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

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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

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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),