



Calycophyllum spruceanum BENTH ameliorates acute inflammation in mice

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ABSTRACT

Ethnopharmacological relevance: *Calycophyllum spruceanum* (Benth.) Hook. F. ex K. Schum. is widely distributed in the Amazonian region of Brazil, where it is popularly known as “mulateiro”, “pau-mulato”, “pau-mulato-de-várzea”, “escorrega-macaco” or “pau-marfim”. Preparations of *C. spruceanum* barks are used in the form of tea, poultice or skin patches to treat stomach diseases, skin inflammation and uterus tumors.

Purpose of the study: To investigate in vivo the antinociceptive and anti-inflammatory activities of the hydro-alcoholic extract of *Calycophyllum spruceanum* barks (HECSb) in order to validate its popular usage in inflammatory conditions.

Materials and methods: Chemical analysis of HECSb was performed using the UHPLC-MS system. Mice were treated *per oral* with HECSb (5–5000 mg/kg) and evaluated for acute toxicity (during 15 days); motor activity (Rota rod test); body weight (up to 72 h); antinociceptive activity: writhes induced by 0.8% acetic acid; paw licking induced by 2.5% formalin; paw withdrawal (von Frey test) induced by carrageenan (300 µg) or PGE2 (100 ng); anti-inflammatory (paw edema model). For histopathological analysis subplantar tissue fragments were collected 1 h after paw edema induction.

Results: HECSb chemical analysis revealed the presence of caffeoylquinic derivatives, small organic acids, and phenolic compounds. HECSb showed antinociceptive effect, reducing the number of acetic acid-induced writhes by 72% at 120 mg/kg, paw licking (phase 2- Formalin test) by 33% at 60 mg/kg and 49% at 120 mg/kg; and paw withdrawal elicited by carrageenan (53% at 120 mg/kg) and PGE2 (120 mg/kg) at 0.5 h (48%) and 1 h (45%). HECSb (120 mg/kg) also inhibited the paw edema elicited both by carrageenan (48%) and PGE2 (92%). Histopathological analysis (leukocyte infiltration, edema, focal areas of hemorrhage, vascular congestion) of HECSb treatment at 120 mg/kg demonstrated normal morphology [median 0 (0,1)] compared to PGE2, showing severe alterations [median 3 (2,3); $p = 0,0035$]. HECSb did not induce acute toxicity nor altered body mass or motor coordination.

Conclusions: HECSb shows antinociceptive and anti-inflammatory effect in mice without inducing apparent acute toxicity.

1. Introduction

Plants of the Brazilian Amazon Forest are extensively used for medicinal purposes. The Rubiaceae family is highly representative,

possessing 124 genera and 1.375 species (Barbosa et al., 2016) and is used to treat syphilis, anemia, malaria, diabetes, hypertension and inflammatory conditions (Karou et al., 2011). In particular, the use of these plants in inflammatory and painful conditions are associated to

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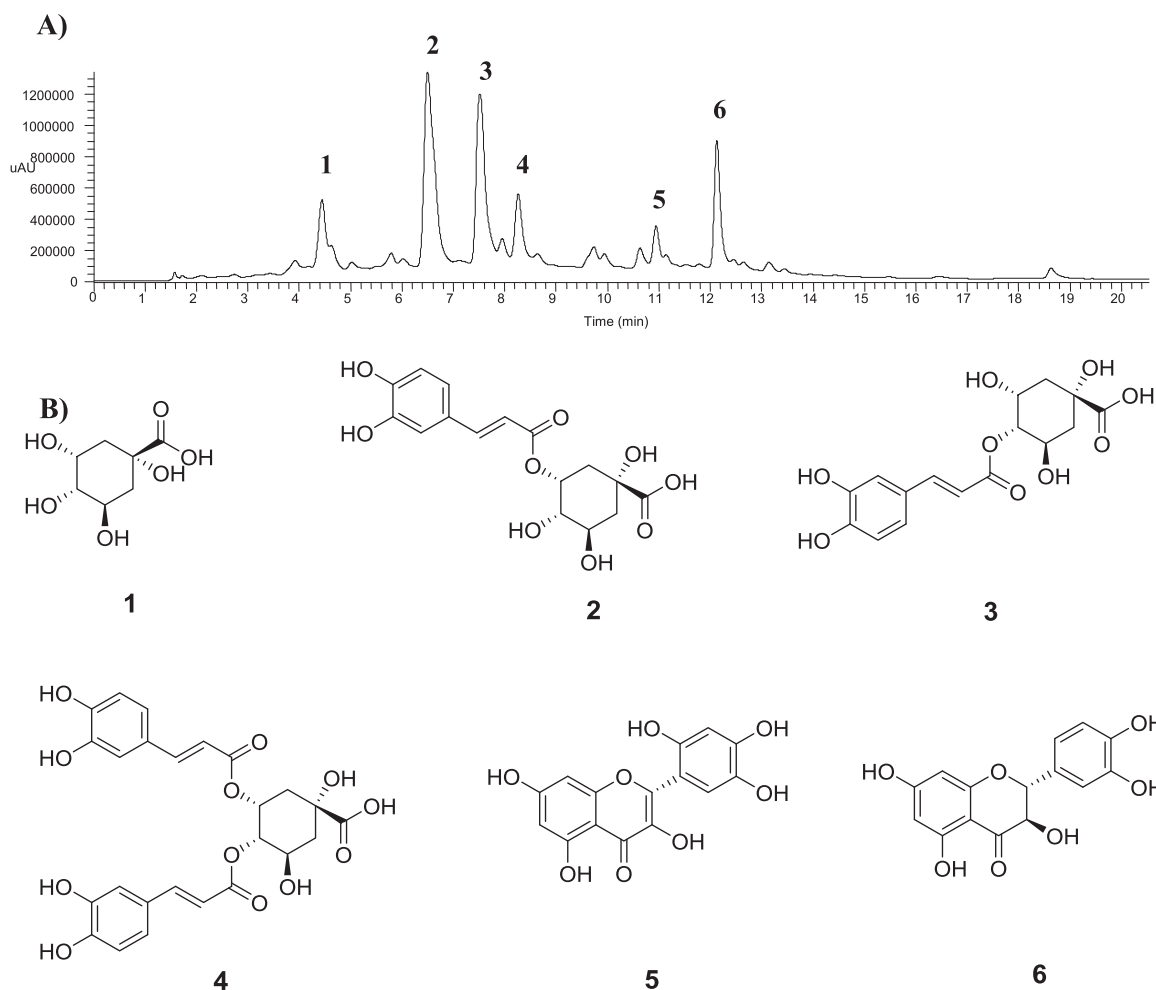


Fig. 1. Phytochemical analysis of *C. spruceanum* bark extract. A) UHPLC-UV-MS/MS chromatogram; B) Identified compounds: quinic acid (1), 3-O-caffeoylquinic acid (2), 4-O-caffeoylquinic acid (3), 3,4-O-dicaffeoylquinic acid (4), 5-hydroxymorin (5), and taxifolin (6).

Table 1

HECSb does not alter the animal permanence in the Rota rod.

| Groups | Doses (mg/kg, v.o) | Permanence time (s) | | | | | | |
|-------------------|--------------------|----------------------------|---------------|---------------|--------------|--------------|---------------|---------------|
| | | 0 | 1 h | 2 h | 3 h | 24 h | 48 h | 72 h |
| Control | – | ^a 38.58 ± 11.97 | 47.80 ± 9.89 | 43.66 ± 10.09 | 45.55 ± 9.62 | 49.18 ± 8.67 | 30.36 ± 12.56 | 40.26 ± 12.09 |
| EHCS _c | 2000 | 36.74 ± 9.95 | 52.58 ± 4.98 | 60.00 ± 0.00 | 60.00 ± 0.00 | 51.87 ± 8.13 | 49.60 ± 10.40 | 60.00 ± 0.00 |
| | 5000 | 24.97 ± 10.08 | 31.66 ± 12.03 | 46.06 ± 10.60 | 49.6 ± 10.40 | 50.04 ± 9.96 | 49.80 ± 10.20 | 47.09 ± 10.22 |

^a Mean ± S.E.M.

the pharmacological effects of the chemical constituents (phenolic and terpenes) of the Rubiaceae plants (Conserva and Ferreira, 2012; Souza et al., 2013), including antioxidant (Silva et al., 2007; De Vargas et al., 2016), anti-inflammatory and antinociceptive (Dongmo et al., 2003) effects.

Calycophyllum spruceanum (Benth.) Hook. f. ex K. Schum. (Rubiaceae) is well distributed in the Amazon area in Brazil, where it is popularly known as “mulateiro”, “pau-mulato”, “pau-mulato-de-várzea”, “escorrega-macaco” or “pau-marfim”. Bark preparations of *C. spruceanum* are utilized in the form of tea, poultice or skin patches to treat stomach diseases, skin inflammation and uterus tumors (Caetano et al., 2014). The phytochemistry study of *C. spruceanum* bark extracts showed the presence of phenols and terpenes, possessing respectively, antioxidant (De Vargas et al., 2016) and in vitro activity against trypanostigote forms of *Trypanosoma cruzi* (Zuleta et al., 2003).

This study aimed to investigate in mice the antinociceptive and anti-

inflammatory activities of the hydroalcoholic extract obtained from *C. spruceanum* barks in order to validate its popular usage in inflammatory conditions.

2. Material and methods

2.1. Preparation of *C. spruceanum* hydroalcoholic extract

Stem barks of *Calycophyllum spruceanum* (Rubiaceae) were collected in Bujari, Acre, Brazil at km 52BR-364 road and a voucher specimen (no 20307) was deposited at UFAC Herbarium. Barks were washed with tap water, dried at 45 °C, grounded into fine particles (food mixer) and weighted in the Laboratory of Food Technology-UFAC. The extract was prepared by percolation using 70% ethanol, left for 72 h at r.t. (procedure repeated after 24 h), filtered (filter paper), concentrated in rota evaporator at 45 °C, lyophilized and named hydroalcoholic extract of *C.*

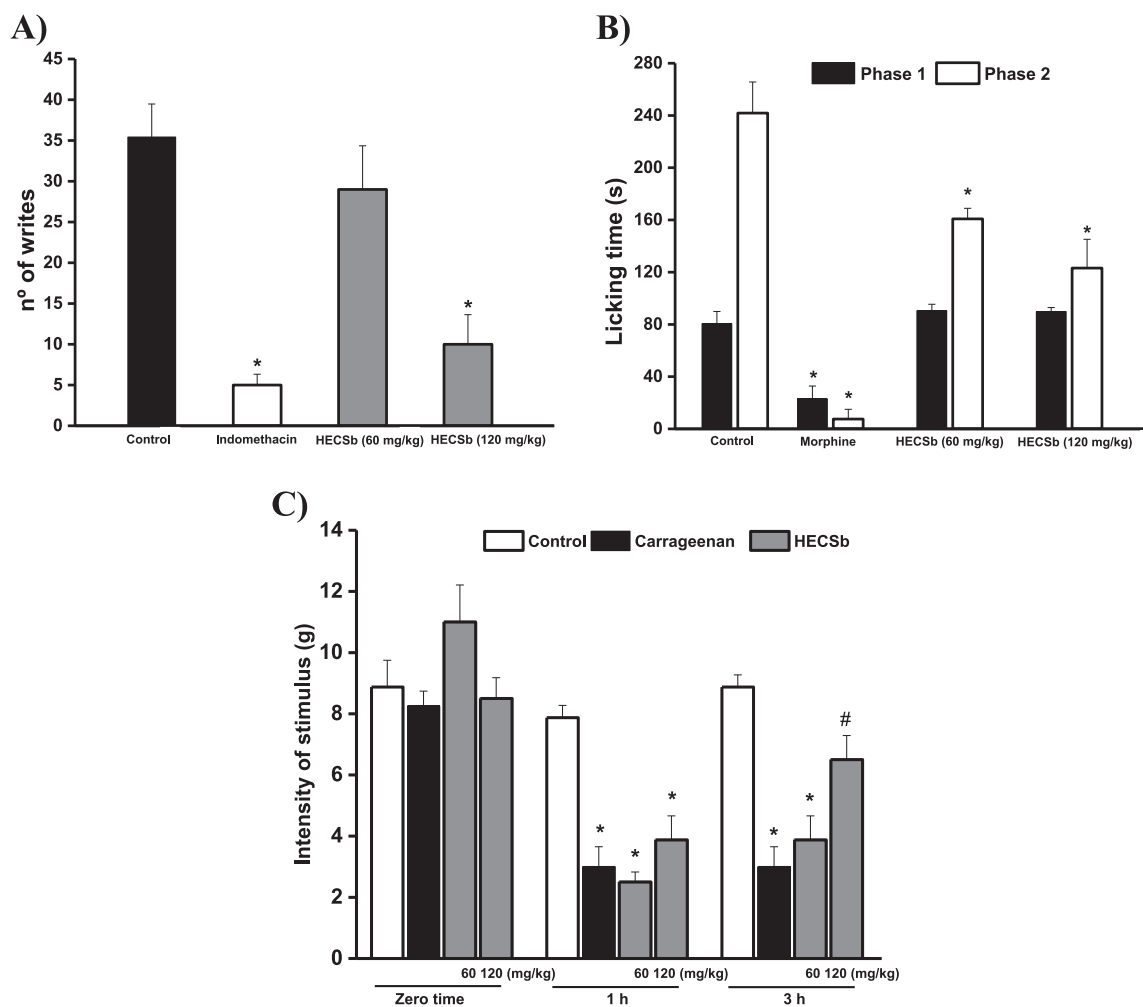


Fig. 2. HECSb inhibits nociceptive behavior in mice. HECSb (60 and 120 mg/kg; p.o.), indomethacin (5 mg/kg; s.c.) was administered in mice 60 min and 30 min, respectively, before nociceptive stimuli. Morphine (13.3 µmol/kg; s.c.) was administered 15 min after HECSb. (A) Writing test: 0.8% acetic acid (v/v; i.p.); (B) Formalin test: 2.5% formalin (s.c.) (C) von Frey test: carrageenan (300 µg/paw). Mean ± S.E.M. (n = 8). *p < 0.05 vs. control; #p < 0.05 vs. stimuli.

spruceanum barks (HECSb). The extraction resulted in 4.07% yield (268.65 g powder).

2.2. Phytochemical characterization

Chemical analysis was performed using the ultra-high pressure liquid chromatography tandem mass spectrometry (UHPLC-MS) system, consisting of an Accela 600 liquid chromatography coupled to a TSQ Quantum Access mass spectrometer bearing a triple-quadrupole (QQQ) mass analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Electrospray ionization in negative mode (ESI⁻) was applied to access the phenolic composition of *Calycophyllum spruceanum*. Mass spectra (MS) was acquired at the *m/z* range from 50 to 1000, and tandem mass spectra (MS/MS) performed by collision-induced dissociation (CID) of previously isolated precursor ions in the QQQ system. Argon was the collisional gas. Tentative identifications were performed by manual interpretation of MS/MS spectra and comparison with data previously published. Chromatographic separations were performed by the use of a Kinetex C18 column (2.6 µm, 30 × 4.6 mm, 100 Å pore size) (Phenomenex, Torrance, CA, USA) using a binary mobile phase (solvent A: ultrapure water; solvent B: methanol). A gradient elution at 28 °C was performed as follows: 0–18 min, 20–100% B and 19–21 min 100% B at a flow rate of 0.4 mL/min. The autosampler temperature was held at 18 °C, and the injection volume was 10 µL. Ionization parameters were capillary voltage 4.3 kV, cone voltage 7 V, sheath gas 28 arb, and auxiliary gas 4 arb. Collision energies were applied from 2 to

40 eV.

2.3. Animals

Male Swiss mice (20–25 g) were maintained with free access to food and water. Experimental protocols were approved by the Animal Care and Use Committee of the State University of Ceara-UECE (n° 6975375.2014), Fortaleza-CE and by the Committee of the State University of Acre-UFAC (n° 32/2014), Rio Branco-AC, Brazil, in accordance with the Guide for the Care and Use of Laboratory Animals of the US guidelines (8th edition, revised 2011).

2.4. Drugs and reagents

Formalin, λ-carrageenan, prostaglandin E₂ (PGE₂) and indomethacin were purchased from Sigma (St. Louis, USA). Morphine was obtained from Cristália (São Paulo, Brazil). Drugs were solubilized directly in sterile saline (NaCl 0.15 M), except for indomethacin, which was dissolved in dimethyl sulfoxide up to 10%.

2.5. Evaluation of acute toxicity, body mass, motor coordination, nociception and inflammation after HECSb treatment in rodents

2.5.1. Acute toxicity and body mass

Animals (n = 5/group) were treated *per oral* with HECSb (5, 50, 300, 2000, 5000 mg/kg) or distilled water after 12 h fasting and

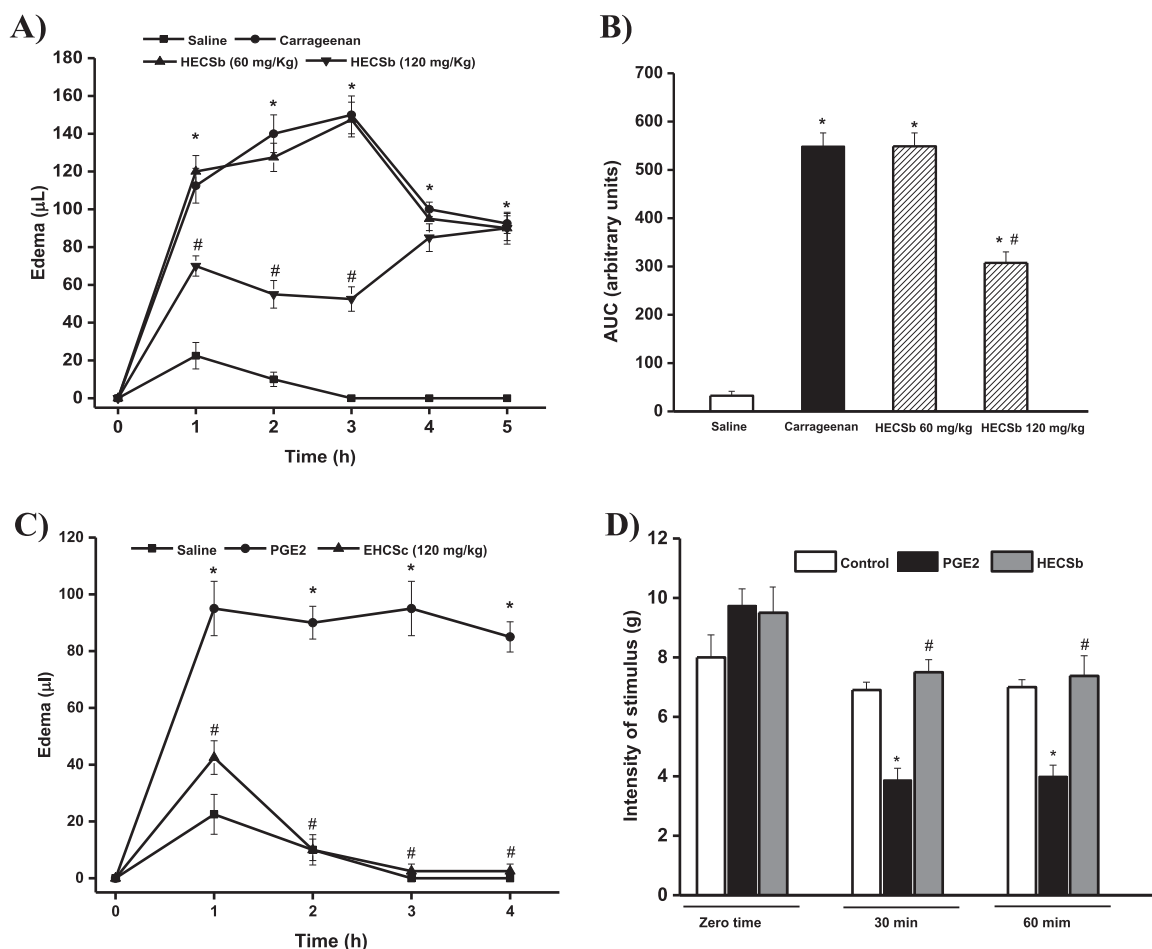


Fig. 3. HECSb inhibits the paw edema induced by carrageenan and PGE2. HECSb (60 and 120 mg/kg; p.o.) was administered 60 min before carrageenan (300 µg/paw) or PGE2 (30 nmol/paw). Edema was measured before (time zero) and at 1–5 after stimuli. (A) increase in paw volume (µL), (B) area under curve (arbitrary units), (C) increase in paw volume (µL) and (D) Hypernociception (g). Mean ± S.E.M. (n = 8). ANOVA and Bonferroni's test; *p < 0.05 vs. control; #p < 0.05 vs. carrageenan or PGE2.

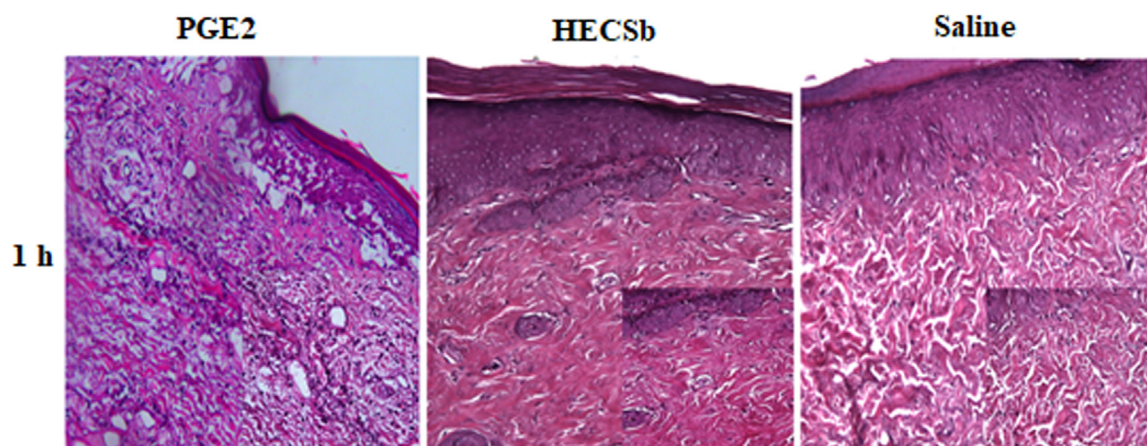


Fig. 4. Saline (0.1 mL/10 g) [median 0 (0,1)] and HECSb (120 mg/kg) [median 0 (0,1)] - Normal tissue (no distinguishable architectural changes), absence of edema, necrosis, inflammatory infiltrate and hemorrhagic areas (HE, 200 e 400 ×). PGE2 (100 ng/paw; s.c.) [median 3 (2,3)]* - edema and inflammatory infiltrate (HE, 200 e 400 ×). Median (maximum and minimum), *p < 0.05 (Kruskal-Wallis followed by Dunn post-test).

evaluated, every 60 min during 3 h, for the following behavioral parameters: hiperactivity, irritability, agressivity, shivering, seizures, piloerection, palpebral ptose, clonic or tonic movements, stereotypes, bizarre behavior, pupil size, excessive grooming, repetitive circling, diarrhea, constipation, defecation, unusual respiratory pattern, lacrimation, salivation, cyanosis and death. At the end of 3 h observation, animals were feed and observed every 24 h during 3 days and at the

15th day. The following scores were adopted: (0) without effect/without behavioral alterations, (-) discrete, (+) moderate, (++) and intense (Almeida et al., 1999; OECD, 2001). Animals were weighted before and 72 h after treatment.

2.5.2. Motor coordination: Rota rod test

Mice were selected 24 h before the experiment, being excluded

those that did not remain in the Rota rod apparatus at 22 rpm for two consecutive periods of 60 s (Dunham and Miya, 1957). The number of falls and the time (s) of permanence on the Rota rod were recorded at the following time: one hour before (zero time), after 60, 120 and 180 min of HECSb treatment, and every 24 h during three days (Malone and Robichaud, 1962).

2.6. Nociception and inflammation

HECSb (60–120 mg/kg) was administered per oral in mice 60 min before nociceptive or inflammatory stimuli. Control animals received distilled water in substitution of HECSb.

2.6.1. Writing test

Acetic acid (0.8%; v/v; 0.1 mL/10 g body weight) was injected in mice by intraperitoneal (i.p.) route. Ten min later the number of abdominal constrictions (writhes) was recorded at 20 min (Koster et al., 1959) (0.9% w/v NaCl). Animals received indomethacin (10 mg/kg) by subcutaneous (s.c.) route 60 min before acetic acid.

2.6.2. Formalin-induced licking test

Formalin (20 μ L; 2.5% v/v) was injected in the right hind paws of mice and the time (s) in which animals spent licking its paws was recorded at phase 1 (0–5 min) and phase 2 (15–30 min) (Hunskar and Hole, 1987). Animals received morphine (13.3 μ mol/kg; s.c.) 15 min before formalin.

2.6.3. von Frey test

Mice were placed individually in boxes of elevated wire-mesh platforms to allow access to the ventral surface of hindpaws (Cunha et al., 2004). The frequency of paw withdrawal in response to 3 applications of von Frey filament (0.8 g) was determined before (basal value) at 0.5, 1 and 3 h after carrageenan (300 μ g/paw; s.c.) or Prostaglandin E2 (PGE2; 100 ng/paw).

2.6.4. Paw edema model

Paw edema was induced by carrageenan (300 μ g/paw; s.c.) or PGE2 (100 ng/paw) and measured by hydroplethysmometry (Pan Lab, Barcelona-Spain) before (zero time) and from 1 to 5 h after stimuli. The differences in paw volume (μ L) or area under curve-AUC (arbitrary units) were compared to that of controls.

2.6.5. Histopathological analysis

Subplantar tissue fragments, collected 1 h after injection of PGE2, were fixed in 10% formalin for 24 h, dehydrated in crescent alcoholic series, diaphanized in xylol, impregnated in paraffin and melted at 60°. Fragments were put into paraffin-forming blocks at r. t., sectioned to 5 μ m in thickness and stained by eosin-hematoxylin (H&E). Morphological changes were observed by light microscopy and graded semi-quantitatively as follows: (0) Normal tissue (no distinguishable change, 0%): absence of edema, necrosis, inflammatory infiltrate or hemorrhagic areas; (1 +) Discrete tissue changes (initiation of changes, up to 30%): edema, necrosis, inflammatory infiltrate and focal areas of hemorrhage; (2 +) Moderate tissue changes (patent changes, 31–60%): edema, necrosis, inflammatory infiltrate and focal areas of hemorrhage; (3 +) Severe tissue changes (wide spread changes, 61–100%): edema, necrosis, inflammatory infiltrate and focal areas of hemorrhage (adapted from Lim et al., 2010).

2.7. Statistical analysis

Results were analyzed by One-way ANOVA followed by Bonferroni test. Histopathological results were expressed as Median (maximum and minimum) and analyzed by Kruskal-Wallis test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Phytochemical characterization

UHPLC-MS of HECSb revealed the presence of caffeoylquinic derivatives, small organic acids, and six phenolic compounds were characterized: compound 1 (r.t. 4.46 min) displayed a deprotonated ion at m/z 191 and fragments at m/z 93 and 85, being identified as quinic acid (De Souza et al., 2016). Caffeoylquinic acids (major constituents), represented by compounds 2 (3-*O*-caffeoylquinic acid, r.t. 6.36 min) and 3 (4-*O*-caffeoylquinic acid, r.t. 7.88 min), both exhibiting a deprotonated molecular ion at m/z 353 with a main fragment at m/z 191, were identified according to the relative abundance of the base peak and elution order in comparison with the literature (Clifford et al., 2005). The identification of compound 4 (3,4-*O*-dicafeoylquinic acid r.t. 8.25 min) a dicafeoyl derivative of the quinic acid (m/z 515 \rightarrow m/z 353), based in the fragmentation and comparison with previously published data (Clifford et al., 2005). Compounds 5 (10.97 min, m/z 319) and 6 (12.08 min, m/z 305) were identified as flavonoids, by means of their fragmentation behavior under CID. 5-hydroxymorin (5) and taxifolin (6) were identified by comparison with the literature (Lin et al., 2003, 2007) (Fig. 1).

3.2. HECSb does not induce acute toxicity nor alters body mass or motor coordination

Treatment with HECSb (60 e 120 mg/kg) did not alter the animal permanence time (zero to 1 h after administration) in the Rota rod, compared to controls (60 \pm 0 s): at 60 mg/kg [HECSb/ 0 h (58.75 \pm 1.25 s); HECSb/ 1 h (58.94 \pm 1.06 s)]; at 120 mg/kg [HECSb/ 0 h (58.25 \pm 1.10 s); HECSb/ 1 h (60 \pm 0 s)].

At higher doses, HECSb (2000 and 5000 mg/kg) did not alter the animal permanence time in the Rota rod (Table 1) or induce lethality or behavioral alterations (hiperactivity, irritability, aggressivity, shivering, seizures, etc.) in the first three hours and from 1 to the 15th days (data not shown). The animal body mass was also unaltered at the end of treatment (Control: 37.37 \pm 1.9; HECSb 2000 mg/kg: 35.59 \pm 1.6; HECSb 5000 mg/kg: 36.94 \pm 1.4).

3.3. HECSb inhibits nociception

HECSb (120 mg/kg) reduced by 72% (10.02 \pm 3.63) the number of writhes elicited by 0.8% acetic acid (35.42 \pm 4.04) (Fig. 2A). In the formalin-induced licking test HECSb (60 and 120 mg/kg) was also inhibitory, decreasing the paw licking time elicited by 2.5% formalin only at phase 2 (241.8 \pm 23.82 s) by 33% (160.83 \pm 8.08) at 60 mg/kg and by 49% (123.16 \pm 22.02 s) at 120 mg/kg (Fig. 2B). HECSb (120 mg/kg) reduced by 53% (6.5 \pm 0.78 g) the paw withdrawal in response to mechanical stimulation with von Frey filaments in animals injected with carrageenan (3.0 \pm 0.65 g) at 3 h, but at 1 h exhibited no alteration (Fig. 1C). HECSb (120 mg/kg) reduced the response elicited by PGE2 at 0.5 h by 48% (HECSb: 7.50 \pm 0.42 g vs. PGE2: 3.87 \pm 0.39 g) and at 1 h by 45% (HECSb: 7.37 \pm 0.67 g vs. PGE2: 4.0 \pm 0.37 g) (Fig. 3D).

3.4. HECSb inhibits paw edema and abolishes inflammatory tissue alterations

HECSb (120 mg/kg) inhibited the paw edema induced by carrageenan (300 μ g/paw; s.c.) from 1 h to 3 h by 48% (Fig. 3A and B) and by PGE2 (100 ng/paw; s.c.) from 1 h to 4 h by 92% (Fig. 3C). Histopathological analysis of paws from animals treated with HECSb (120 mg/kg) or saline (0.1 mL/10 g) demonstrated normal morphology [median 0 (0,1)] compared to non-treated animals stimulated with PGE2, that showed severe alterations [median 3 (2,3); $p = 0,0035$] in the following parameters: leukocyte infiltration, edema, focal areas of

hemorrhage and vascular congestion (Fig. 4).

4. Discussion

This study revealed that the hydroalcoholic extract of *Calycophyllum spruceanum* (HECSb) elicits antinociceptive and anti-inflammatory effect in rodents with no alteration in the animal's body weight, motor performance nor induced apparent acute toxicity.

It is well known that drugs presenting depressant activity in the central nervous system (CNS), such as anxiolytic, sedative, muscle relaxant and hypnotic analgesic may cause motor incoordination (Pultrini et al., 2006) and influence nociceptive behavior (Soja et al., 2002). In our study HECSb treatment did not alter the animal permanence on the Rota rod bar, thus excluding muscle relaxant effect or any other significant deleterious influence commonly present in the profile of CNS depressant drugs. The lack of toxicity of Rubiaceae plants, such as *Posoqueria acutifolia* (Souza et al., 2007) and *Coutarea hexandra* (Santos et al., 2006) had been already described.

In addition, HECSb showed antinociceptive effect in the writhing test that although of low selectivity, is very sensitive for evaluation of peripheral antinociceptive activities (Gené et al., 1998). In this test, acetic acid irritation triggers the release of bradykinin, prostaglandins and cytokines (Corrêa et al., 1996; Ribeiro et al., 2000) that activate chemosensitive nociceptors leading to inflammatory pain. Accordingly, HECSb inhibited the inflammatory phase (phase 2) of formalin-induced licking test (Hunskar and Hole, 1987) and the hypernociception induced by carrageenan. This data, could be associated to the inhibition of inflammatory mediators, since carrageenan initiates the release of the cytokine tumor necrosis factor-alpha (TNF- α) and other mediators, including PGE2, considered the final hypernociceptive mediator (Cunha et al., 2005).

It is well known that carrageenan induces a time-course and biphasic paw edema in which, the first phase (zero to 2 h) is associated to the release of histamine and serotonin, and the second phase (2–5 h) is mediated by prostaglandins and cytokines (Di Rosa et al., 1971; Rocha et al., 2006). The HECSb inhibitory effect on the paw edema induced either by carrageenan and PGE2 corroborate the effect demonstrated in experimental models that evaluate inflammatory nociception (writhing test induced by acetic acid, formalin-phase 2 and hypernociception – von Frey test). The histological data corroborated this effect, since HECSb reduced the inflammatory parameters cell infiltrate, hemorrhage and vascular congestion observed in the paw edema induced by PGE2. In this line, several species of the Rubiaceae family have been widely used in the folk medicine as anti-inflammatory (Souza et al., 2013). Experimental data also demonstrated that *Mitragyna ciliata* (Rubiaceae) reduces the paw edema elicited by carrageenan possibly via inhibition of kinins and prostaglandins release (Dongmo et al., 2003). Moreover, the phytochemical analysis of HECSb revealed the presence of chlorogenic acid (CGA) as the major constituent. It has been demonstrated that CGA possess anti-inflammatory and analgesic effects in rats (Santos et al., 2006; Upadhyay and Rao, 2013) and inhibits the production of PGE2 in a model of arthritis (Chen and Wu, 2014).

The present study is coherent with the popular use of tea preparations of *C. spruceanum* barks. In addition, the literature favors our study in which hydroalcoholic preparation was used, suggesting superior anti-inflammatory effect compared to tea preparations, since extractions using hydroalcoholic solvents result in secondary metabolites of varied polarities, preserving components other than those present in the tea preparation (Aguilar et al., 2002).

In conclusion, HECSb presents antinociceptive and anti-inflammatory effect in mice without inducing apparent acute toxicity.

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2007-1). Assrey AMS is senior investigator of CNPq.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

All authors listed on this paper have made significant contributions to the study. To ensure clarity, we detailed author's contribution below, which substantiate the inclusion of each author in the manuscript:

Ana Paula Azevedo Barros da Silva: study design, experimental protocols, acquisition, analysis and interpretation of data, critical review and wrote the manuscript first version

Renata Moraes Ferreira Amorim: study design, experimental protocols of inflammation and nociception, acquisition, analysis and interpretation of data, critical review and wrote the manuscript first version.

Roberta de Freitas Lopes: analysis and interpretation of data, critical review.

Mário Rogério Lima Mota: performed histopathological analysis, interpretation of data, critical review, and wrote the manuscript.

Felipe Silva: Phytochemical characterization.

Hector Koolen: Phytochemical characterization.

Emerson Lima: Phytochemical characterization.

Ana Maria S. Assrey: performed final writing, study design, analysis and interpretation of data, and coordinated the manuscript construction.

Renildo Moura da Cunha: performed final writing, study design, analysis and interpretation of data, and coordinated the manuscript construction

We declare that all authors discussed the results and commented on the manuscript.

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Glossary

- HECSb:** Calycophyllum spruceanum barks
- UHPLC-MS:** Ultra-high pressure liquid chromatography tandem mass spectrometry
- ESI:** Electrospray ionization in negative mode
- MS:** Mass spectra
- CID:** collision-induced dissociation
- PGE₂:** Prostaglandin E₂
- AUC:** Area under curve
- CGA:** Chlorogenic acid
- CNS:** Central nervous system