REVISTA DELOS DESARROLLO LOCAL SOSTENIBLE

Phytochemical Profile, toxicity assessment, and biological activities of hydroalcoholic extract of *Calycophyllum spruceanum* leaves

Perfil fitoquímico, avaliação de toxicidade e atividades biológicas do extrato hidroalcoólico das folhas de *Calycophyllum spruceanum*

Perfil fitoquímico, evaluación de toxicidad y actividades biológicas del extracto hidroalcohólico de las hojas de *Calycophyllum spruceanum*

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ABSTRACT
Pharmacotherapy for the treatment of inflammation based on non-steroidal anti-inflammatory drugs (NSAIDs) and opioids can cause gastrointestinal discomfort, ulcers, renal, cardiac and metabolic dysfunctions. Studies on natural products containing constituents with anti-inflammatory and analgesic activity remain important and quite justified. Plants belonging to the *Rubiaceae* family are examples of source of bioactives. The hydroalcoholic extract of *Calycophyllum spruceanum* (Benth.) Hook. F. ex K. Schum (Mulateiro) leaves (HECsL), native to the Amazon biomes, when subjective to liquid chromatography in UHPLC-MS, revealed the presence of four phenolic constituents: caffeoylquinic derivatives, small organic acids and flavonoids, constituents that have antioxidant and anti-inflammatory activity. HECsL did not show acute toxicity, nor did it change the motor coordination or body mass of the experimental animals. HECsL at a dose of 60 mg/kg reduced the number of writhings induced by 0.8% acetic acid (63%), increased the time spent on the hot plate (30%), and increased the tail withdrawal latency at a dose of 120 mg/kg (49%). The stimulation of hypernociception by carrageenan in the animals abdomen was reversed by HECsL (59%) by decreasing the number of total leukocytes (67%). HECsL has anti-inflammatory and antinociceptive prophylactic effects on the peripheral and central nervous system of *Swiss* mice, being devoid of acute toxicity.

Keywords: mulateiro, nociception, inflammation, natural products, Rubiaceae, phytochemistry.

RESUMO
A farmacoterapia para o tratamento da inflamação à base de anti-inflamatórios não esteroides (AINEs) e de opioides, podem desencadear desconfortos gastrintestinais, úlceras, disfunções renais, cardíacas e metabólicas. Estudos sobre produtos naturais contendo constituintes com atividade anti-inflamatória e analgésica, continuam importantes e bastante justificados. As plantas pertencentes à família Rubiaceae são exemplos de fontes desses bioativos. O extrato hidroalcoólico das folhas de *Calycophyllum spruceanum* (Benth.) Hook. f. ex K. Schum (Mulateiro) (HECsL), planta da família Rubiaceae, natural do bioma amazônico, quando submetido à cromatografia líquida em UHPLC-MS apresentou quatro constituintes fenólicos: derivados cafeoilquínicos, pequenos ácidos orgânicos e flavonoides, em seu conjunto possuidores de atividades antioxidante e anti-inflamatória. O HECsL não evidenciou toxicidade aguda, não alterou a coordenação motora e nem a massa corporal dos animais experimentais. O HECsL na dose de 60 mg/kg reduziu o número de contorções induzidas pelo ácido acético 0,8% (63%), aumentou o tempo de permanência na placa quente (30%) e aumentou a latência de retirada da cauda na dose de 120 mg/kg (49%). A estimulação da hipernocicepção pela carragenana no abdômen dos animais foi revertida pelo HECsL (59%) diminuindo o número de leucócitos totais (67%). O HECsL apresenta efeitos anti-inflamatórios e antinociceptivos no sistema nervoso periférico e central de camundongos *Swiss*, sendo desprovido de toxicidade aguda.

Palavras-chave: mulateiro, nocicepção, inflamação, produtos naturais, Rubiaceae, fitoquímica.

RESUMEN
La farmacoterapia para el tratamiento de la inflamación a base de antiinflamatorios no esteroides (AINE) y de opioides, pueden desencadenar molestias gastrointestinales, úlceras, disfunciones renales, cardíacas y metabólicas. Estudios más profundos de productos naturales poseedores de activos con actividad antiinflamatoria y analgésica, siguen siendo importantes y bastante justificados. Las plantas pertenecientes a la familia Rubiaceae son ejemplos de fuentes de estos bioactivos. El extracto hidroalcohólico de las hojas de *Calycophyllum spruceanum* (Benth.) Hook. f. ex K. Schum (HECsL), planta de la familia Rubiaceae, natural del bioma amazónico, cuando sometido a la cromatografía líquida en UHPLC-MS presentó cuatro constituyentes fenólicos: derivados cafeoilquínicos, pequeños ácidos orgánicos y flavonoides, en su conjunto posuidores de actividades antioxidante y antiinflamatoria. El HECsL no mostró toxicidad aguda, no alteró la coordinación motora ni la masa corporal de los animales experimentales. El HECsL en la dosis de 60 mg/kg redujo el número de contorsiones inducidas por el ácido acético 0,8% (63%), aumentó el tiempo de permanencia en la placa caliente (30%) y aumentó la latencia de retirada de la cola en la dosis de 120 mg/kg (49%). La estimulación de la hipernocicepción por carragenina en el abdomen de los animales fue revertida por el HECsL (59%) disminuyendo el número de leucocitos totales (67%). HECsL tiene efectos antiinflamatorios y antinociceptivos en el sistema nervioso periférico y central de los ratones suizos y carece de toxicidad aguda.

Palabras clave: mulateiro, nocicepción, inflamación, productos naturales, Rubiaceae, fitoquímica.

1 INTRODUCTION

Inflammation is a complex physiological response of the body to biological, physical or chemical stimuli. Despite being a physiological event, inflammation is associated with increased tissue damage and pain. This process involves resident cells, such as macrophages, and release of leukocyte chemotactic factors (prostaglandin E2-PGE2; tumor necrosis factor alpha-TNF-α, nitric oxide-NO) (Kulkarni et al., 2016).

Inflammatory pain is an event that results from the release of inflammatory mediators that activate/sensitize nociceptors (Millan, 1999). When stimulated, nociceptors become hypersensitized to mild pain stimuli, generating increased sensitivity response or hypernociception (Campbell; Meyer, 2006). TNF-α is a primary cytokine that plays central role in hypernociception, stimulating a cascade of cytokines and production of prostaglandins, important target for analgesic drugs (Kawabata, 2011).

The current pharmacotherapy for the treatment of inflammation, such as non-steroidal anti-inflammatory drugs (NSAIDs) and opioids, has been shown to be disadvantageous when used in the long-term regimen and may cause gastrointestinal ulcers, renal, cardiac and methabolic dysfunctions, (Bindu *et al.*, 2020; Judd *et al.*, 2014). Thus, the study of natural substances that may have anti-inflammatory and analgesic effects is of enormous relevance.

Plants belonging to the family Rubiaceae represent important source of bioactive products. *Calycophyllum spruceanum* (Benth.) Hook. F. ex K. Schum (Mulateiro) is native from the Amazonian region and is commonly used by the population as a rejuvenating, healing, antimicrobial and for the treatment of inflammatory diseases (Santos *et al.,* 2016; Sarquis *et al.,* 2019; Peixoto *et al.*,2018).

Its barks contain flavonoids, caffeoylquinic derivatives (Da Silva *et al*., 2018), terpenes (Zuleta *et al*., 2003), tannins (Da Costa *et al*., 2011) and alkaloids (Dookie *et al*., 2021) presenting antioxidant (De Vargas *et al*., 2016), antimicrobial (Dookie *et al*., 2021), antiparasitic (Zuleta *et al*., 2003), antinociceptive and anti-inflammatory effects (Da silva *et al*., 2018).

Considering the pharmacological studies described for the barks and the lack of experimental studies for *C. spruceanum* leaves, considering the abundant availability of plant leaves, therefore a more rational and ecologically correct exploitation and less harmful to the plant's health, the aim of this study was to characterize and evaluate the effect of its hydroalcoholic extract of *C. spruceanum* leaves in mice models of nociception and acute inflammation.

2 MATERIALS AND METHODS

2.1 HYDROALCOHOLIC EXTRACT PREPARATION

Fresh leaves of *C. spruceanum* (Rubiaceae) were collected in Bujari, Acre, Brazil at km 52BR-364 road (voucher UFAC Herbarium nº 20307), washed, dried at 45 °C, grounded into fine particles and weighted. The extract was prepared by the percolation method using 70% ethanol for 72 h at r.t. (procedure repeated after 24 h). The final extract was filtered, concentrated at 45 °C, lyophilized and named hydroalcoholic extract of *C. spruceanum* leaves (HECsL).

2.2 CHEMICAL CHARACTERIZATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC-MS/MS)

The chemical composition of HECsL was performed by liquid chromatography (UHPLC-MS system), consisting of an Accela 600 coupled to TSQ Quantum Access mass spectrometer bearing a triple-quadrupole (QqQ) mass analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The phenolic composition was accessed by Electrospray Ionization in negative mode (ESI^-).

Mass spectra (MS) was acquired at the *m/z* range from 50 to 1000, and tandem mass spectra (MS/MS) performed by collisional-induced dissociation (CID) of previously isolated precursor ions in the QqQsystem, using argon as the collisional gas. Tentative identifications were performed by manual interpretation of MS/MS spectral and comparison with data previously published. Chromatographic separations were performed on a Kinetex C18 column (2.6 µm, 30 × 4.6 mm, 100 Å pore size; solvent A: ultrapure water and solvent B: methanol) (Phenomenex, Torrance, CA, USA) using a binary mobile phase. The elution was performed at 28°C 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 10µL. Ionization parameters were capillary voltage 4.3 kV, cone voltage 7 V, sheath gas 28arb, and auxiliary gas 4 arb. Collision energies were applied as an increasing ramp from 2 to 40 eV.

2.3 ANIMALS AND TREATMENT

Female *Swiss* mice (25 - 35 g), maintained at 22 ± 2 ºC, light/dark cycle of 12:12h with free access to water and food, were treated per oral with HECsL (60 - 5000 mg/kg). After 60 minutes, 0.9% NaCl (saline) or the phlogistic agent carrageenan was administered by intraperitoneal (IP) route. The antinociceptive and anti-inflammatory effects were assessed in the models of abdominal writhing, hot plate, tail dipping and peritonitis.

2.4 EVALUATION OF TOXICITY AND MOTOR COORDINATION

For evaluation of toxicity, mice were previously fasted for 12 h, randomly separated into 5 groups (n=5), and treated per oral (p.o.) with sterile saline (0.1 mL/10 g body weight) as control. The HECsL was administered at the initial dose of 2000 mg/kg, or in case of no occurrence of toxicity or lethality, a new group recived HECsL at 5000 mg/kg (OECD, 2001).

Animals were observed every 60 min for 3 h before beind feed, and after receiving food and water every 24 hours for 3 days and at the 15th day. The behavioral response was observed by the following parameters: no effect/no behavioral changes, diminished effect, mild, and intense effects (Almeida *et al*., 1999).

The motor coordination of the animals was evaluated in-the Rota rod test by the permanence time (s) in the apparatus (9-10 r.p.m.) at 0, 60, 120 and 180 min after treatment with HECsL (60 and

120 mg/kg; p.o.) (Carter *et al*., 2001). The weight of the animals was also recorded before and 72 hours after the treatment.

2.5 EVALUATION OF THE ANTINOCICEPTIVE EFFECT: WRITHING, HOT PLATE AND TAIL DIPPING TESTS

For evaluation of visceral nociception in the Writhing test, mice received acetic acid (0.8%; v/v; 0.1 mL/10 g body mass; I.P) and the number of contortions of the abdominal musculature was recorded from 10 to 30 min. after treatment (Koster *et al*., 1959).

For evaluation of the sensorial response to thermal stimuli, mice were placed in the hot plate (53 C° ± 1 °C) up to 25s and the time of the reactions (jumping, licking or shaking hind paws) to thermal stimulus was registered before (basal value) and up to 60, 120, 180 and 240 min after treatment. (Gupta *et al*., 2005).

In order to evaluate the medullary nociceptive reflex in the tail dipping model, mice had their tails immersed in a water bath (50ºC ± 1ºC), and the tail withdrawal latency (sec) was recorded before (zero time) and 60, 120 and 180 min after treatment (D'amour; Smith, 1941).

2.6 EVALUATION OF THE ANTI-INFLAMMATORY EFFECT: PERITONITIS MODEL

Peritonitis was induced by carrageenan (500 µg; IP) (Levy, 1969) and the inflammatory parameters (abdominal hypernociception and leukocyte migration) was evaluated 4 hours later.

For the abdominal hypernociception, the animals were placed in acrylic boxes with mesh floor, stimulated in the peritoneum (average of 4 values) with a rigid tip connected to an electronic analgesimeter and the response (peritoneum withdrawal) expressed in g (Cunha *et al*., 2005). After euthanasia the peritoneal cavity was washed (5 UI heparine + saline) and the peritoneal fluid collected for quantification of total leukocytes performed in Neubauer chamber (20 µL peritoneal fluid + 80 µL Turk reagent) (Souza *et al*., 1985).

2.7 STATISTICAL ANALYSIS

Data were expressed as Mean ± SEM and analyzed by ANOVA followed by Tukey test. The data of behavioral tests was expressed by Student t-test. Values of $p<0.05$ were considered significant.

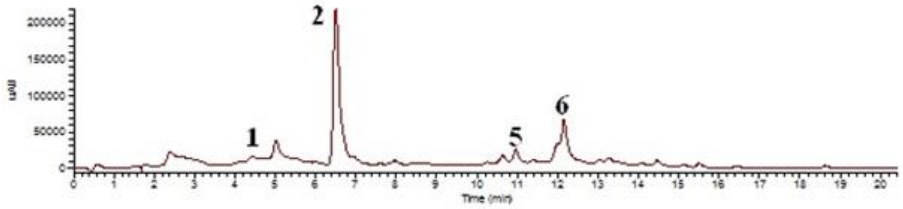
3 RESULTS AND DISCUSSION

3.1 HECSL CHARACTERIZATION BY UHPLC-UV-MS/MS

The UHPLC-UV-MS/MS of HECsL, performed by the relative abundance of the base peak and elution order (Clifford, 2005), revealed the presence of 4 phenolic constituents (caffeoylquinic derivatives, small organic acids, and flavonoids): compound 1 (quinic acid - r.t 4.46 min) displaying deprotanated ion at m/z 191 and fragments at m/z 93 and 85 (De Souza, 2016); compound 2 (3-O-caffeoylquinic acid - r.t. 6.36 min), exhibiting deprotonated molecular ion at 353 with main fragment at m/z 191; compounds 5 (5-hydroxymorin - 10.97 min m/z 319) and 6 (taxifolin - 12.08 min, m/z 305), were identified by comparison with the literature as flavonoids, by means of their fragmentation behavior under CID (Lin *et al*, 2003) (Figure 1).

These chemical components have a variety of effects including the anti-inflammatory effects of the caffeoylquinic acid (Liu *et al*., 2015) and taxofolin (CECHINEL-FILHO *et al*., 2000) and antinociceptive effects of taxofolin (Cechinel-Filho *et al*., 2000). In addition, quinic acid, one of the most common phenolic components among polyphenols, has established antioxidant function (DE SOUZA, 2016). The antioxidant effect is related to the anti-inflammatory due to the increase in leukocyte infiltration the and the amount of reactive oxygen species resulting in oxidative stress (Hussain *et al*., 2016). Inflammation and nociception are present in a variety of pathologies, such as cancer, arthropathies, lupus and psoriasis (Hussain *et al*., 2016). Thus, we can suggest that natural products such as the one investigated in this study, which contain these components in their composition, could in the future be validated as alternatives for reducing the signs and symptoms of inflammatory pathologies.

Figure 1- UHPLC-UV-MS/MS chromatogram of the HECsL. 1- quinic acid; 2- 3-O-caffeoylquinic acid; 5- 5-hydroxymorin; 6 taxifolin.



Peak	tR	*m/z*
1	4.46 min	191 – 9 – 85
2	6.36 min	353 – 191
5	10.97 min	319
6	12.08 min	305

Source: data from the authors

3.2 EFFECT OF HECSL ON ACUTE TOXICITY, MOTOR COORDINATION AND BODY MASS

HECsL given at 60 and 120 mg/kg did not alter the motor coordination of animals evaluated in the Rota rod by the permanence time. Also, HECsL administered at 2000 or 5000 mg/kg did not alter this behavior at 3, 24, 48, 72 hours, and after 15 days compared to controls (data not shown).

Moreover, the body mass of the animals, evaluated before and after 72 hours from the treatment with HECsL did not show alterations compared to the control animals (Table 2).

Acute toxicity studies have the main objective to evaluate the safety of the use of biomolecules from natural source, including medicinal plants via identification of its adverse effects. The lack of change in motor coordination has being described to indicate no sedative effect, and possibly no excessive stress (Ibrahim *et al*., 2016). Therefore, our results indicate that the prophylactic use of HECsL is safe at the rage of doses. Similar effects had been demonstrated for the hydroethanolic extract obtained from the leaves of *C. spruceanum,* of similar composition that HECsL (Da Silva *et al*., 2018).

Table 1 – HECsL does not alter the animal permanence in the Rota rod.

Groups	**Doses**	**Permanence time**						
		0	**1h**	**2h**	**3h**	**24h**	**48h**	**72h**
Control	-	38 ± 11	35 ± 10	46 ± 10	37 ± 10	48 ± 11	46 ± 10	46 ± 12
HECsL	**2000**	33 ± 25	45 ± 20	53 ± 15	52 ± 17	47 ± 17	51 ± 19	49 ± 22
	5000	11 ± 5	15 ± 7	15 ± 7	21 ± 9	33 ± 11	52 ± 7	23 ± 9

Source: Prepared by the authors.

Table 2- HECsL does not alter the animals body mass

	Body mass (g)		
	Initial	**Final**	**Weight Evolution (%)**
Control	37.58 ± 0.61	37.37 ± 1.90	-0.56
HECsL (2000 mg/kg)	35.54 ± 0.36	33.53 ± 1.03	-5.66
HECsL (5000 mg/kg)	40.32 ± 2.80	40.31 ± 2.80	-0.025

Mean ± S.E.M. (n=8). Two-Way ANOVA/Tukey test.
Source: Prepared by the authors.

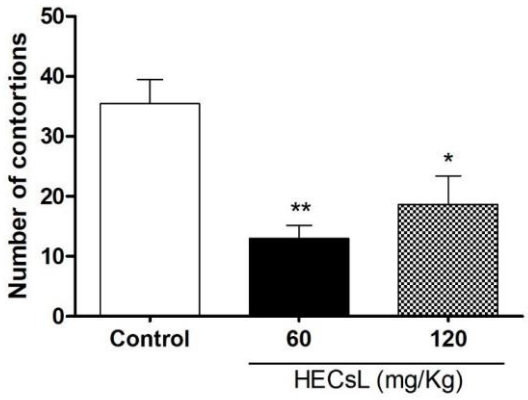
3.3 EFFECT OF HECSL ON THE NOCICEPTION INDUCED BY ACETIC ACID

HECsL at 60 mg/kg reduced by 63% (13 ± 5) the number of writhes induced by 0.8% acetic acid (35 ± 10) and at 120 mg/kg by 47% (18 ± 11 *vs* 35 ± 10 control) (Figure 2).

These data, although the nociception assessment using abdominal contortions is non-specific for nociception in the peripheral nervous system (PNS) and central nervous system (CNS) (Koster *et al*., 1959) may suggest the antinociceptive action of HECsL, since the nociceptive stimuli, whether in the PNS or CNS, are part of the physiology of pain.

Pain is one of the most reported symptoms and the main complaint of patients suffering from the most diverse conditions, whether inflammatory or not (Rocha *et al*., 2007). In this context, therapeutic or prophylactic alternatives such as the one proposed in this study, may be promising for improving the clinical profile of various painful conditions. Similar to our results, taxofolin, a constituent present in HECsL exhibits potent and dose-dependent antinociceptive action in the model of acetic acid-induced abdominal constriction when administered per oral (Cechinel-Filho *et al*., 2000).

Figure 2: EHCsL inhibits abdominal contortions induced by acetic acid. Mean ± S.E.M. One-way ANOVA/Tukey tests. *p<0,05 *vs.* control, **p<0,01 *vs* control.



Source: data from the authors

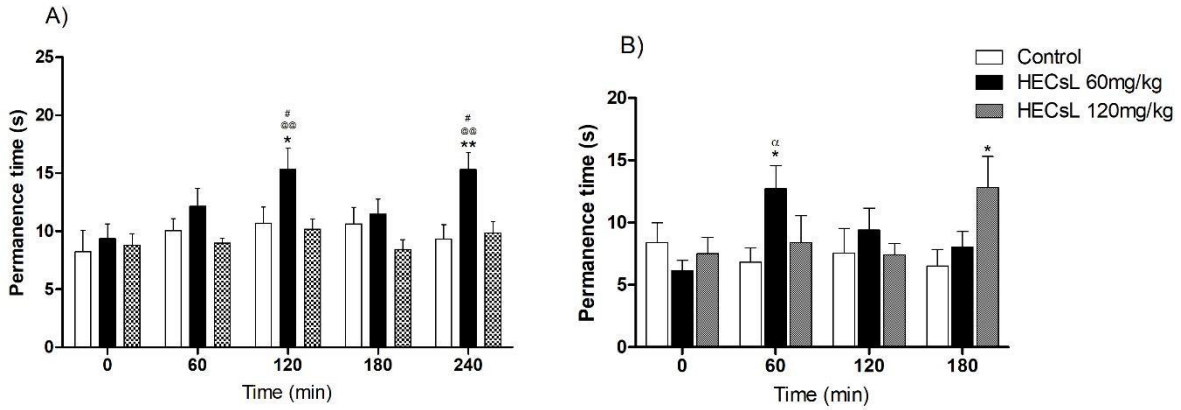
3.4 EFFECT OF HECsL ON NOCICEPTION: HOT PLATE AND TAIL DIPPING TESTS

The analysis of latency of permanence time showed that HECsL at 60 mg/kg increased this behavior in the hot plate by 30% at 120 min (15.30 ± 1. 86 s *vs.* control: 10,7 ± 1,37 s), by 39% at 240 min (15.30 ± 1.45 s *vs.*control: 9,32 ± 1,24 s) (Figure 3A).

In respect to the withdrawal latency, HEScL 60 mg/kg increased the behavior by 23% at 60 min (12.70 ± 1,86 s *vs.* control: 6.83 ± 1.12 s), while at 120 mg/kg it increased the tail withdrawal latency by 49% at 180 min (12.79 ± 2.5 s *vs.* control: 6.50 ± 1.31 s) (Figure 3B).

The tests hot plate (Gupta *et al*., 2005) and tail (D'Amour; Smith, 1941) evaluate nociception in the CNS. Nociception arising from direct sensitization in the CNS is more intense and lasting, being related to chronic pain (Arendt-Nielsen *et al*., 2015). Chronic pain is difficult to be treated (Arendt-Nielsen *et al*., 2015) and its treatment includes non-steroidal anti-inflammatory drugs and glucocorticoids, that may cause a variety of adverse effects, such as dysfunction of the digestive, renal, cardiac, respiratory and nervoussystems, along with in the bone tissue (Dutra *et al*., 2016). In this line, the prophylactic use of HEScL is a protective alternative for chronic pain, showing the advantageus antinociceptive effect on the CNS and reduced toxicity.

Figure 3. EHCsL inhibits hot plate latency and tail dipping. Mean ± S.E.M. Two-way ANOVA/Tukey tests. *p<0,05 vs. control, **p<0,01 vs. control, αp<0,05 vs. time 0, #p<0,05 vs. treatments.



Source: data from the authors

3.5 EFFECT OF HECSL ON PERITONEAL LEUKOCYTE MIGRATION AND HYPERNOCICEPTION STIMULATED BY CARRAGEENAN

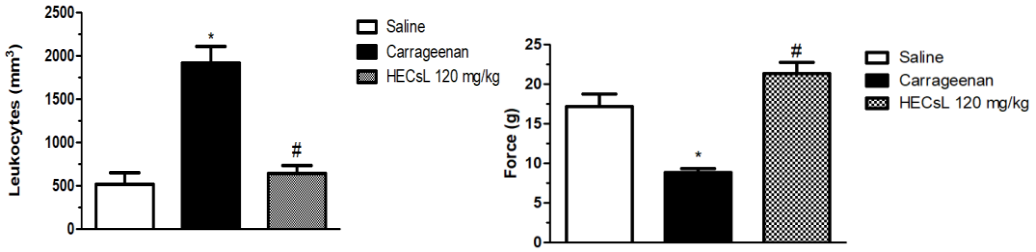
Intraperitoneal administration of carrageenan increased the n° of total leukocytes which migrated to the peritoneal fluid in 3.7-fold (1919 ± 532.5 *vs.* saline: 512.5 ± 392.6 cells/mm^3). HECsL (120 mg/kg, p.o.) reduced the n° of total leukocytes by 67% (637.5 ± 272.2 cells/mm^3) (Fig. 4A). In addition, subcutaneous administration of carrageenan induced paw edema (65. 42 ± 15.39 µl), being reduced by HCsL (43.33 ± 10.99 µl) (data not shown).

Moreover, carrageenan reduced the abdomen nociceptive threshold in response to the mechanical stimulation 3h later (8.83 ± 1.33 *vs.* control: 17.13 ± 4.43 g), inducing hypernociception, which was reversed by HECsL (21.31 ± 4.03 g) (Fig. 4B).

Anti-inflammatory effects were also obtained for the hydroalcoholic extract of *C. spruceanum* bark (HECsB), that possess similar chemical composition to HECsL demonstrated by the reduction of leukocyte migration and paw edema induced by carrageenan. In addition, HECsB presents non-specific antinociceptive effect for the PNS and SNC (Da Silva *et al*., 2018).

Aqueous extract *Calycophyllum spruceanum* bark which contains taxifolin and 5-hydroxymorin, as HECsL, has an antioxidant effect that is related to the anti-inflammatory effect (Peixoto *et al*., 2018). In addition, caffeoylquinic acid, the major component of HECsL, reduced the expression of inflammatory markers including interleukin (IL)-6, monocyte chemotactic protein 1 (MCP-1) and tumor necrosis factor-alpha (TNF-α) in rats (Liu *et al*., 2015) and the leukocyte infiltrate in human kidney cells (Kim *et al*., 2017); and taxofolin had an anti-inflammatory effect demonstrated by the reduction of paw edema elicited by carrageenan, dextran and bradykinin in rats (Cechinel-Filho *et al*., 2000). Given this, we can suggest that the antinociceptive and anti-inflammatory effects of HECsL are associated to its chemical components. However, additional studies are needed to evaluate the molecular mechanisms involved in the effects of HECsL.

Figure 4. EHCsL inhibits peritoneal leukocyte migration and hypernociception induced by carrageenan. Mice received HECsL (120 mg/kg; P.O.) 1 h before carrageenan (500 µg; I.P.). Control animals received saline (0.9% NaCl; I.P.). After 4 h, peritoneal fluid was collected for evaluation of (A) Total leukocytes (cells/mm^3), (B) Hypernociception (g). Mean ± S.E.M. (n=8). One-way ANOVA/Bonferroni. *p<0,05 *vs.* control, *p<0,05 *vs.* control, $^{\#}$p<0,05 *vs.* treatments.



Source: data from the authors

4 CONCLUSIONS

The hydroalcoholic extract of *C. spruceanum* leaves, with a chemical composition similar to alcoholic extracts from other parts recorded in the literature was also presented anti-inflammatory and antinociceptive effects on peripheral and central nervous systems in *Swiss* mice, being devoid of acute toxicity. The foliage is the most renewable part of the plant. Rational removal of part of the leaves is less harmful, making the choice less impactful on the integrity of the plant, when large-scale production is intended.

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