice, the phenomenon is biphasic and, immediately after intraplantar injection of carrageenan, several mediators act sequentially to elicit an inflammatory response (Posadas et al., 2004).

Lasting 30–60 min (Hunskaar and Hole, 1987), the initial phase of edema has been attributed to release of histamine, serotonin, and bradykinin, which promote vascular permeability (Di Rosa et al., 1971). The subsequent phase, in which the edema grows in volume, is associated with increased levels of prostaglandins (Hunskaar and Hole, 1987) and cyclooxygenase (COX2) (Posadas et al. 2004), the latter of which can be blocked by NSAIDs (Hunskaar and Hole, 1987).

Pretreatment with MECu reduced the edema response to carrageenan at the lowest dose, extract effect was similar to that of indomethacin. The extract may have reduced the release of histamine, serotonin, and bradykinin, as well as prostaglandins, probably by inhibiting COX activity.

As non-selective COX inhibitors, NSAIDs inhibit the generation of prostaglandins, and hence the manifestation of gastroprotective factors such as mucus formation, bicarbonate secretion, proliferation

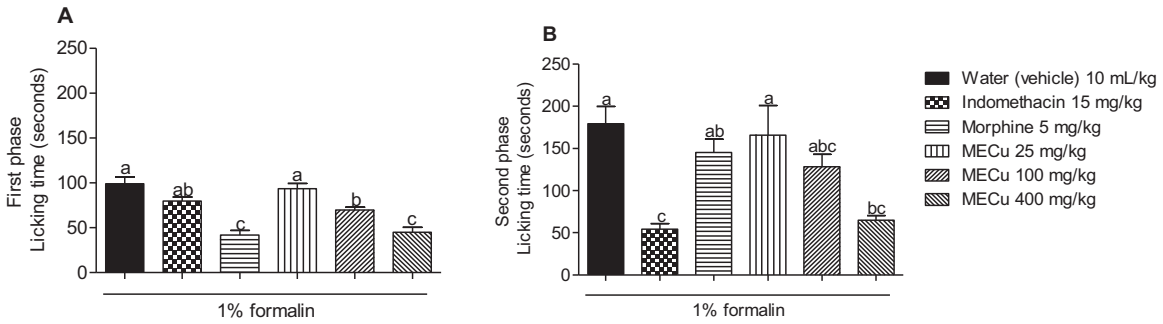

Fig. 5. Effect of methanol extract of *Croton urucurana* bark (MECu) on duration of formalin-induced paw-licking (seconds) in the first 5 min (first phase, *A*) and from 15–30 min (second phase, *B*) of nociception in mice. One hour after treatment, 40 μL of formalin (1.2%) was injected intraplantarly in the right hind paw. Columns represent means ± SEM ($n=8$) of licking time. Data were subjected to one-way ANOVA followed by Tukey's post-test. Different letters indicate significant differences ($p < 0.05$).

of gastric epithelial cells, while also inhibiting increased blood flow. By inhibiting COX-1, NSAIDs promote the appearance of gastrointestinal ulcers (Takeuchi, 2012). Drawing on Wolff Cordeiro et al. (2012), who showed that the methanol extract of *Croton urucurana* bark, tested at different doses, inhibited indomethacin-induced gastric lesions in rats, it can be inferred that the anti-inflammatory activity of MECu occurs via selective COX-2 blockade.

Also, the extract reduced leukocyte recruitment into the peritoneal cavity (except when administered at 400 mg/kg) – an effect possibly explained by the presence of phenolic antioxidants such as gallocatechin (**1**), proanthocyanin B3 (**2**), catechin (**3**), and epicatechin (**4**) in MECu, as well as gallic acid, found by Peres et al. (1997). Despite their antioxidant properties, these compounds can act as pro-oxidants at higher doses (Sandoval-Acuña et al., 2014).

Evidence suggests that oxidative stress caused by generation of reactive oxygen species (ROS) in endothelial cells promotes leukocyte recruitment during inflammation. Oxidative stress enhances the expression of endothelial cell adhesion molecules (CAMs) by activating these on the endothelial surface or via a transcription-dependent mechanism involving redox-sensitive factors such as nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) transcription factors (Mittal et al., 2014).

At the highest dose, MECu may therefore have promoted ROS generation, exacerbating the inflammatory process, whereas lower doses inhibited this effect.

Phenolic compounds such as **1**, **2**, **3**, and **4** are known to block the NF-κB and AP-1 activation pathways, inhibiting the expression of ICAM-1 (an important adhesion molecule for leukocyte migration) by the vascular endothelium (Mittal et al., 2014). Moreover, terpenes **8**, **9**, and **10** are known to inhibit pro-inflammatory transcription factors (de las Heras and Hortelano, 2009).

Lipid mediators such as leukotrienes, thromboxanes, platelet-activating factor (PAF), cytokines, and chemokines (Bishayee and Khuda-Bukhsh, 2013; Muller, 2002) are also involved in cell recruitment. The reduction in polymorphonuclear cell recruitment promoted by MECu may therefore have occurred via blockage of enzymes involved in the production of mediators such as phospholipases, COX, and LOX or in the synthesis and release of cytokines, or even in receptors involved in cell recruitment.

The reduction of polymorphonuclear cell migration promoted by MECu may also be related to the presence of sulfhydryl compounds, reported by Wolff Cordeiro et al. (2012) as involved in the antiulcerogenic activity of a *C. urucurana* bark methanol extract. Noteworthy among sulfhydryl compounds is γ-glutamylcysteinylglycine glutathione peroxidase (a precursor enzyme responsible for protection against oxidative damage), which at low cellular levels promotes activation of NF-κB (Staal et al., 1990), and consequently leukocyte recruitment (Mittal et al., 2014).

In the present study, MECu also reduced writhing induced by acetic acid. Administered to the peritoneal cavity, acetic acid causes abdominal writhing by direct activation of primary afferent nociceptors or, indirectly, by stimulating the release of endogenous mediators such as histamine, serotonin, bradykinin, prostaglandins, and cytokines (IL-1β, IL-6, IL-8, TNF) that enhance hyperalgesia (Dray, 1995). This activation and peripheral sensitization of nociceptors triggers writhing, causing the animal to contract and extend its hind limbs (Le Bars et al., 2001). This suggests that the antinociceptive activity of MECu may involve reduction of the nociceptive effect caused by cytokines such as TNF-α, IL-1β, and IL-8 (Dray, 1995) and/or prostaglandins PGE_2, $PGF_{2\alpha}$, and PGI_2 (Deraedt et al., 1980).

MECu administration reduced the duration of formalin-induced paw-licking in both nociception phases. In the first phase, which involves the opioid receptor pathway (Rao et al., 2007), this effect was confirmed by comparison with morphine. This suggests that the effect of the extract may be related to the presence of alkaloids **5**, **6**, and **7** (among other possible metabolites), whose activity may be similar to that of morphine. Accordingly, the activity of aporphinic alkaloids such as tembetarine (**5**) and magnoflorine (**6**) can occur via adrenergic and serotonergic transmission (Stévigny et al., 2005) involved in the propagation of nociceptive information (Amaya-Castellanos et al., 2011; Stone et al., 1998).

The second, inflammatory phase of nociception, involves the release of mediators such as histamine, serotonin, bradykinin, and prostaglandin (Hunskaar and Hole, 1987), possibly explaining the activity displayed by MECu. Terpenoids are known to exhibit antinociceptive activity in both phases, albeit more pronounced in the second phase (de Moraes et al., 2012; Santos et al., 2005). The activity of the extract may therefore stem from diterpenes **8**, **9**, and **10**.

In addition, plant-derived terpenes have been shown to participate in a number of biological activities, including anti-inflammatory (Salae et al., 2012) and antinociceptive processes (Calixto et al., 2000).

Considering that gastric and duodenal ulcers are the predominant side effects of commercially available NSAIDs (Yuan et al., 2006), the phytochemical diversity exhibited by *C. urucurana* stimulates the search for novel anti-inflammatory and analgesic compounds that prove less toxic to the gastrointestinal tract, or even gastroprotective. Adding to the anti-inflammatory and antinociceptive activities demonstrated in the present investigation, *C. urucurana* has proved antiulcerogenic in rats, with no acute or subacute toxicity when administered orally (Wolff Cordeiro et al., 2012).

5. Conclusions

The methanol extract of *C. urucurana* bark exhibited anti-inflammatory and antinociceptive properties. Its antinociceptive

action involves both central and peripheral pathways. These effects may be related to the presence of diterpenes, alkaloids, and/or flavonoids in the methanol extract obtained from its bark. These findings, in addition to the antiulcerogenic properties already reported for the extract of *C. urucurana* bark corroborate the popular use of the species and highlight its therapeutic, yet not fully explored, potential.

Acknowledgments

The authors thank the Laboratory of Natural Products and Mass Spectrometry (LaPNEM) of the Universidade Federal de Mato Grosso do Sul for the UFLC-DAD-ESI-TOF-MS/MS data. Thanks are also given to CAPES, CNPq, and FUNDECT-MS for the grants awarded.

References

Al-Saeed, A., 2011. Gastrointestinal and cardiovascular risk of nonsteroidal anti-inflammatory drugs. Oman Med. J. 26, 385–391.
Alves, E.O., Mota, J.H., Soares, T.S., Vieira, Md.C., 2008. Crescimento e distribuição espacial de *Croton urucurana Baill.* em Dourados-MS. Rev. Caatinga, 83–88.
Amaya-Castellanos, E., Pineda-Farias, J.B., Castañeda-Corral, G., Vidal-Cantú, G.C., Murbartián, J., Rocha-González, H.I., Granados-Soto, V., 2011. Blockade of 5-HT7 receptors reduces tactile allodynia in the rat. Pharmacol. Biochem. Behav. 99, 591–597.
Arevalo, C., Lotti, C., Piccinelli, A.L., Russo, M., Ruiz, I., Rastrelli, L., 2009. Magnoflorine and phenolic derivatives from the leaves of *croton xalapensis* L.(Euphorbiaceae). Nat. Prod. Commun. 4, 1697–1700.
Bishayee, K., Khuda-Bukhsh, A.R., 2013. 5-lipoxygenase antagonist therapy: a new approach towards targeted cancer chemotherapy. Acta Biochim. Biophys. Sin. 45, 709–719.
Calixto, J.B., Beirith, A., Ferreira, J., Santos, A.R., Filho, V.C., Yunes, R.A., 2000. Naturally occurring antinociceptive substances from plants. Phytother. Res. 14, 401–418.
Callemien, D., Collin, S., 2008. Use of RP-HPLC-ESI (-)-MS/MS to differentiate various proanthocyanidin isomers in lager beer extracts. J. Am. Soc. Brew. Chem. 66, 109–115.
de las Heras, B., Hortelano, S., 2009. Molecular basis of the anti-inflammatory effects of terpenoids. Inflamm. Allergy Drug Targets 8, 28–39.
de Moraes, W.F., Galdino, P.M., Nascimento, M.V., Vanderlinde, F.A., Bara, M.T., Costa, E.A., de Paula, J.R., 2012. Triterpenes involved in the anti-inflammatory effect of ethanolic extract of *Pterodon emarginat*us Vogel stem bark. J. Nat. Med. 66, 202–207.
de Souza, G.E., Ferreira, S.H., 1985. Blockade by antimacrophage serum of the migration of PMN neutrophils into the inflamed peritoneal cavity. Agents Actions 17, 97–103.
Deraedt, R., Jouquey, S., Delevallée, F., Flahaut, M., 1980. Release of prostaglandins E and F in an algogenic reaction and its inhibition. Eur. J. Pharmacol. 61, 17–24.
Di Rosa, M., Giroud, J.P., Willoughby, D.A., 1971. Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. J. Pathol. 104, 15–29.
Dray, A., 1995. Inflammatory mediators of pain. Br. J. Anaesth. 75, 125–131.
Esmeraldino, L.E., Souza, A.M., Sampaio, S.V., 2005. Evaluation of the effect of aqueous extract of *Croton urucurana* Baillon (Euphorbiaceae) on the hemorrhagic activity induced by the venom of *Bothrops jararaca*, using new techniques to quantify hemorrhagic activity in rat skin. Phytomedicine 12, 570–576.
Gurgel, L.A., Silva, R.M., Santos, F.A., Martins, D.T., Mattos, P.O., Rao, V.S., 2001. Studies on the antidiarrhoeal effect of dragon's blood from *Croton urucurana*. Phytother. Res. 15, 319–322.
Hunskaar, S., Fasmer, O.B., Hole, K., 1985. Formalin test in mice, a useful technique for evaluating mild analgesics. J. Neurosci. Methods 14, 69–76.
Hunskaar, S., Hole, K., 1987. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 30, 103–114.
Jaiswal, R., Jayasinghe, L., Kuhnert, N., 2012. Identification and characterization of proanthocyanidins of 16 members of the *Rhododendron genus* (Ericaceae) by tandem LC-MS. J. Mass Spectrom. 47, 502–515.
Koster, R., Andersons, M., Debber, E.J., 1959. Acetic acid analgesic screening. Fed. Proc. 18, 418–420.
Le Bars, D., Gozariu, M., Cadden, S.W., 2001. Animal models of nociception. Pharmacol. Rev. 53, 597–652.
Mittal, M., Siddiqui, M.R., Tran, K., Reddy, S.P., Malik, A.B., 2014. Reactive oxygen species in inflammation and tissue injury. Antioxid. Redox Signal. 20, 1126–1167.
Muller, W.A., 2002. Leukocyte-endothelial cell interactions in the inflammatory response. Lab. Invest. 82, 521–533.
Peres, M.T., Delle Monache, F., Cruz, A.B., Pizzolatti, M.G., Yunes, R.A., 1997. Chemical composition and *anti*microbial activity of *Croton urucurana* Baillon (Euphorbiaceae). J. Ethnopharmacol. 56, 223–226.
Peres, M.T.L.P., Monache, F.D., Pizzolatti, M.G., Santos, A.R.S., Beirith, A., Calixto, J.B., Yunes, R.A., 1998a. Analgesic compounds of *Croton urucurana* Baillon. Pharmaco-chemical criteria used in their isolation. Phytother. Res. 12, 209–211.
Peres, M.T.L.P., Pizzolatti, M.G., Yunes, R.A., Monache, F.D., 1998b. Clerodane diterpenes of *Croton ururucana*. Phytochemistry 49, 171–174.
Pieters, L., de Bruyne, T., Claeys, M., Vlietinck, A., Calomme, M., vanden Berghe, D., 1993. Isolation of a dihydrobenzofuran lignan from South American dragon's blood (*Croton* spp.) as an inhibitor of cell proliferation. J. Nat. Prod. 56, 899–906.
Posadas, I., Bucci, M., Roviezzo, F., Rossi, A., Parente, L., Sautebin, L., Cirino, G., 2004. Carrageenan-induced mouse paw oedema is biphasic, age-weight dependent and displays differential nitric oxide cyclooxygenase-2 expression. Br. J. Pharmacol. 142, 331–338.
Queiroz, M., Queiroz, E., Zeraik, M., Marti, G., Favre-Godal, Q., Simoes-Pires, C., Marcourt, L., Carrupt, P., Cuendet, M., Paulo, M., 2014. Antifungals and acetylcholinesterase inhibitors from the stem bark of *Croton heliotropiifolius*. Phytochem. Lett. 10, 88–93.
Rao, V.S., Gurgel, L.A., Lima-Júnior, R.C., Martins, D.T., Cechinel-Filho, V., Santos, F.A., 2007. Dragon's blood from *Croton urucurana* (Baill.) attenuates visceral nociception in mice. J. Ethnopharmacol. 113, 357–360.
Salae, A.-W., Rodjun, A., Karalai, C., Ponglimanont, C., Chantrapromma, S., Kanjana-Opas, A., Tewtrakul, S., Fun, H.-K., 2012. Potential anti-inflammatory diterpenes from *Premna obtusifolia*. Tetrahedron 68, 819–829.
Salatino, A., Salatino, M.L.F., Negri, G., 2007. Traditional uses, chemistry and pharmacology of *Croton species* (Euphorbiaceae). J. Braz. Chem. Soc. 18, 11–33.
Sandoval-Acuña, C., Ferreira, J., Speisky, H., 2014. Polyphenols and mitochondria: an update on their increasingly emerging ROS-scavenging independent actions. Arch. Biochem. Biophys. 559, 75–90.
Santos, F.A., Jeferson, F.A., Santos, C.C., Silveira, E.R., Rao, V.S.N., 2005. Antinociceptive effect of leaf essential oil from *Croton sonderianus* in mice. Life Sci. 77, 2953–2963.
Staal, F.J., Roederer, M., Herzenberg, L.A., 1990. Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. Proc. Natl. Acad. Sci. 87, 9943–9947.
Stone, L.S., Broberger, C., Vulchanova, L., Wilcox, G.L., Hökfelt, T., Riedl, M.S., Elde, R., 1998. Differential distribution of alpha2A and alpha2C adrenergic receptor immunoreactivity in the rat spinal cord. J. Neurosci. 18, 5928–5937.
Stévigny, C., Bailly, C., Quetin-Leclercq, J., 2005. Cytotoxic and antitumor potentialities of aporphinoid alkaloids. Curr. Med. Chem. 5, 173–182.
Takeuchi, K., 2012. Pathogenesis of NSAID-induced gastric damage: importance of cyclooxygenase inhibition and gastric hypermotility. World J. Gastroenterol. 18, 2147–2160.
Tomita, M., Lee, K., Nakano, T., 1965. Alkaloid of *Croton cumingii* Muell. Et Arg. Yakugaku Zasshi: J. Pharm. Soc. Jpn. 85, 276–277.
Vaisberg, A.J., Milla, M., Planas, M., Cordova, J.L., de Agusti, E.R., Ferreyra, R., Mustiga, M., Carlin, L., Hammond, G.B., 1989. Taspine is the cicatrizant principle in Sangre de Grado extracted from *Croton lechleri*. Planta Med. 55, 140–143.
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. Exp. Biol. Med. 111, 544–547.
Wolff Cordeiro, K., Aparecida Pinto, L., Nazari Formagio, A.S., Faloni de Andrade, S., Leite Kassuya, C.A., de Cássia Freitas, K., 2012. Antiulcerogenic effect of *Croton urucurana* Baillon bark. J. Ethnopharmacol. 143, 331–337.
Yan, R., Wang, W., Guo, J., Liu, H., Zhang, J., Yang, B., 2013. Studies on the alkaloids of the bark of *magnolia officinalis*: isolation and on-line analysis by HPLC-ESI-MSn. Molecules 18, 7739–7750.
Yuan, Y., Padol, I.T., Hunt, R.H., 2006. Peptic ulcer disease today. Nat. Clin. Pract. Gastroenterol. Hepatol. 3, 80–89.