



Original Article

 Anti-inflammatory action of ethanolic extract and clerodane diterpenes from *Casearia sylvestris*

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ABSTRACT

The present study aimed to investigate the anti-inflammatory activity of ethanolic extract from *Casearia sylvestris* Sw., Salicaceae, leaves and to identify the compounds responsible for this activity. The ethanolic extract from *C. sylvestris* leaves was fractionated by solid phase extraction and the chemical composition of extract and fractions were assessed by chromatographic techniques. Casearin-like clerodane diterpenes were quantified in ethanolic extract (27.4%, w/w) and in fraction 2 of solid phase extraction (50.6%, w/w). Carrageenan-induced paw edema and carrageenan-induced pleurisy assays (rats) were used to evaluate anti-inflammatory activity of ethanolic extract, its fractions and clerodane diterpenes from *C. sylvestris* – caseargrewiin F and casearin B. The ethanolic extract was tested in the rat paw edema model and the doses tested (10 and 100 mg/kg) had no effect. In the pleurisy model, the extract doses of 300 and 500 mg/kg showed inhibitory effect. The fraction 2 of solid phase extraction (10 mg/kg), caseargrewiin F and casearin B (0.5 mg/kg) showed a significant reduction ($p < 0.05$) of the carrageenan-induced paw edema in rats compared to indomethacin. Gastric ulcers were not observed in animals treated with samples from *C. sylvestris*. In conclusion, the ethanolic extract from *C. sylvestris*, its enriched fraction of clerodane diterpenes, casearin B and caseargrewiin F exhibited anti-inflammatory activity on *in vivo* models in rats. Casearin-like clerodane diterpenes may be considered active chemical markers for *C. sylvestris* leaves. On the other hand, these diterpenes are promising compounds in the development of new drugs with anti-inflammatory action without gastric side effects.

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Introduction

Casearia sylvestris Sw., Salicaceae, is a tree or shrub widely used in Brazilian folk medicine in which is primarily known as “guaçatonga”, a Tupi-Guarani name. This species is geographically distributed throughout Latin America and in Brazil occurs in many biomes as Atlantic Forest, Cerrado and Amazon Forest. In a review of the plant, Ferreira et al. (2011) described many traditional uses and pharmacological properties emphasizing anti-ulcer, anti-inflammatory, anti-tumor and anti-venom serum activities

of *C. sylvestris*. The Brazilian Health Surveillance Agency (Anvisa) included *C. sylvestris* in herbal form of the Brazilian Pharmacopoeia (Formulário de Fitoterápicos, 2011) – an infusion of dried leaves indicated as anti-dyspeptic. The hydroethanolic or aqueous extracts of its fresh leaves have been widely used in Brazil as topical wound healing and anti-inflammatory agent (Silva, 1926; Hoehne, 1939; Correa, 1975).

Regarding the anti-inflammatory activity, the aqueous extract (Ruppelt et al., 1991), the hydroethanolic extract (Silva et al., 2004; Albano et al., 2013) and the essential oil (Esteves et al., 2005) from *C. sylvestris* leaves showed inhibitory effect in inflammation models. However, none of these studies used the ethanolic extract from the leaves that presented antiulcerogenic effect in models of acute and chronic ulcers in rats (Sertié, 1990). Thus, it became relevant

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to evaluate the anti-inflammatory activity of ethanolic extract from the leaves because one of the main constraints of anti-inflammatory non-steroidal drugs is the appearance of peptic ulcers (Wallace, 2008). Furthermore, these studies did not investigate the possible compounds involved in anti-inflammatory activity.

In scientific literature there are a diversity of secondary metabolites described for *C. sylvestris* leaves: monoterpenes, sesquiterpenes (Sousa et al., 2007), nor-isoprenoids (Santos, 2008; Wang et al., 2009), diterpenes (Itokawa et al., 1990; Morita et al., 1991; Santos et al., 2007), triterpenes, lapachol, caffeic acid, chlorogenic acid and vanillic acid, flavonoids (Raslan et al., 2002), neolignans (Wang et al., 2010), ellagic and gallic acid derivatives (Silva et al., 2008) among other compounds. In terms of chemotaxonomy *Casearia* genus has been characterized by the presence of typical highly oxygenated tricyclic *cis*-clerodane diterpenes with tetrahydrofuran ring with two acyloxy groups (Xia et al., 2015). Twenty eight clerodane diterpenes of this type were isolated from *C. sylvestris* including casearins A–X and caseargrewiin F that showed pronounced antitumor activity (Ferreira et al., 2011). Clerodane diterpenes also showed antiulcerogenic activity as demonstrated by Santos (2008). In the same study, quantification of total casearin-like diterpenes in ethanolic extract was determined as 18.1% (w/w) using HPLC-PDA.

Therefore, the present study aimed to investigate the anti-inflammatory activity of ethanolic extract from *C. sylvestris* leaves and to identify the compounds responsible for this activity. This information is fundamental to the development of traditional herbal medicine from *C. sylvestris*.

Materials and methods

Phytochemical procedures

Plant material and extraction

Leaves of *Casearia sylvestris* Sw., Salicaceae, were collected at the Medicinal Garden of the School of Pharmaceutical Sciences of São Paulo State University (Araraquara, SP, Brazil) in March 2010 (S 21.81466, W 48.20215). Voucher specimen is deposited with the herbarium “Maria Eneida Kaufmann” of the Botanical Institute of São Paulo State (São Paulo, Brazil) with the reference number AGS102.

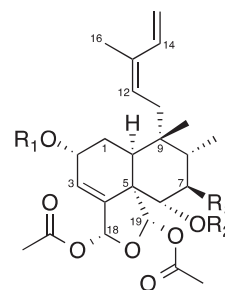
Dried and powdered leaves of *C. sylvestris* (2 kg) were extracted by maceration with ethanol (241) at 40 °C for seven days under occasional stirring. The pooled extractive solutions were concentrated under reduced pressure at 40 °C and dried in a desiccator (silica gel) to give a residue (290.5 g) named dried ethanolic extract from *C. sylvestris* leaves (EEC).

Fractionation of EEC by solid-phase extraction (SPE)

EEC (20 g) was separated by SPE from silica gel (60–200 μm)/activated charcoal (1:1, w/w) by elution with hexane:ethyl acetate (95:5, v/v), ethyl acetate and methanol to afford three fractions named SPECs1, SPECs2 and SPECs3, respectively (Santos et al., 2010).

Purification and structural determination of clerodane diterpenes from SPECs2

Caseargrewiin F (**1**), casearins B (**2**) and X were purified and identified by Santos et al. (2010).



1 R₁=*n*-C₃H₇CO; R₂=R₃=H
2 R₁=CH₃; R₂=CH₃CO; R₃=*n*-C₃H₇-COO

TLC and HPLC-PDA analyzes of EEC, SPECs1, SPECs2 and SPECs3

TLC analysis was developed in silica gel plate aluminum backed (20 cm × 20 cm; 200 μm) from Sorbent® Technologies using hexane:ethyl acetate:isopropanol 70:28:02 (v/v) as eluent and sulfuric anisaldehyde as spray reagent (110 °C; 10 min). Sample concentration (EEC and SPECs1–3) was 10 mg/ml (ethyl acetate). Caseargrewiin F (**1**), casearin B (**2**) and X were used as standards for identification purposes (1 mg/ml; ethyl acetate).

HPLC analyzes and sample pretreatment were developed with HPLC grade solvents and ultrapure water (18.2 MΩ cm). Sample pretreatment included a solid phase extraction on reversed-phase silica cartridge (Phenomenex® Strata™ C18-E; 15 mm × 10 mm; 55 μm). Samples (EEC: 7.3 mg; SPECs1: 8.2 mg; SPECs2: 8.9 mg) were dissolved in 1 ml of methanol:water 98:2 (v/v) and applied in SPE cartridge. Elution was developed with 4 ml of methanol:water 98:2 (v/v). The obtained solutions were dried in a desiccator (silica gel under reduced pressure) and the residues were dissolved with 1 ml of methanol, and submitted to membrane filtration (0.22 μm, PVDF Millipore®). Caseargrewiin F, casearins B and X (1 mg/ml; methanol; membrane filtration) were used as standards for identification purposes. Analytical reversed-phase HPLC-PDA was performed using a Shimadzu® system (Kyoto, Japan) comprising a model Prominence® LC-20AT pump, SIL-20A autosampler, DGU-20A5 degasser, CTO-20A column oven, SPD-M20A photodiode array detector and CBM-20A communication bus module, fitted with Hypersil Gold® C18 (Thermo Scientific, USA) column (250 × 4.6 mm, 5 μm), with control and data handling managed by Lcsolution® multi-PDA software. Samples were eluted with methanol:acetonitrile:water 22:44:34 (v/v), changing by linear gradient to 47:53:00 over 42 min, and isocratically with 47:53:00 for 15 min. The solvent flow rate was 0.8 ml/min and detection was at 200–700 nm for all samples. Aliquots of 20 μl were injected (Claudino et al., 2013). The analytical curve for quantification of total casearin-like clerodane diterpenes was obtained by using solutions of caseargrewiin F at concentrations of 0.035, 0.070, 0.140, 0.280 and 0.560 mg/ml (methanol) that were injected in triplicate; both regression equation and linearity factor were determined.

Pharmacological experiments

Animals and bioassays to evaluate anti-inflammatory activity

Male rats Wistar weighing 250–280 g were obtained from the Central Biotery of São Paulo State University (Botucatu, SP, Brazil) and were maintained in a room with controlled temperature (24 ± 1 °C) at 12 h light/dark cycle. The animals received food and water *ad libitum*, except during the 12 h before the experiments in which there was restriction of food. Two bioassays were performed to evaluate the anti-inflammatory activity, the paw edema

according to the protocol approved by the Ethics Committee on Animal Use of the School of Pharmaceutical Sciences, UNESP, Araraquara-SP (CEUA/FCF/CAR n^o 10/2012) and pleurisy according to the protocol approved by the Ethics Committee on Animal Use of the University Center of Rio Preto (CEUA/UNIRP n^o 16/2013). The structure of the proposal was conducted according to ethical principles of animal experimentation of the Brazilian College of Animal Experimentation.

Carrageenan-induced paw edema in rat

A carrageenan-induced paw edema assay was evaluated according to the method described by Winter et al. (1962). The animals were treated by oral route with vehicle, indomethacin (10 mg/kg), EEC (10 and 100 mg/kg), SPE1 and SPE2 (10 mg/kg), caseargrewiin F and casearin B (0.5 and 2.5 mg/kg). One hour after administration of the agents, edema was induced by injection of 100 μ l of λ -carrageenan 1% (w/v, Sigma-Aldrich[®], St. Louis, USA) into the sub-plantar tissue of the right hind paw. The left paw was used as control, which was injected with the same volume of the vehicle (saline). Paw thickness were measured before and 1, 2, 3, 4, 5 and 6 h after carrageenan injection. Inflammatory swelling was expressed as thickness variation. Indomethacin was used as reference drug while control group received the vehicles that were used to dissolve the samples. The group treated with vehicle was considered as maximum of inflammation and all others treatments were compared to this group. Gastric ulcerogenesis was also evaluated by macroscopic visualization of the rat stomachs after the end of the experiments.

Carrageenan-induced pleurisy in rat

Pleurisy was performed as described by Vinegar et al. (1973) with minor modifications. Initially, the animals ($n=6$ per group) were orally administered by gavage to EEC (100, 300 and 500 mg/kg), SPECs1 (30 mg/kg), SPECs2 (30 mg/kg), dexamethasone (0.5 mg/kg, positive control) and vehicle (8% Tween 80 and 0.1% carboxymethylcellulose in water, negative control and migration control). One hour later, the animals were submitted to anesthesia/analgesia by intraperitoneal administration of ketamine (80 mg/kg) associated with xylazine (7 mg/kg) and then λ -carrageenan (4 mg/ml in saline) was injected into the pleural cavity in a volume of 0.1 ml (400 μ g/pleural cavity). The negative control received intrapleural saline (0.1 ml/pleural cavity). After 4 h, the animals were sacrificed in a CO₂ chamber and the pleural cavity rinsed with 2 ml of phosphate buffered saline (PBS) containing heparin. An aliquot of 50 μ l of the pleural fluid was diluted in Turk solution to lyse the erythrocytes and used to count the number of total leukocytes (LT) in a Neubauer chamber. The number of LT by pleura was calculated by means of correction factors. For differential count, another aliquot of lavage fluid was used for obtaining smear stained with May–Grunwald–Giemsa in which the percentage of mononuclear (MN) and polymorphonuclear (PMN) leukocytes were determined. Applying the percentages obtained at the respective numbers of LT, it has reached the number of MN and PMN by pleura.

Statistical analyses

The results were presented as mean \pm standard error of the mean. Statistical analyzes of data were performed using analysis of variance (ANOVA), Tukey test. For this we used the InStat3 software (GraphPad[®], San Diego, CA, USA) assuming minimal levels of significance of $p < 0.05$.

Results

Chemical profile of EEC and its fractions and purification of clerodane diterpenes

EEC, SPECs1, SPECs2 and SPECs3 yields were 11.8, 3.9, 29.6 and 15.6% (w/w), respectively. TLC analysis showed the presence of casearin X ($R_f=0.23$) only in EEC and SPECs2. Caseargrewiin F and casearin B showed similar R_f values (0.17 and 0.18). Thus, a spot in EEC and SPECs2 with $R_f=0.18$ could be attributed to one of these diterpenes or both of them. Clerodane diterpenes were not identified in SPECs1 and SPECs3. HPLC-PDA identification of clerodane diterpenes was based on peak UV spectra (λ_{max} 232–238 nm) as described by Claudino et al. (2013) and a typical UV spectra of casearin-like clerodane diterpenes was presented in Fig. 2. Caseargrewiin F (**1**) and casearins B (**2**) and X were identified both in EEC and SPECs2 by t_R and UV spectra comparison (Figs. 1 and 2). The quantification of total casearin-like clerodane diterpenes equivalents to caseargrewiin F was performed using analytical curve of caseargrewiin F ($y = 53,760,775.275 x + 557,587.117$; $R^2 = 0.9987$) and considering the sum of the areas of the peaks identified as casearin-like clerodane diterpenes. The results obtained in quantitative analysis of total casearin-like clerodane diterpenes equivalents to caseargrewiin F were 27.4 and 50.6% (w/w) for EEC and SPECs2, respectively. Caseargrewiin F was also quantified in EEC and SPECs2 and the obtained values were 1.1 and 1.4% (w/w), respectively.

Bioassays to evaluate anti-inflammatory activity

Carrageenan-induced paw edema in rat

Edema was measured as the difference between the initial (before the treatment, 0 h) and final hind paw thickness obtained after different periods of time (1, 2, 3, 4, 5 and 6 h) after administration of the agent in the right hind paw of the animals (100 μ g/paw, intraplantar).

The extract at doses of 10 and 100 mg/kg did not present significant anti-inflammatory activity in the model of paw edema in rat (Fig. 3A and B).

SPECs2 (10 mg/kg), caseargrewiin F (**1**) and casearin B (**2**) (0.5 and 2.5 mg/kg) showed a significant reduction of the carrageenan-induced paw edema in rats compared to control group. The effect of SPECs2 (Fig. 4) began in the second hour after carrageenan administration and persisted throughout the experiment in a dose dependent manner similarly or higher than indomethacin that also reduced significantly the edema compared to control group.

The effect of caseargrewiin F (**1**) started at the second hour after carrageenan administration at doses of 0.5 and 2.5 mg/kg (Fig. 5A and B). Caseargrewiin F was able to reduce significantly the edema in both doses evaluated from the second hour and remained throughout the experiment.

The effect of casearin B (**2**) (0.5 and 2.5 mg/kg) (Fig. 6A and B) showed similar activity profile or significantly higher than indomethacin.

Animals treated with *C. sylvestris* samples (EEC, SPECs2, cas B and casgw F) did not present gastric lesions whereas indomethacin group showed evident lesions in the rat stomachs.

Carrageenan-induced pleurisy in rat

In the evaluation of EEC on pleurisy model, the intrapleural injection of carrageenan (migration control) caused scores of LT, MN and PMN significantly higher than those found for the negative control. Comparing the groups receiving the EEC with the migration control, the dose of 100 mg/kg had no effect, but the doses of 300 and 500 mg/kg significantly reduced the number of LT (36 and 42%, respectively) and PMN (41 and 49%, respectively). Dexamethasone

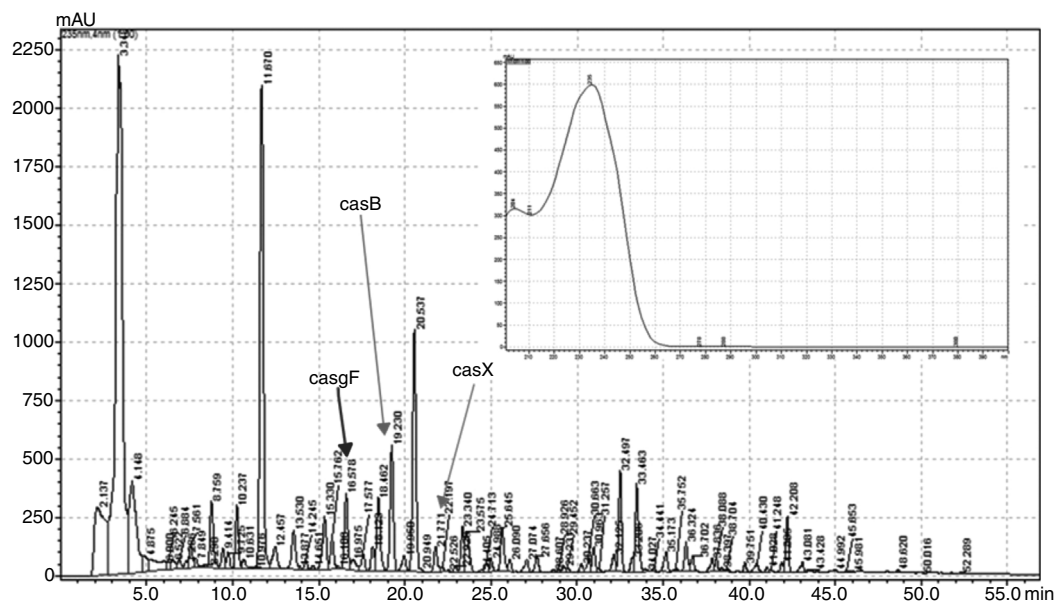


Fig. 1. HPLC-PDA chromatogram of EEC and UV spectra of caseargrewiin F peak ($t_r = 16.58$ min). Chromatographic conditions: C18 column (250 mm \times 4.6 mm, 5 μ m); methanol:acetonitrile:water 22:44:34 (v/v) to 47:53:00 over 42 min (linear gradient) and 47:53:00 for 15 min (isocratic); flow rate 0.8 ml/min; 235 nm; injection volume 20 μ l.

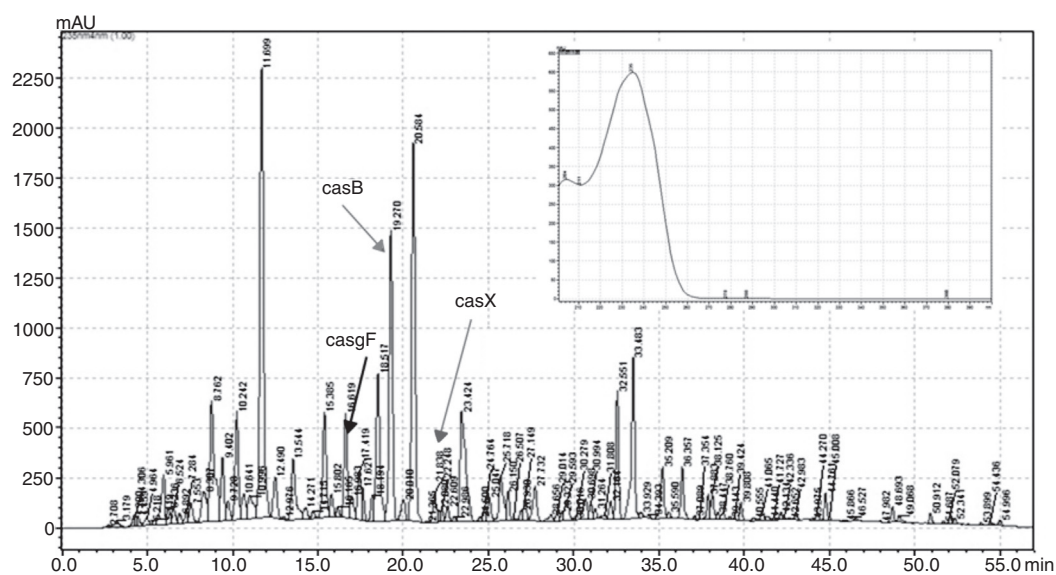


Fig. 2. HPLC-PDA chromatogram of SPECS2 and UV spectra of caseargrewiin F peak ($t_r = 16.62$ min). Chromatographic conditions: C18 column (250 mm \times 4.6 mm, 5 μ m); methanol:acetonitrile:water 22:44:34 (v/v) to 47:53:00 over 42 min (linear gradient) and 47:53:00 for 15 min (isocratic); flow rate 0.8 ml/min; 235 nm; injection volume 20 μ l.

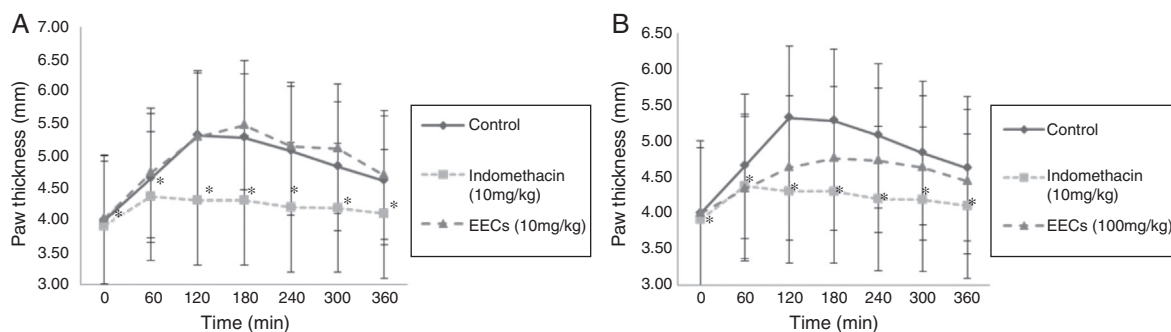


Fig. 3. Influence of EEC (10 mg/kg) (A) and (100 mg/kg) (B) on carrageenan-induced hind paw edema in rats. Values expressed as mean \pm SEM ($n = 6$). Data were analyzed by two-way Tuckey's test for comparisons between groups. *Statistical significance: $p < 0.05$ when compared with the control.

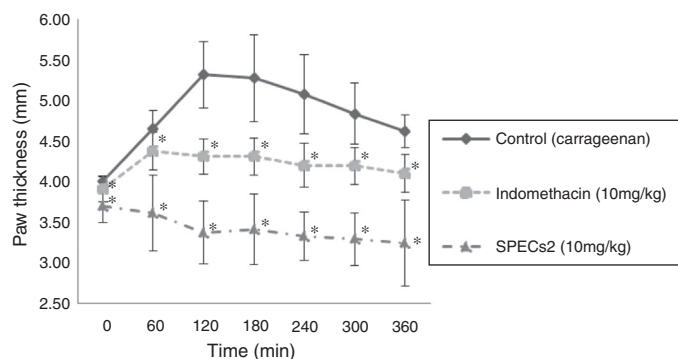


Fig. 4. Influence of SPECS2 (10 mg/kg) on carrageenan-induced hind paw edema in rats. Values expressed as mean \pm SEM ($n=6$). Data were analyzed by two-way Tuckey's test for comparisons between groups. *Statistical significance: $p < 0.05$ when compared with the control.

(positive control) reduced by 57, 45 and 60% the number of LT, PMN and MN, respectively, compared to control migration (Table 1).

Also in the evaluation of SPECS1 and SPECS2, the streptococcal injection of carrageenan (migration control) caused scores of LT, MN and PMN significantly higher than those found for the negative control. Comparing the groups receiving the fractions with the control of migration, SPECS1 had no effect, but SPECS2 significantly reduced the number of PMN and LT (47 and 48%, respectively). Dexamethasone (positive control) reduced by 61, 51 and 65% of the number of LT, PMN and MN respectively, compared with migration control (Table 2).

Discussion

The present study supports that ethanolic extract, its enriched diterpene fraction and isolated clerodane diterpenes from *C.*

sylvestris leaves exhibit anti-inflammatory activity reinforcing previous results obtained by other authors as discussed below.

Ruppelt et al. (1991) demonstrated that the aqueous extract from *C. sylvestris* leaves presents analgesic and anti-inflammatory actions in mice that received intraperitoneal injection of acetic acid. The analgesic action was confirmed by the reduction in the number of writhes and anti-inflammatory action by reducing of the Evans blue extravasation into the peritoneal cavity. Silva et al. (2004) showed that the hydroethanolic extract from *C. sylvestris* leaves exhibits anti-inflammatory effects in skin edema model in rats. Esteves et al. (2005) demonstrated that the essential oil from *C. sylvestris* leaves (EOCS) exhibits anti-inflammatory activity on carrageenan-induced paw edema model and granulomatous tissue test, both in rats. These authors also demonstrated that the EOCS exhibits analgesic action by reducing the number of writhes after intraperitoneal injection of acetic acid in mice. Mattos et al. (2007) evaluated the effect of hydroethanolic extract from *C. sylvestris* leaves on three models of nociception in mice, one model of pain not associated with inflammation (hot-plate test) and two models of pain associated with inflammation (acetic acid-induced abdominal writhes and ovalbumin-induced licking behavior in sensitized animals). About the last two tests, this extract showed inhibitory effect with all doses tested whereas in the hot plate test, showed an inhibitory effect only at the highest dose. More recently, Albano et al. (2013) demonstrated that the hydroethanolic extract from *C. sylvestris* leaves reduced paw edema in mice and leukocyte migration in pleurisy in rats, both induced by carrageenan. However, none of these studies used the ethanolic extract from *C. sylvestris* leaves (EEC) which presented antiulcerogenic effect in models of acute and chronic ulcers in rats (Sertié, 1990). Also, these studies did not investigate the possible compounds involved.

In the present study, in addition to using the EEC, its fractions and isolated clerodane diterpenes were also tested to investigate the possible compounds involved with the anti-inflammatory activity of EEC in two models, one to evaluate the formation of

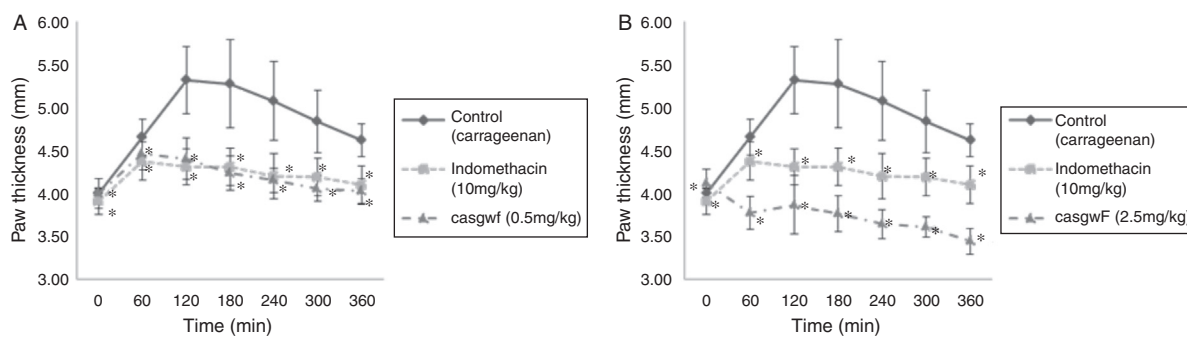


Fig. 5. Influence of casgwF (0.5 mg/kg) (A) and (2.5 mg/kg) (B) on carrageenan-induced hind paw edema in rats. Values expressed as mean \pm SEM ($n=6$). Data were analyzed by two-way Tuckey's test for comparisons between groups. *Statistical significance: $p < 0.05$ when compared with the control.

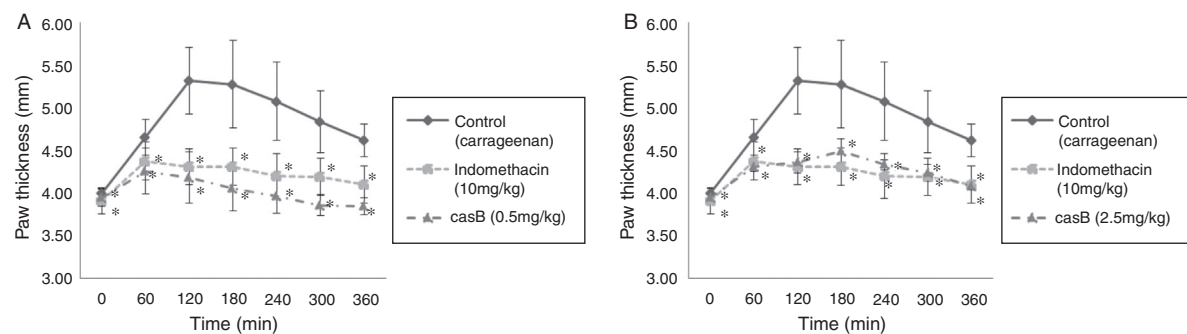


Fig. 6. Influence of casB (0.5 mg/kg) (A) and (2.5 mg/kg) (B) on carrageenan-induced hind paw edema in rats. Values expressed as mean \pm SEM ($n=6$). Data were analyzed by two-way Tuckey's test for comparisons between groups. *Statistical significance: $p < 0.05$ when compared with control.

Table 1
Effect of EEC orally administrated on the number of leukocytes present in the pleural cavity 4 h after intrapleural injection of carrageenan (400 µg/pleural cavity) in rats.

Group	(Cells/pleural cavity) × 10 ⁶		
	Total leukocytes	Mononuclear leukocytes	Polymorphonuclear leukocytes
Negative control	8.29 ± 1.27	5.78 ± 0.94	2.51 ± 0.50
Migration control	47.47 ± 4.17 ^a	10.85 ± 1.58 ^a	36.62 ± 5.01 ^a
Positive control	20.58 ± 1.09 ^{a,b}	5.96 ± 0.82 ^b	14.62 ± 1.44 ^{a,b}
EEC			
100 mg/kg	43.33 ± 2.84 ^a	7.76 ± 2.03	35.57 ± 2.87 ^a
300 mg/kg	30.37 ± 3.84 ^{a,b}	8.74 ± 2.01	21.63 ± 1.87 ^{a,b}
500 mg/kg	27.40 ± 4.47 ^{a,b}	8.68 ± 2.27	18.72 ± 2.45 ^{a,b}

Results are expressed as mean ± standard error of the mean (n = 6 animals per group). Negative control, vehicle orally + intrapleural saline (0.1 ml/pleural cavity); migration control, vehicle orally; positive control, dexamethasone orally (0.5 mg/kg).

^a p ≤ 0.05 compared to the negative control.

^b p ≤ 0.05 compared with migration control (ANOVA, Tukey's test).

Table 2
Effect of SPECS1 and SPECS2 orally administrated on the number of leukocytes present in the pleural cavity 4 h after intrapleural injection of carrageenan (400 µg/pleural cavity) in rats.

Group	(Cells/pleural cavity) × 10 ⁶		
	Total leukocytes	Mononuclear leukocytes	Polymorphonuclear leukocytes
Negative control	7.41 ± 0.78	5.18 ± 0.60	2.23 ± 0.40
Migration control	46.82 ± 3.45 ^a	11.15 ± 2.23 ^a	35.66 ± 3.95 ^a
Positive control	17.88 ± 1.19 ^{a,b}	5.41 ± 1.10 ^b	12.47 ± 0.92 ^{a,b}
SPECS1 (30 mg/kg)	44.03 ± 2.47 ^a	8.26 ± 1.29	35.77 ± 2.22 ^a
SPECS2 (30 mg/kg)	24.74 ± 3.86 ^{a,b}	6.23 ± 0.95	18.44 ± 3.32 ^{a,b}

Results are expressed as mean ± standard error of the mean (n = 6 animals per group). Negative control, vehicle orally + intrapleural saline (0.1 ml/pleural cavity); migration control, vehicle orally; positive control, dexamethasone orally (0.5 mg/kg).

^a p ≤ 0.05 compared to the negative control.

^b p ≤ 0.05 compared with migration control (ANOVA, Tukey's test).

edema and the other to evaluate the cell migration (pleurisy). Initially, the EEC was tested in the rat paw edema model and the highest dose tested (100 mg/kg) had no effect (Fig. 3B). Thus, the doses of 100, 300 and 500 mg/kg were used in the pleurisy model and the two higher doses showed inhibitory effect.

In this way, the effects of SPECS1 and SPECS2 fractions were investigated in these models. Only SPECS2 showed anti-inflammatory effect. Santos (2008) characterized SPECS1 as a low polarity fraction containing volatile compounds from the leaves essential oil and spathulenol was the main volatile compound (17.5%, w/w). In the fraction SPECS1 obtained in the present study, spathulenol was also the main volatile compound, representing 60.6% (w/w) of volatiles in SPECS1. Spathulenol was also identified in the leaves essential oil from *C. sylvestris* (Esteves et al., 2005; Sousa et al., 2007). Clerodane diterpenes were not identified in SPECS1 (TLC and HPLC-PDA).

On the other hand, clerodane diterpenes are the main compounds in SPECS2. These results suggested that the compounds responsible for the anti-inflammatory activity of EEC were clerodane diterpenes. Thus, casearin B and caseargrewiin F were evaluated in paw edema model and demonstrated activity compared to indomethacin. Furthermore, through bioguided fractionation, Santos (2008) showed that the concentrated fraction in clerodane diterpenes (SPECS2) and clerodane diterpenes (casearins B, D, O and X and caseargrewiin F) showed antiulcerogenic activity in acute ulcer model induced by ethanol in rats.

Regarding of probable mechanisms of anti-inflammatory activity of *C. sylvestris*, the literature is relatively sparse. Some studies have suggested that they might be associated with reduced production of prostaglandins (PG) by inhibition of cyclooxygenase (COX) or secreted phospholipase A₂ (sPLA₂). In the first case, there are few and weak evidences, one suggested by Sassioto et al. (2004) who found that the decoction from *C. sylvestris* leaves showed a similar effect to non-steroidal anti-inflammatory drugs (COX classical inhibitors) in a model of osteogenesis. Esteves et al. (2005)

suggest a similar mechanism since EOCS reduced paw edema induced by carrageenan, but not the increased vascular permeability induced by substances that degranulate mast cells. Furthermore, this hypothesis raises the following question: how can the same extract or clerodane diterpene from *C. sylvestris* leaves inhibit COX and at the same time provide antiulcerogenic effect? In addition, SPECS2, cas B and casgw F showed anti-inflammatory action in paw edema model without gastric effects. One possible response to such inquiry would be the highly selective inhibition of COX-2, isoform involved in the production of PG in the site of inflammation. Thus, the production of PG in the stomach for COX-1 was not affected.

With respect to the inhibition of sPLA₂, Borges et al. (2000) showed that the aqueous extract from *C. sylvestris* leaves inhibited *in vitro* enzymatic activity of sPLA₂ isolated from the venom of *Bothrops pirajai* and *B. jararacussu*. In addition, studies have demonstrated that extracts from *C. sylvestris* show inhibitory effect on biological activities of sPLA₂ isolated from poisons (Raslan et al., 2002; Cavalcante et al., 2007). However, it is important to note that there are ten different groups of sPLA₂ and the main sPLA₂ involved in the inflammatory reaction in humans belong to the group V while sPLA₂ found in poisons belong to other groups (Dennis et al., 2011). Regarding of probable mechanisms, Albano et al. (2013) suggested that anti-inflammatory activity of *C. sylvestris* may be related to the inhibition of nitric oxide production because the hydroethanolic extract from the leaves of this plant reduced levels of nitrite and nitrate in pleural lavage of rats subjects to pleurisy induced by carrageenan. They also suggested a relationship with antioxidant activity, because this study demonstrated the reduction of various parameters of oxidative stress in the lung of these animals.

Conclusion

The results of the present study demonstrated that ethanolic extract from *C. sylvestris* leaves (EEC), its enriched fraction of clerodane diterpenes (SPECS2), casearin B and caseargrewiin F exhibited

anti-inflammatory activity on *in vivo* models in rats. These results are in agreement with Brazilian traditional use of the species. Thus, casearin-like clerodane diterpenes may be considered chemical markers for *C. sylvestris* leaves, its derived plant drug or extracts used for anti-inflammatory therapeutic purposes. Therefore this study contributed for standardization of a further herbal medicine based on Brazilian traditional medicine data. On the other hand, casearin-like clerodane diterpenes are promising compounds in the development of new drugs with anti-inflammatory action without gastric side effects.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

EGP, RCC, EOV, CMRF participated in the evaluation of anti-inflammatory activity, acquisition and interpretation of data. EGP realized phytochemical procedures and analysis. AJC, AGT, CMC and AGS participated in the design and coordination of the work and helped to draft the manuscript. All the authors have contributed to critical reading of the final manuscript and approved its submission.

Conflicts of interest

The authors declare that there are no conflict of interest.

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