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**ORIGINAL ARTICLE**

# Pharmacological properties of lichen *Cladonia clathrata*

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**Abstract**

*Cladonia clathrata* Ahti & L. Xavier-Filho (Cladoniaceae) is a lichen; several *Cladonia* species extracts have been used for various remedies in folk medicine. In order to evaluate the actions of this lichen, studies were performed on antinociceptive, anti-inflammatory, and antioxidant activities. The hydroalcoholic extract (HE) of *C. clathrata* stems was used in the following experiments. Oral treatment with the HE of *C. clathrata* elicited inhibitory activity ($p < 0.001$) on acetic acid-induced abdominal writhes at 100 (47.2%), 200 (47.2%), and 400 mg/kg (86.4%), and reduced the formalin-induced nociception on both the neurogenic (400 mg/kg, $p < 0.01$) and inflammatory phases (200 and 400 mg/kg, $p < 0.01$). It was not associated with non-specific effects, such as muscle relaxation or sedation. The HE reduced the carrageenan-induced edema formation at 100, 200, and 400 mg/kg ($p < 0.05$) and inhibited neutrophil migration into the peritoneal cavity at 400 mg/kg ($p < 0.001$). The HE of *C. clathrata* reacted with the DPPH radical and reduced the same by 50.19%, and exhibited an $IC_{50}$ value of $69.25 \pm 0.65$ μg/mL. The HE of *C. clathrata* stems shows antinociceptive and anti-inflammatory activities, with a moderate antioxidant potential.

**Keywords:** *Anti-inflammatory activity; antinociceptive activity; antioxidant potential; Cladonia clathrata; lichen*

## Introduction

Lichens are symbiotic organisms composed of fungi (mycobionts) and algae and/or cyanobacteria (photobionts) (Ingólfsdóttir, 2002). They produce characteristic secondary metabolites that are unique with respect to those of higher plants. Several lichen extracts have been used for various remedies in folk medicine, and screening tests with lichens have indicated the frequent occurrence of metabolites with antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antiproliferative, antipyretic, and cytotoxic properties (Kumar & Muller, 1999; Muller, 2001; Süleyman et al., 2003; Bucar et al., 2004; Omarsdottir et al., 2007). In addition to their reputed effectiveness in the treatment of many diseases, some lichens have also been used traditionally as medicinal plants to treat bronchitis and inflammatory disorders (Kumar & Muller, 1999; Bucar et al., 2004). Leukotrienes (LT), the products of 5-lipoxygenase (5-LO) metabolism

of arachidonic acid, play an important role in a variety of pathophysiological states in humans, particularly those involving inflammation, and it has previously been shown that these mediators might be influenced by lichen-derived compounds (Bucar et al., 2004). Other literature reports of biological activities of these naturally occurring compounds are scarce. This may partly be due to the difficulties encountered with collecting substantial amounts of plant material, as most lichen species grow as scattered patches, mainly on stones or on tree trunks.

Of the hundreds of known secondary lichen metabolites, the dibenzofuran derivative usnic acid is without doubt the most extensively studied. Usnic acid is widely distributed in species of *Cladonia* (Cladoniaceae), *Usnea* (Usneaceae), *Lecanora* (Lecanoraceae), *Ramalina* (Ramalinaceae), *Evernia*, *Parmelia* (Parmeliaceae), and other lichen genera (Ingólfsdóttir, 2002). Studies have been conducted in which the anti-inflammatory activity of (+)-usnic acid was compared to

that of ibuprofen using the rat paw edema assay (acute effects) and the cotton pellet assay (chronic effects) (Vijayakumar et al., 2000). Usnic acid and the depside diffractaic acid were identified as active components of an *Usnea diffracta* Vain. methanol extract, showing analgesic and antipyretic activities in mice (Okuyama et al., 1995; Ingólfsdóttir, 2002). Polysaccharides from lichens have shown various biological activities, such as antitumor, anticoagulant, antithrombotic, and immunomodulating activities, and studies were carried out in an attempt to show the importance of polysaccharides as an additional tool in chemotyping (Carbonero et al., 2001; Martinichen-Herrero et al., 2005; Omarsdottir et al., 2007). Many *Cladonia* species have been used in the treatment of pulmonary tuberculosis (Ingólfsdóttir, 2002). Anti-inflammatory, analgesic, and antipyretic effects have been suggested to be linked to the inhibition of prostaglandin synthesis, owing to the uncoupling effects on oxidative phosphorylation (Ingólfsdóttir, 2002).

The goal of the present study was to evaluate the antinociceptive, anti-inflammatory, and antioxidant effects of the hydroalcoholic extract (HE) from *Cladonia clathrata* Ahti & L. Xavier-Filho stems.

## Materials and methods

### Plant material

*Cladonia clathrata* was collected in March 2007, in Itabaiana county, Sergipe State, northeastern Brazil (10°44′S, 37°23′W). The lichen was identified by Professor Marcelo Pinto Marcelli (Botanical Institute of São Paulo/SP, Brazil), with voucher number SP 393248. Prior to extraction, stems were dried at 40°C in a forced air oven (Marconi MA 037) for 24 h.

### Extraction of Cladonia clathrata

The dried stems of *C. clathrata* (200 g) were powdered, and extracted by maceration at room temperature with 70% alcohol (1000 mL) for 10 days. The extract of *C. clathrata* was filtered under vacuum, the solvent was removed using a rotary evaporator, and the resulting semisolid mass was vacuum-dried in a desiccator; the solid residue (hydroalcoholic extract) was stored at 5°C until being re-suspended in saline at different concentrations.

### Total phenolics and polysaccharides

The quantitative analysis of total phenolics was based on the method described by Bonoli et al. (2004), with minor modifications. *C. clathrata* hydroalcoholic extract

(HE) (10 mg) was dissolved in 10 mL of methanol. An aliquot of 7.5 mL was diluted in 42.5 mL of methanol. One hundred microliters of this solution were added to 500 μL of Folin–Ciocalteu reagent diluted 1:10 with distilled water and mixed during 1 min, then 2 mL of 20% $Na_2CO_3$ solution was added and mixed again during 30 s. After 2 h, the absorbance at 750 nm was read. Gallic acid was used to obtain the calibration curve. The measurements were carried out in triplicate. The results are expressed as mg gallic acid/g of the HE ± standard deviation.

The polysaccharide content was measured by the phenol–sulfuric acid colorimetric method (Dubois et al., 1956), using a standard curve of glucose solution. *C. clathrata* HE (10 mg) was dissolved in 1 mL of water and afterward diluted in 1 mL of a phenol solution, then 5 mL of concentrated sulfuric acid was added and the absorbance at 490 nm was read. The measurements were carried out in triplicate. The results are expressed as mg polysaccharide/g of the HE ± standard deviation.

### Animals

Swiss mice (20–30 g) and Wistar rats (120–180 g) of both sexes were obtained from the Central Biotery of the Federal University of Sergipe (São Cristóvão, Brazil). Animals were randomly assigned to groups and maintained in plastic boxes at controlled room temperature (25–28°C) with free access to food and water, under a 12:12 h light/dark cycle. All the experimental procedures were carried out during the light period of the day (08:00 a.m. to 05:00 p.m.) and complied with the guidelines on animal care of the Federal University of Sergipe Ethics Committee for Animal Use in Research, conducted in accordance with the internationally accepted principles for laboratory animal use and care. Animals submitted to oral administration of the extract or drugs were fasted for 12 h before the experiments and acclimatized for at least 2 h before the experiments. All efforts were made to minimize the number of animals used and their suffering.

### Chemicals and drugs

The following chemicals and drugs were used: aspirin (acetylsalicylic acid, ASA), butylated hydroxytoluene (BHT), carrageenan, dexamethasone, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), glucose, and morphine hydrochloride from Sigma Chemical Co. (St. Louis, MO, USA); acetic acid from Merck (Damstadt, Germany); formalin from Baker (Santo Amaro, SP, Brazil); solvents from Vetec (Rio de Janeiro, RJ, Brazil). All substances used were dissolved in saline solution, with the exceptions of ASA which was dissolved in 5%

Tween 80 in 0.9% NaCl solution, and BHT, gallic acid, and DPPH that were dissolved in methanol. The final concentration of Tween 80 did not exceed 5% and did not cause any effect *per se*.

## Nociceptive tests

### Acetic acid-induced abdominal writhes
Abdominal writhes consisted of a contraction of the abdominal muscle together with a stretching of the hind limbs (Tonos et al., 1999), induced by intraperitoneal (i.p.) injection in mice of acetic acid (0.6% solution, 0.1 mL/10 g), the nociceptive agent (Koster et al., 1959).

The animals were pre-treated with *C. clathrata* HE (100, 200, or 400 mg/kg) orally (per os, p.o.) 60 min before initiating algesic stimulation, or with aspirin (ASA, 300 mg/kg, p.o., 60 min before the administration of acetic acid), used as positive control ($n = 6$/group). The abdominal writhes were observed, in separate individual chambers, for a period of 20 min, starting after the administration of acetic acid.

### Formalin test
The formalin test was applied according to the method of Dubuisson and Dennis (1977), modified by Hunskaar and Hole (1987).

Mice were pre-treated with *C. clathrata* HE (100, 200, or 400 mg/kg, p.o., 60 min before the administration of formalin), or with morphine hydrochloride (10 mg/kg, i.p., 30 min before the administration of formalin) or ASA (300 mg/kg, p.o., 60 min before the administration of formalin), used as positive controls, before intraplantar injection of 1% formalin solution (20 μL) into the right hindpaw of the animal ($n = 6$/group). The control group received vehicle, saline (0.1 mL/10 g, p.o., 60 min before the administration of formalin, $n = 6$).

The time that the animal spent licking or biting its paw was measured during the first phase (0–5 min) and the second phase (20–25 min) of the test.

## Anti-inflammatory activity

### Measurement of paw edema in rats
The anti-inflammatory activity was studied using the paw edema model induced by 1% carrageenan, administered at a volume of 0.1 mL/animal into the subplantar region of the right hindpaw of the rat (Winter et al., 1962). The volume of the paw was measured by removal of the water column using a hydroplethysmometer (model 7150; Ugo Basile, Varese, Italy), at time 0 and the intervals of 1, 2, 3, and 4 h immediately after the subplantar injection of carrageenan (Harris & Spencer, 1962).

The HE of *C. clathrata* at the dose of 100, 200, or 400 mg/kg, ASA (300 mg/kg), and vehicle were administrated p.o. 1 h before the edematogenic agent to different groups of animals for each treatment ($n = 6$/group).

The data obtained for the various groups are reported as mean ± SEM and expressed in milliliters. The percentage inhibition in the edema experiment was calculated based on the area under the curve (AUC) after 4 h.

### Neutrophil migration into the peritoneal cavity
Leukocyte migration was induced by injection of carrageenan (1%, 250 μL, i.p.) into the peritoneal cavity of mice 1 h after administration of the HE of *C. clathrata* (100, 200, and 400 mg/kg, p.o., $n = 6$) or dexamethasone [2 mg/kg, subcutaneous (s.c.), $n = 6$] by modification of the technique previously described by Matos et al. (2003). The animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and were euthanized by cervical dislocation 4 h after carrageenan injection. Shortly after, saline containing ethylenediaminetetraacetic acid (EDTA; 1 mM, i.p., 3 mL) was injected. Immediately a brief massage was done for further fluid collection, which was centrifuged (2000 rpm, 5 min) at room temperature. The supernatant was disposed of and the precipitate was re-suspended in 300 μL of saline. An aliquot of 10 μL from this suspension was dissolved in 200 μL of Turk solution and the total cells were counted in a Neubauer chamber, under optical microscopy. The results are expressed as the number of neutrophils/mL. The percentage of leukocyte inhibition $= (1 - T/C) \times 100$, where $T$ represents the treated group leukocyte count and $C$ represents the control group leukocyte count.

## Measurement of locomotor activity

In order to evaluate a possible non-specific muscle-relaxant or sedative effect of the extract, mice were submitted to the open-field test. The ambulatory behavior was assessed in an open-field test as previously described (Rodrigues et al., 2002). The apparatus consisted of a wooden box measuring $40 \times 60 \times 50$ cm. The floor of the arena was divided into 12 equal squares, and the numbers of squares crossed with all paws were counted in a 6-min session. Mice were pre-treated with *C. clathrata* HE (100, 200, or 400 mg/kg, p.o., $n = 6$) or vehicle (0.1 mL/10 g, p.o., $n = 6$) 60 min beforehand.

## Quantitative assay of antioxidant activity

The quantitative analysis of antioxidant activity was based on the method described by Brand-Williams et al. (1995) and Sánchez-Moreno et al. (1998), with minor modifications. The scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was followed by monitoring the decrease

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in absorbance at 515 nm, which occurred due to reduction by the antioxidant.

The calibration curve was established by preparing dilutions of a DPPH radical stock solution (100 μg/mL) to obtain final concentrations of 5, 10, 15, 20, 25, 30, 60, 65, and 70 μg/mL. The absorbance of each standard concentration was then monitored in a spectrophotometer (UV BEL Photonics 1105) at 515 nm. The measurements were carried out in triplicate with intervals of 1 min. The equation of the concentration vs. absorbance calibration curve for the DPPH radical was $C = -0.01923 + 0.0284A$, where $C$ is the concentration of the DPPH radical in the medium, and $A$ is the absorbance at 515 nm. The correlation coefficient was $R = 0.9918$.

A solution containing 500 μg/mL of *C. clathrata* HE was prepared in methanol, and diluted in concentrations of 30, 60, 90, 120, 150, 180, 360, 390, and 420 μg/mL. The disappearance of the DPPH radical was monitored by the decrease in absorbance at 515 nm, which was recorded after 0, 1, 5, and 10 min, and subsequently every 10 min up to 60 min (Sousa et al., 2007). The negative control was pure methanol used for dissolving the samples, while the positive controls were gallic acid and butylated hydroxytoluene (BHT) dissolved in methanol at concentrations of 30, 60, 90, 120, 150, 180, 360, 390, and 420 μg/mL. The mixture of methanol and HE was used as a blank.

The concentration of DPPH radical in the reaction mixture was calculated based on the calibration curve, where [DPPH] is expressed in μg/mL. The percentage of remaining DPPH (%DPPH$_{REM}$) was calculated according to Brand-Williams et al. (1995), as follows: %DPPH$_{REM}$ = [DPPH]$_T$/[DPPH]$_{T0}$ × 100, where $T$ is the time when absorbance was determined (1–60 min) and $T_0$ is time zero. The amount of antioxidant necessary to decrease the initial concentration of DPPH radical by 50% (IC$_{50}$) was calculated by plotting %DPPH$_{REM}$ at time 60 min with 420 μg/mL of extract. The results are expressed as μg antioxidant/mL DPPH ± standard deviation.

The absorbance values observed in all samples at 60 min (420 μg/mL) were transformed into percentage of inhibition (IP), determined by the equation: IP = {[Abs$_{control}$ – (Abs$_{sample}$ – Abs$_{blank}$)] × 100}/Abs$_{control}$, where Abs$_{control}$ is the initial absorbance of the methanol solution of the DPPH radical and Abs$_{sample}$ is the absorbance of the reaction mixture (DPPH + sample). The results are expressed as % of inhibition.

### Statistical analysis

The results for antinociceptive, anti-inflammatory, and locomotor activities are presented as the mean ± SEM of $n$ animals per group. The antioxidant activity was determined using Origin version 7.5 (Microcal, Northampton, MA, USA), and values are presented as the mean ± standard deviation of three assays. Statistical evaluation of the data was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's or Tukey's test; $p$ values less than 0.05 were considered significant.

## Results

### Total phenolics and polysaccharides

The concentration of total phenolics expressed as mg gallic acid/g of HE was 371.8 ± 57.3, and the concentration of total polysaccharides was 198.0 ± 5.5 mg/g of HE.

### Acetic acid-induced writhing in mice

The results in Figure 1 show that *C. clathrata* stem HE given p.o. (100–400 mg/kg, $n$ = 6/group) 1 h before the administration of acetic acid caused an inhibition of 47.2, 47.2, and 86.4% ($p < 0.001$) on acetic acid-induced writhes at the doses of 100, 200, and 400 mg/kg, respectively. ASA (300 mg/kg, $n$ = 6) exhibited significant (90.4%, $p < 0.001$) inhibition of the control writhes in acetic acid-induced writhing.

### Formalin reaction time in mice

As shown in Figure 2, *C. clathrata* HE administered orally (100–400 mg/kg, 1 h before the administration of formalin, $n$ = 6/group) produced marked inhibition on intraplantar injection of formalin in mice both against neurogenic pain (first phase) at the dose of 400 mg/kg
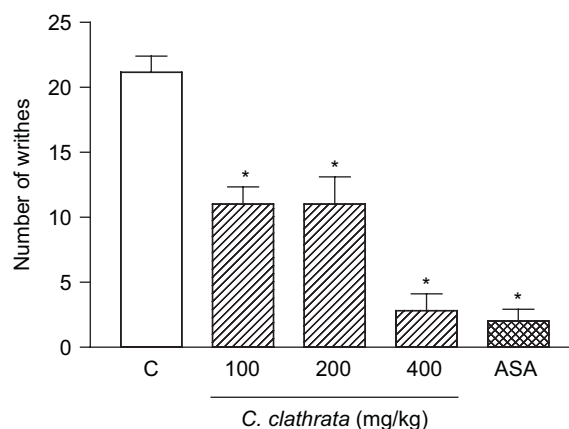


**Figure 1.** Influence of *C. clathrata* hydroalcoholic extract (HE) in nociceptive behavior of mice evaluated in acetic acid-induced abdominal writhing model. Nociception was registered by the number of writhes that the animal presented 20 min following i.p. acetic acid (0.6%) injection. Groups of animals were pre-treated with vehicle (C, control group, $n$ = 6, open column), aspirin (acetylsalicylic acid, ASA, 300 mg/kg, $n$ = 6, cross-hatched column), or HE (100–400 mg/kg, $n$ = 6/group, right-hatched columns), p.o., 60 min before acetic acid. Each column represents the mean ± SEM. Asterisks denote statistical significance, *$p < 0.001$, in relation to control group. ANOVA followed by Bonferroni's test.
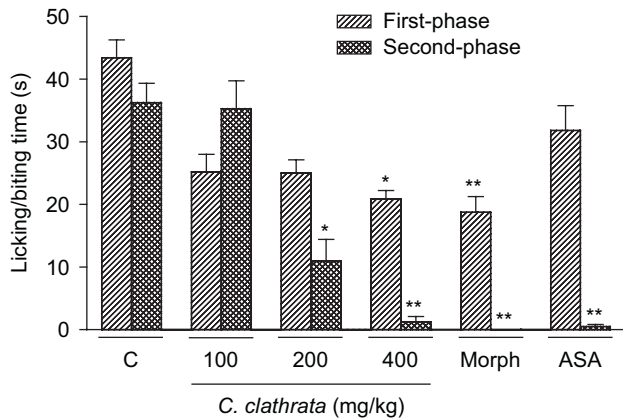
**Figure 2.** Effect of *C. clathrata* HE in nociceptive behavior of mice evaluated in formalin-induced nociception model. Groups of mice were pre-treated with vehicle (column C, control group, 10 mL/kg, p.o., 60 min before the administration of formalin, *n* = 6), aspirin (acetyl-salicylic acid, ASA, 300 mg/kg, p.o., 60 min before the administration of formalin, *n* = 6), morphine (Morph, 10 mg/kg, i.p., 30 min before the administration of formalin, *n* = 6), or *C. clathrata* HE (100–400 mg/kg, p.o., 60 min before the administration of formalin, *n* = 6/group) against the early phase (0–5 min, right-hatched columns) or late phase (20–25 min, cross-hatched columns) of formalin-induced nociception in mice. Each column represents the mean ± SEM. Asterisks denote statistical significance, *$p < 0.01$ and **$p < 0.001$, in relation to control group. ANOVA followed by Bonferroni's test.

($p < 0.01$) and against inflammatory pain (second phase) at the doses of 200 ($p < 0.01$) and 400 mg/kg ($p < 0.001$). ASA (300 mg/kg, p.o., 60 min before the administration of formalin) caused inhibition of the second phase of formalin-induced nociception ($p < 0.001$, *n* = 6, Figure 2). Morphine (10 mg/kg, i.p., 30 min before the administration of formalin) caused significant inhibition of both phases of formalin-induced nociception ($p < 0.001$, *n* = 6, Figure 2).

### Carrageenan-induced paw edema in rats

As observed in Figure 3, the single oral treatment of rats with the HE of *C. clathrata* (100–400 mg/kg, 1 h before the administration of carrageenan, *n* = 6/group) was capable of reducing ($p < 0.05$) the edema formation induced by carrageenan (1%, 100 μL/paw), an effect observed at 3 and 4 h after the administration of this phlogistic agent. Likewise, ASA (300 mg/kg, p.o., 1 h before the administration of carrageenan, *n* = 6) significantly inhibited ($p < 0.001$) the edematogenic response evoked by carrageenan in rats, at 1, 2, 3, and 4 h (Figure 3).

The mean AUC found in carrageenan-treated rats was $1.67 \pm 0.04$ mL × h. (*n* = 6). Based on AUC values, the HE at 100, 200, and 400 mg/kg caused 21.6, 22.2, and 28.1% ($p < 0.05$) of inhibition on the edema response, respectively (*n* = 6/group). ASA at 300 mg/kg (*n* = 6) caused an inhibition of 47.9% ($p < 0.001$).
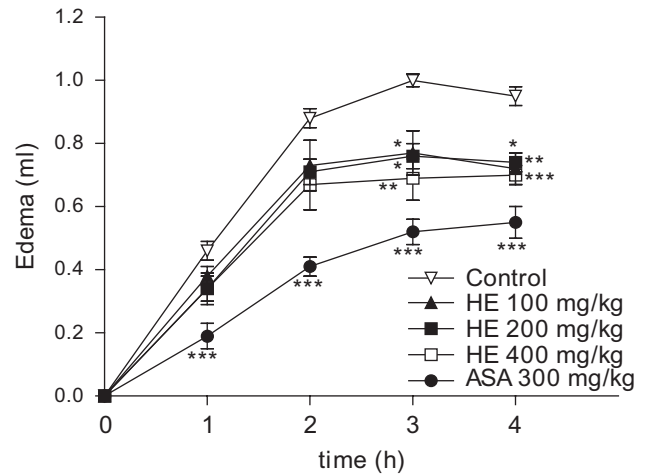


**Figure 3.** Effect of *C. clathrata* HE on rat paw edema induced by carrageenan. Groups of rats were pre-treated with vehicle (control group, 10 mL/kg, p.o., *n* = 6), aspirin (acetylsalicylic acid, ASA, 300 mg/kg, p.o., *n* = 6), or *C. clathrata* HE (100–400 mg/kg, p.o., *n* = 6/group) 60 min before carrageenan-induced paw edema. Measurements were performed at times 0, 1, 2, 3, and 4 h after the subplantar injection of carrageenan (1%, 100 μL). Each value represents the mean ± SEM. Asterisks denote statistical significance, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, in relation to control group. ANOVA followed by Bonferroni's test.
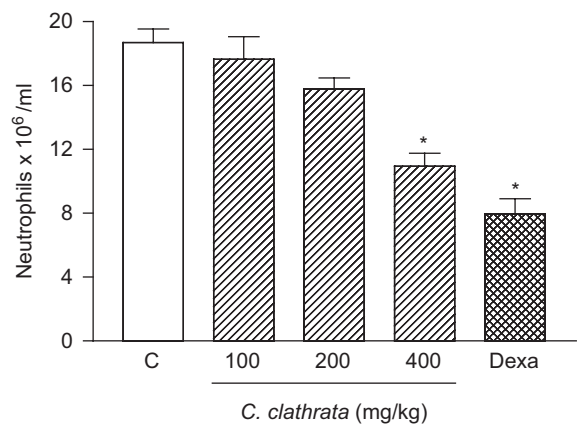


**Figure 4.** Effect of *C. clathrata* HE on leukocyte migration into the peritoneal cavity induced by carrageenan in mice. Groups of animals were pre-treated with vehicle (C, control group, 10 mL/kg, p.o., open column), dexamethasone (Dexa, 2 mg/kg, s.c., cross-hatched column), or HE at the doses of 100, 200, and 400 mg/kg (p.o., right-hatched columns) 60 min before carrageenan (1%, 250 μL, i.p.)-induced peritonitis. Cell counts were performed at time 4 h after the injection of carrageenan. Each value represents the mean ± SEM (*n* = 6/group). Asterisks denote statistical significance, *$p < 0.001$, in relation to control group. ANOVA followed by Bonferroni's test.

### Carrageenan-induced peritonitis in mice

Carrageenan (1%, 250 μL) induced neutrophil migration into the peritoneal cavity 4 h after stimulus (Figure 4). Figure 4 shows the inhibitory effect of the HE of *C. clathrata* (41.5% at the dose of 400 mg/kg, $p < 0.001$, *n* = 6) on the carrageenan-induced response.

The results obtained with the control group support the effect of the HE of *C. clathrata* stems, since the vehicle presented no activity, and the control drug dexamethasone (2 mg/kg, s.c., 1 h before the administration of carrageenan, $n = 6$) inhibited (57.4%, $p < 0.001$) carrageenan-induced neutrophil migration into the peritoneal cavity (Figure 4).

### Evaluation of locomotor activity

The HE of *C. clathrata* (100–400 mg/kg, p.o.) did not affect the locomotor activity in the open-field test when compared with animals that received vehicle (control group). The mean numbers ± SEM of crossed squares were $121.0 \pm 3.1$, $120.2 \pm 2.7$, $121.7 \pm 3.3$, and $126.2 \pm 2.8$ for the control, 100, 200, and 400 mg/kg groups, respectively.

### Antioxidant activity

The amount of DPPH radical that reacted with the stem HE (420 μg/mL, 60 min, $n = 3$) was 50.19%, while for the positive controls BHT and gallic acid (420 μg/mL, 60 min, $n = 3$) the amount of DPPH radical used was higher at 96% (96.91 and 96.24%, respectively).

The $IC_{50}$ value for the HE of stems was higher ($69.25 \pm 0.65$ μg/mL DPPH) as compared with the $IC_{50}$ values for the reference compounds BHT and gallic acid ($12.33 \pm 3.25$ and $9.33 \pm 0.22$ μg/mL DPPH, respectively, $n = 3$).

## Discussion

The present study demonstrates that *C. clathrata* HE displays antinociceptive and anti-inflammatory properties with a moderate antioxidant potential, and provides some evidence on the mechanisms implicated in these effects.

For the first time, this work shows that *C. clathrata* HE p.o. produces significant antinociception according to assessment of the abdominal writhes elicited by acetic acid and of formalin-induced pain. In addition, the doses of the extract that caused significant antinociception did not produce any statistically significant motor dysfunction.

It has been suggested that acetic acid acts by releasing endogenous mediators that stimulate the nociceptive neurons (Collier et al., 1968). This method is sensitive to non-steroidal anti-inflammatory drugs (NSAIDs) and to narcotics and other centrally acting drugs (Collier et al., 1968; Santos et al., 1998; Reichert et al., 2001).

Ribeiro et al. (2000) have demonstrated that the nociceptive activity of acetic acid may be due to the release of cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-8, by resident peritoneal

macrophages and mast cells. Thus, the previous findings and the results presented herein might indicate that the antinociceptive action of the HE of *C. clathrata* in the acetic acid-induced writhing test could be due to inhibition of the release of TNF-α, IL-1β, and IL-8 by resident peritoneal cells. However, this possibility remains to be tested in future studies.

The results of the present study have also shown that morphine is largely effective in preventing both the first and second phases of formalin-induced pain. Morphine and opioid drugs exert their antinociceptive activities by performing in receptors (μ, κ, and δ) and thereby modulating the stimulus transmission without interfering in the cause. It is well known that NSAIDs (such as aspirin, acetaminophen, and diclofenac), known to inhibit cyclooxygenase (COX) activity, are largely ineffective or cause very weak inhibition at the first phase of the formalin test (Hunskaar & Hole, 1987; Malmberg & Yaksh, 1992; Santos et al., 1998). In addition, NSAIDs can attenuate, in a dose-related manner, the second phase of formalin-induced licking (Hunskaar & Hole, 1987; Malmberg & Yaksh, 1992; Santos et al., 1998). Previous studies have shown that formalin releases various inflammatory mediators such as substance P, neurokinins A and B, and prostaglandins (Hunskaar et al., 1986; Hunskaar & Hole, 1987; Santos & Calixto, 1997). Our results show, however, that *C. clathrata* HE p.o. produced inhibition on both the first (neurogenic nociception) and the second phase (inflammatory nociception) of the formalin test in mice, similar to the morphine effect. These data suggest that the HE of stems can produce antinociceptive action through a contribution of opioid receptor-mediated mechanisms and/or inhibition of COX, and consequently prostaglandin synthesis. However, the possibility that *C. clathrata* acts on COX remains to be tested in future studies.

The anti-inflammatory effect of *C. clathrata* HE was evaluated in carrageenan-induced paw edema and peritonitis models. In rats, the inflammatory response induced by carrageenan in the paw edema model is characterized by a biphasic response with marked edema formation resulting from the rapid production of several inflammatory mediators such as histamine, serotonin, and bradykinin, which is subsequently sustained by the release of prostaglandins and nitric oxide (NO) with a peak at 3 h, produced by inducible isoforms of COX (COX-2) and nitric oxide synthase (iNOS), respectively (Seibert et al., 1994; Nantel et al., 1999). In the present work, previous oral treatment with the HE of *C. clathrata* was effective in reducing the edematogenic response evoked by carrageenan in rats between the third and fourth hours after the injection. This evidence allows us to suggest that the anti-inflammatory action of the HE of *C. clathrata* is related to the inhibition of one or more intracellular signaling pathways involved in the effects of several inflammatory mediators.

Cell recruitment during inflammation depends on the orchestrated release of local mediators which are responsible for local vascular and tissue changes as well as for the recruitment of host defense cells (Luster et al., 2005). The inflammation induced by carrageenan involves cell migration, plasma exudation, and production of mediators, such as NO, prostaglandin $E_2$, IL-1β, IL-6, and TNF-α (Salvemini et al., 1996; Loram et al., 2007). These mediators are able to recruit leukocytes, such as neutrophils, in several experimental models. The HE inhibited leukocyte migration induced by i.p. injection of carrageenan (in a peritonitis model). A putative mechanism associated with this activity may be inhibition of the synthesis of many inflammatory mediators whose involvement in cell migration is well established.

The DPPH test is a very convenient method for screening small antioxidant molecules because the intensity of the reaction may be analyzed by a simple spectrophotometric assay (Sánchez-Moreno et al., 1998; Soler-Rivas et al., 2000). The DPPH radical is scavenged by antioxidants through the donation of hydrogen to form the stable reduced DPPH molecule. The radicals are stabilized through the formation of non-radical products by reaction with antioxidant agents (Argolo et al., 2004).

The capacity for scavenging free radicals was evaluated for the HE of lichen *C. clathrata*. IP and $IC_{50}$ values are considered to be good measures of the antioxidant efficiency of extracts and pure compounds. Typical IP values for plant materials with acknowledged potent antioxidant activities are around 70–90%, as observed for the stem bark (93%) and leaves (75%) of *Cassia fistula*, a traditional Indian medicine (Siddhuraju et al., 2002), and for the edible seeds (52–80%) of *Rosa rubiginosa* (Moure et al., 2001). Less potent materials show IP values in the range of 9–14%, as for example the seeds of *Gevuina avellana* (Moure et al., 2001). Our results showed that the HE of *C. clathrata* stems has a moderate antioxidant potential. It is thus suggested that this antioxidant effect is unrelated to the functional (antinociceptive and anti-inflammatory) activities of the HE.

Polysaccharides stimulate a wide range of immune responses, such as cytokine release, generation of reactive oxygen species, generation of NO, release of arachidonic acid metabolites, and activation of NF-κB by macrophages (Schepetkin & Quinn, 2006). Thus, it is clear that polysaccharides from lichen have significant therapeutic potential that can be related to the antinociceptive and anti-inflammatory effects.

In conclusion, our study revealed that the HE of *C. clathrata* stems shows antinociceptive and anti-inflammatory activities that do not depend on its antioxidant activity. The identification and the isolation of such bioactive components are in progress, which could elucidate the antinociceptive and anti-inflammatory properties of *C. clathrata*.

## Declaration of interest

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