Research Article

CASEARIA SYLVESTRIS SW. ESSENTIAL OIL ACTIVITY IN INFLAMMATION IN RATS INDUCED BY *BOTHROPS ALTERNATUS* VENOM

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ABSTRACT

Casearia sylvestris Sw. (Flacourtiaceae) has been used in Brazilian folk medicine in different preparations for many uses, including healing of skin problems, anti-inflammatory, gastric ulcer treatment and herpes. The anti-inflammatory effects of the essential oil extracted from the leaves of *C. sylvestris* were investigated through experimental models in rats (rat paw edema, vascular permeability) using *Bothrops alternatus* venom as the inflammatory stimulus. The administration of 200 mg/kg i.p. of essential oil significantly inhibited *B. alternatus* venom-induced edema, when compared to control group. The essential oil was more effective than dexamethasone and promethazine in decreasing the edema. In the same experiment, cyproheptadine was more effective than the essential oil. The combination of the essential oil with methysergide more effectively inhibited edema formation than cyproheptadine or the essential oil itself. The increased vascular permeability induced by *B. alternatus* venom decreased the exuded dye content with the essential oil treatment when compared to control group. It was also more effective than dexamethasone or indomethacin. These results suggest that the essential oil has an anti-inflammatory activity in inflammatory models evoked by bothropic venom and histamine and serotonin receptors may be involved in the mechanism of action.

Keywords: *Casearia sylvestris;* essential oil; *Bothrops alternatus;* anti-inflammatory.

INTRODUCTION

Casearia sylvestris Sw. (Flacourtiaceae) grows wild in tropical America. In Brazil it occurs as a small tree or as a bush, popularly known as "guaçatonga", "erva-de-bugre" or "pau-de-lagarto"[1]. In folk medicine it has been used as anti-snake venom[2], antiseptic and wound healing[3], topical anesthetic[4] and ulceration process[5].

The ethanolic extracts of *C. sylvestris* leaves protect the stomach mucosa without changing the gastric pH and, in a chronic model of ulcer, presented a curative effect in rats[6,7]. Previous studies described the isolation of clerodane diterpenes with cytotoxic, antitumor and antifungic activities[8,9].

Several plants or their extracts are claimed to be antidotes for snakebites in traditional medicines, mostly by rural populations in many parts of the world[10].

The aqueous extract of *C. sylvestris* reduced vascular permeability, lethality and edema induced by *Bothrops jararaca* venom in mice[11,12]. *In vitro* studies showed that the aqueous extract is able to inhibit the anticoagulant activity of phospholipase A_2 enzymes isolated from bee or snake venoms and decreased hemorrhagic, coagulant and proteolytic activity induced by bothropic venoms [13,14].

The genus *Bothrops* (Crotalidae) has caused the majority of snake accidents in Central and South America. The envenomation is well characterized by local inflammation, pain, edema and necrosis. Extensive local tissue damage and systemic effects such as hemorrhage, defibrination and thrombocytopenia can also be noted[15,16,17].

The aim of this study was to investigate the anti-inflammatory activity of the essential oil extracted from the leaves of *C. sylvestris* using inflammatory animal models induced by *Bothrops alternatus* venom.

MATERIALS AND METHODS

Plant material

Leaves of *C. sylvestris* were collected at Alfenas, State of Minas Gerais, Brazil. A voucher specimen is deposited at Laboratório de Fitofármacos, Universidade de Alfenas, under registration number LF-258.

Isolation and analysis of composition of the essential oil of C. sylvestris

The essential oil was obtained using Method I of the Brazilian Pharmacopoeia[18]. Air-dried and powdered leaves of *C. sylvestris* were steam distilled for 3h using a Clevenger apparatus. The essential oil of *C. sylvestris* (EOCS) obtained was stored in glass container at –5 ^{0}C until use.

Phytochemical analysis by combined gas chromatography-mass spectrometry (GC-MS)

The essential oil was submitted to quantitative analysis in a Finnigan Varian 3400 automated gas chromatograph mass spectrometer data system with selective mass detector Finnigan INCOS-XL.

GC conditions: carrier gas, helium at flow rate of 1.0 $mL.min^{-1}$; sample size, 2 μL injected using the splitless injection technique; fused capillary silica column DB-5MS (30 m x 0.25 mm x 0.25 μm). Temperatures: injector = 220°C, detector = 280°C, column = 60°C, 4°C.min^{-1}, 240°C (7 min). The MS were taken at 70 eV.

The main constituents were identified by comparison with the mass spectrums from Wiley and Nist 98 spectrum library.

Drugs and reagents

The animals were treated with 200 mg/kg of EOCS by oral or intraperitoneal route[19]. An emulsion of EOCS was prepared using polisorbate 80 (Tween), subsequently diluted in 0.9% saline solution. Control groups were treated with solution of polisorbate 80 in saline. Indomethacin, dexamethasone and cyproheptadine were obtained from Prodome Co.. Promethazine and methysergide were obtained from Rhodia Co. and Sandoz Co..

Snake venom

The desiccated *B. alternatus* venom (BAV) used in this study was obtained from Universidade de Alfenas Serpentarium. The venom was dissolved in 0.9% saline solution (0.5 mg/ml or 0.125 mg/ml) just before use. The administrated dose was 50 μg/animal[20].

Animals

The Project was approved for the Ethic Commission of the Universidade de Alfenas, Minas Gerais, Brazil, Protocol Number 05A/2005. Male rats (*Rattus norvegicus* – albinus, Wistar), weighing 150 – 200 g, acquired from the Animal Experimental Center of Universidade de Alfenas were used. The animals were kept in five animal groups in polyethylene boxes, in a climatic environment (23 ± 2°C), air humidity control (53%), in 12 hour/shifts with dark/light control, with food and water "ad libitum". Animals were fasted overnight prior to each experiment.

BAV-induced rat paw edema

The inhibitory activity of EOCS on BAV-induced edema was compared with dexamethasone, cyproheptadine and promethazine in the same experiment, in order to verify the influence of these drugs on the mechanisms involved in the oedematogenic effect of the bothropic venom[21]. Animals were treated with EOCS in association with serotonin and histamine H_1 antagonist cyproheptadine or a non-specific serotonin antagonist, methysergide. One hour after administration of saline solution (2 ml/kg, i.p.), EOCS (200 mg/kg, i.p.), dexamethasone (2 mg/kg, i.p.), cyproheptadine (10 mg/kg, p.o.), promethazine (10 mg/kg, p.o.), EOCS and cyproheptadine (200 and 10 mg/kg, i.p.) or EOCS and methysergide (200 and 10 mg/kg, i.p.), BAV solution (0.1 ml, 0.05% in 0.9% saline) was injected into the sub-plantar area of the right hind-paw of rats. An equal volume of saline was injected into the left hind-paw. At different times after BAV injection, the volume of each paw, up to the tibio-tarsal articulation, was determined using a plethysmometer (model 7140, Ugo Basile, Italy). The results were expressed as mean difference between the two paw volumes. The dorso-ventral diameters of the paws were also determined with a precision caliper rule. In this case, the results were expressed as mean difference between the two paw diameters[22].

Increase of vascular permeability induced by BAV

The effect of EOCS was verified on BAV-induced increased vascular permeability[20,23,24]. Thirty minutes after treatments with control solution (2 ml/kg, i.p.), EOCS (200 mg/kg, i.p.), indomethacin (10 mg/kg, *p.o.*) or dexamethasone (2 mg/kg, i.p.), a dose of 25 mg/kg of 2.5% Evans blue dye in 0,9% saline solution was administered intravenously. Ten minutes later, each animal received 4 intracutaneous injections of BAV (12.5 μg in 0.1 ml of saline solution) on the dorsal region. Thirty minutes later, the animals were sacrificed and the fragments of dorsal epidermis removed. The exuded dye was extracted from epidermis with formamide solution (37°C for 24 hours), centrifuged (2500 rpm, 15 minutes) and the content in the supernatant was measured on a spectrophotometer at 620 nm.

Statistical analysis

Results are expressed as mean ± SEM. The statistical analysis was done using Analysis of Variance (ANOVA) followed by Dunnett test. Results with $p < 0,05$ were considered significant.

RESULTS

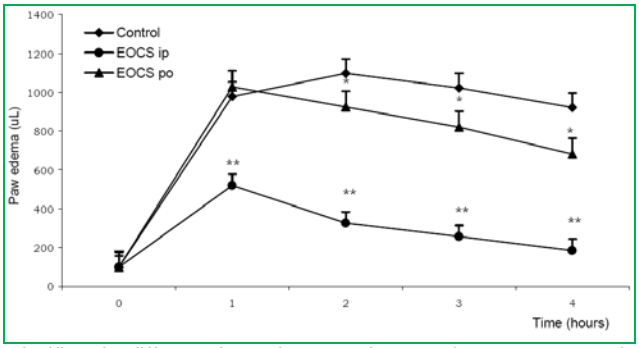
Analysis of EOCS

The essential oil extraction procedure resulted in 2,5% yield. The oil was yellow. The following compounds were identified by GC/MS: bicyclogermacrene (40,9%), β-acoradiene (20,8%), spathulenol (12,6%), thujopsene (5,2%), germacrene B (3,9%), caryophyllene (3,8%), α-humulene (3,7%), globulol (2,2%), germacrene D (1,9%) and calamenene (1,5%).

BAV-induced rat paw edema

BAV induced a hemorrhagic edema within a period of 24 hours at least (data not show). The group treated with 200 mg/kg of EOCS had a significant reduction of the paw volume compared to control group, both by oral and intraperitoneal route, with the latter showing a larger inhibitory effect at all time intervals measured (Figure 1). At the peak of the edema (2 h), the i.p. administration of EOCS caused an inhibition of paw volume about 70%.

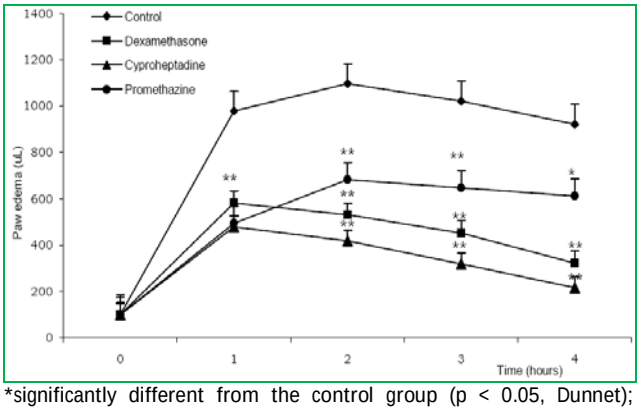
Figure 1: Effect of oral and intraperitoneal administration of EOCS (200 mg/kg) on the rat paw edema induced by BAV.



*significantly different from the control group ($p < 0.05$, Dunnet); **significantly different from the control group ($p < 0.01$, Dunnet).

The administration of standard drugs (dexamethasone, cyproheptadine and promethazine) produced a significant reduction of this edema, at all intervals measured (Figure 2). The treatment with cyproheptadine was the more effective, showing a reduction of the edema of 62 %, in the peak of this edema.

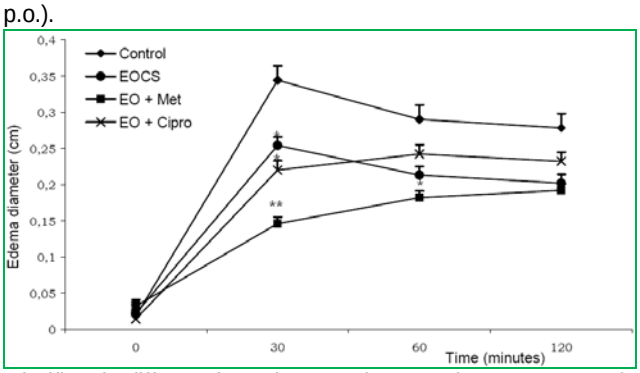
Figure 2: Effect of administration of dexamethasone (2 mg/kg, i.p.), cyproheptadine (10 mg/kg, p.o.) and promethazine (10 mg/kg, i.p.) on rat paw edema induced BAV.



*significantly different from the control group ($p < 0.05$, Dunnet); **significantly different from the control group ($p < 0.01$, Dunnet).

Except for the first measure (30 min), the administration of EOCS plus cyproheptadine and methysergide did not show a significant reduction of the edema induced by BAV, when compared with the administration of EOCS itself (Figure 3).

Figure 3: Effect of administration of EOCS (200 mg/kg i.p.) plus methysergide (10 mg/kg, i.p.) and cyproheptadine (10 mg/kg, p.o.).

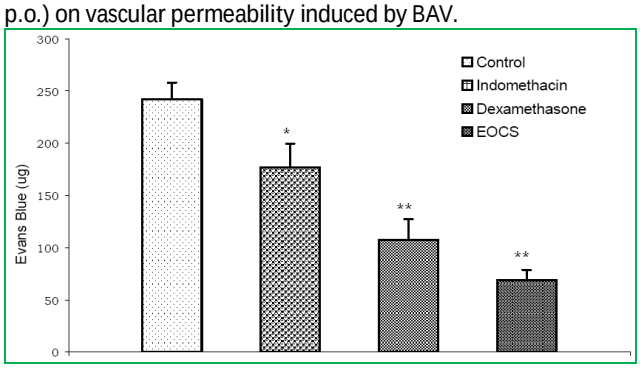


*significantly different from the control group ($p < 0.05$, Dunnet); **significantly different from the control group ($p < 0.01$, Dunnet).

Increased vascular permeability induced by BAV

The vascular permeability induced by intracutaneous injection of BAV was significantly reduced by EOCS (200 mg/kg, i.p.), also by 2.0 mg/kg of dexamethasone and 10 mg/kg of indomethacin. EOCS showed the most effective inhibition (71.4 %) of the exuded dye content when compared with control group followed by dexamethasone (55.5 %) and by indomethacin (27 %). These results are shown in figure 4.

Figure 4: Effect of administration of EOCS (200 mg/kg i.p.), indomethacin (10 mg/kg, p.o.) and dexamethasone (2 mg/kg, p.o.) on vascular permeability induced by BAV.



*significantly different from the control group ($p < 0.05$, Dunnet); ** significantly different from the control group ($p < 0.01$, Dunnet).

DISCUSSION

According to ethnobotanical data[2], the bark, root and leaves of *C. sylvestris* are used against snakebites in some regions of Brazil. A mixture of polysaccharides extracted from the bark of this plant show protection against bothropic venoms[25]. The aqueous extract of it leaves showed the ability of neutralize the haemorrhagic activity induced by bothropic venoms and isolated toxins[13]. The present study was carried out to evaluate the activity of *C. sylvestris*, this time using the essential oil against BAV.

Snake venoms are complex mixtures of proteins. Some of these proteins are myotoxins, haemorrhagins, phospholipase A_2 and proteases. They cause changes in haemostatic mechanism, interfering with platelet function, damaging blood vessels and activating coagulation factors[26]. Several inflammatory mediators such like histamine, bradikinin, leukotrienes and serotonin are released as well[27].

Phospholipase A_2 is one of the major component responsible for lethality of BAV, since the lethal potency of the purified enzyme is 46-fold greater than the whole venom[28]. The neutrophil migration induced by BAV seemed to be related to phospholipase A_2 activity of this venom, since dexamethasone and NDGA, but not indomethacin, strongly reduced neutrophil migration, suggesting that arachidonate-derived lipoxygenase metabolites, such as leukotriene B_4, act as chemotactic mediators[29]. The injection of BAV in mouse footpad caused hemorrhagic, edema-forming activities and histopathological alterations, including myonecrosis[30].

Dexamethasone, a steroidal anti-inflammatory drug that inhibits the products of arachidonic acid during its synthesis and release, showed a significant inhibition of the vascular permeability and of the edema induced by BAV. In this experiment, the edema inhibition was greater in animals treated with cyproheptadine, which was administered orally (10 mg/kg), suggesting that histamine and serotonin, besides phospholipase A_2, play a important role in this model of inflammation, since the inhibition observed with the anti-histaminic drug promethazine was lower than cyproheptadine, administered in the same dose and route.

The treatment with EOCS produced a significant reduction of the edema and of vascular permeability when compared to control group. In the edema induced by BAV, the combination with standard drugs suggests that the action of EOCS interferes with serotonin and histamine receptors, at least in the first half hour after the injection of BAV, showing high capacity of distribution, considering dose and route, which was orally, with lower absorption than intraperitoneal route.

The sesquiterpenes are the major components present in the sample of EOCS. Some of them, like bicyclogermacrene, germacrene B, caryophyllene, α-humulene, germacrene D, are known for anti-inflammatory activity of several essential oils[31,32,33,34]. These compounds can also be found in aqueous extracts, since other compounds (e.g. flavonoids, saponins) may stabilize them in water[33].

A sample of essential oil of *C. sylvestris* colected in São Paulo, Brazil, composed mainly by sesquiterpenes showed anti-inflammatory activity in acute, sub-acute and sub-chronic models of inflammation[33]. Therefore, we suggest that the naturally occurring sesquiterpenes present in the EOCS seems to be responsible for the anti-inflammatory action against BAV reported in this work, which would involves arachidonate-derived mediators, serotonin and histaminic receptors. Further experiments are in progress to determinate which components are responsible for such activity.

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