Research Article

EFFECT OF CINNAMIC ACID ON THE ACUTE INFLAMMATORY RESPONSE

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ABSTRACT

To determine the effect of the Cinnamic acid (CA) on the acute inflammatory response by evaluation the *in vitro* leukocyte chemotaxis, cróton oil-induced ear edema and paw edema induced by carrageenan and dextran. The anti-inflammatory activity of CA was evaluated ear edema induced by croton oil, paw edema induced by carrageenan or dextran and leukocyte chemotaxis *in vitro*. Was also evaluated the activity of the enzyme myeloperoxidase (MPO) and the concentration of nitric oxide (NO) in the section of the ears and the plantar tissue. The CA showed low cytotoxicity and showed no activity when used as a chemotactic agent *in vitro* in the concentrations 2 and 15 μg/mL; but the concentration of 150 μg/mL was observed a significant chemotactic activity. The antiedematogenic topic effect (ear edema) was observed at a concentration of 5.0 mg/ear, while the systemic (paw edema) at all doses tested. The CA inhibited leukocyte infiltration evaluated by the reduction in MPO activity and reduced levels of NO in inflamed tissue. The results suggested that CA possessed anti-inflammatory effects. The possible anti-inflammatory mechanism of CA be related to inhibition of the synthesis/release of inflammatory mediators such as NO and histamine.

Keywords: Inflammation, Topical inflammation, Cinnamic acid, ear edema, paw edema.

INTRODUCTION

Medicinal plants have been used as therapeutic alternative for the treatment many conditions and diseases[1]. Among the medicinal plants, *Cinnamomum cassia* has been the target of several pharmacological studies. *C. cassia* has been reported to have antifungal, antimicrobial, anti-cancer, anti-oxidant and anti-inflammatory properties[2-6]. Chemical studies this plant revealed the presence of phenylpropanoids, terpenoids, flavonoids, polyphenols and volatile oil[7].

Cinnamic acid ((E)-3-phenylprop-2-enoic acid) is a phenylpropanoid found in the *Cinnamomum cassia* essential oils[8]. This compound has a long history of human use as flavoring agent[9]. Researches have demonstrated several pharmacological properties of Cinnamic acid (CA) such as, antimicrobial[10], antiproliferative[11], antifungal[12] and antitumor activity[13-14]. Moreover, CA induce angiogenesis *in vivo* and *in vitro*[15], it has antidiabetic[16] and antioxidant activity[17], and might act as a depigmenting agent by its effect on melanin synthesis pathway[18]. It was recently reported its *in vitro* anti-inflammatory activity on LPS-induced in RAW264.7 macrophages[19, 20].

The inflammation is a response of the vascularized tissues against a harmful stimuli, involving an immune system coordinated action and of the injured tissue[21]. This response is characterized by vascular, cellular and lymphatic events intended to eliminate the aggressive agent and restore damaged tissue[22, 23]. These processes are mediated and integrated by several inflammatory mediators produced by neutrophils, macrophages, mast cells, lymphocytes and platelets[24, 25]. However, persistence of the inflammatory response as well as the constant production of inflammatory mediators can cause tissue damage and consequent loss the function[26]. Under such conditions, the use of the drugs or medicinal plants that are able to modulate this process may present an interesting therapeutic possibility.

This study was designed to determine the effect of the CA on the acute inflammatory response by evaluation the *in vitro* leukocyte chemotaxis, croton oil-induced ear edema and paw edema induced by carrageenan and dextran.

MATERIALS AND METHODS

Drugs and reagents

CA was obtained from Carlo Erba of Brasil S.A. Indomethacin, zymosan, fMLP (formyl-methionyl-leucyl-phenylalanine), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide], croton oil and carrageenan were purchased from Aldrich Chemical Co.

Animals

Male mice Swiss strain (25-35 g) were maintained under a controlled temperature of 22◦C on a 12h light/dark cycle and receiving food and water *ad libitum*. The experimental protocols were approved by the Ethical Committee in Animal Experimentation of the State University of Maringá (CEUA/UEM 3657210315).

Chemotaxis in vitro

To evaluate the chemotactic effect of CA, leukocytes were obtained from the peritoneal cavity of mice 4 hours after injection zymosan (1 mg/cavity, i.p.). The cell number was adjusted to 1 × 10^6 cells/mL in RPMI medium that contained 0.1% bovine serum albumin (BSA). The assay of leukocyte chemotaxis was performed using Boyden micro chambers (48 wells; Neuro Probe, Inc., Cabin John, MD, USA); with the use of a polycarbonate filter (5 µm, 25×80 mm, 50 Pk; GE Water & Process Technologies). The chemoattractant N-formyl-methionyl-leucyl-phenylalanine (fMLP; 10^{-6} M), CA (2; 15 and 150 µg/mL) and a negative control (RPMI 1640) were placed in the lower compartment of the chamber. Then, a suspension of leukocytes (1×10^6 cells/mL) was put in the upper compartment of the chamber. The cells were allowed to migrate into the membrane for 1 h at 37◦C in 5% CO_2. After incubation period, the membrane was washed and stained using Instant Prov (Newprove). The membrane area of each well was scored using light microscopy to count the intact cells present in five random fields. The results were expressed as the mean number of leukocytes per field and representative of three separate experiments[27].

Dimethylthiazol Diphenyltetrazolium Bromide (MTT) assay

The MTT assay is based on the mitochondrial enzyme reduction of the tetrazolium dye to detect and determine cell viability. Leukocytes were obtained from the peritoneal cavity of male mice four hours after injection of 200 µL zymosan solutions (1 mg/cavity, i.p.). The leukocytes were placed at a density of 5 x 10^5 cells/well in a volume of 100 µL RPMI medium (supplemented with 10% of fetal bovine serum (FBS) and penicillin 100 U/ml + streptomycin 100 µg/ml) into 96-well plates. After 90 min exposure to CA (10, 30, 60 and 90 µg/mL) or vehicle (0.1% Tween 80 solution, used as control), 10 µL of MTT (5 mg/ml, Sigma) stock solution was added to each well. After 2 h of incubation at 37 °C, the supernatant was removed and 100 µL of DMSO (dimethyl sulfoxide) were added to each well. The cells were incubated for 10 min at 25 °C. The resulting absorbance was measured at 540 nm. The values of the blank wells were subtracted from each well of treated and control cells. The percentage of viability was determined by the following formula:

$$\% \text{ Viable cells} = \frac{(\text{Abs. of the treated cells} - \text{Abs. of the blank})}{(\text{Abs. of the control} - \text{Abs. blank})} \times 100$$

Croton oil-induced ear edema

Cutaneous inflammation was induced by application of 20 µL of croton oil (200 µg) diluted in acetone/water solution (vehicle) to the inner surface of the right ear in mice. The left ear received equal volume of the vehicle. CA (0.5, 1.0, 2.5 and 5.0 mg/ear), dexamethasone (0.1 mg/ear, anti-inflammatory reference drug) or vehicle were topically applied to the right ear 1 hour before croton oil application. Four hours after the inflammatory stimulation, the mice were euthanized and a section of 6 mm diameter tissue was removed from both the treated and untreated ears. Edema was determined by weight difference between the two sections[28]. Data were expressed as mean ± standard error of the mean (S.E.M.) of the weight of the ears.

Paw Edema Induced by Carrageenan

Paw edema was induced in the right hind paw of the mice by a subplantar injection of 20 µL carrageenan suspension (500µg/paw) in sterile saline (0.9%). The contralateral paw was injected with saline (i.e., negative control). CA (50, 125 or 250 mg/kg), indomethacin (10 mg/kg, reference drug) or vehicle (saline) was administered orally 60 min before carrageenan administration in the hind paw. The degree of edema was measured 1, 2, and 4 h after carrageenan injection using plethysmography (Ugo-Basile, Comerio, Italy). Edema was expressed in terms of the increase in paw

volume, subtracting the rates of the paw injected with saline (control paw) from the rates of the paw injected with carrageenan.

Paw Edema Induction by Dextran

Mice male were treated with CA (50, 125 and 250 mg/kg) or vehicle (saline) or promethazine (10 mg/kg, reference drug) by the oral route. After 30 minutes, they received an intraplantar injection of 20 µL dextran solution (500 mg/paw), dissolved in 0.9 % saline in the right hind paw and the same volume of vehicle (0.9 % saline) into the left hind paw according to the technique of Winter et al.[29]. The paw volume was measured 30, 60, 120 and 240 minutes after dextran injection using plethysmography (Ugo-Basile, Comerio, Italy). Edema was expressed in terms of the increase in paw volume, subtracting the rates of the paw injected with saline (control paw) from the rates of the paw injected with dextran.

Determination of Myeloperoxidase Activity – MPO

The activity of MPO was evaluated in the supernatant of homogenates obtained from sections of ears (controls and treated) and plantar tissue of mice. The sections of the ears or plantar tissue were placed in 50 mM potassium phosphate buffer, pH 6.0; containing 0.5 % hexadecyltrimethyl-ammonium bromide (1 mL/50 mg tissue) in a Potter homogenizer. The homogenate was shaken in a vortex mixer and centrifuged for 5 min. Ten microliters of the supernatant were added to each well of a 96-well microplate, in triplicate. Two hundred microliters of the buffer solution containing 16.7 mg O-dianisidine dihydro-chloride (Sigma-Aldrich, St. Louis, MO, USA), 90 ml double distilled water, 10 ml potassium phosphate buffer, and 50 ml of 1% H2O2 was added. The enzyme reaction was stopped by addition of ethyl acetate. Enzyme activity was determined by the absorbance measured at 460 nm using a microplate spectrophotometer (Spectra Max Plus).

Determination of the Concentration of Nitric Oxide – NO

The concentration of NO was determined by the Griess method, in which the nitrite production is determined as an indirect measure of the production of the gas. The supernatant of the homogenate sections of ears or plantar tissue (50 µL) were incubated with equal volumes of Griess reagent mixtures (1% sulfanilaminide in 5% phosphoric acid and 0,1% N-1-naphthylethylenediamine dihydrochloride in water) at room temperature for 10 min. The absorbance was measured in a microplate reader at 550nm[30]. The NO concentrations were calculated from a sodium nitrite standard curve. Data were expressed as µM concentration of NO^{2-}.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data were subjected to analysis of variance (ANOVA) followed by Tukey's post hoc test. Values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The chemoattractant effect of CA was tested at concentration 2; 15 and 150 µg/mL. The results obtained are shown in the Figure 1. fMLP induced a significant leukocyte migration when compared with the control group (RPMI 1640). CA at concentrations of 2 and 15 µg/mL did not stimulated the leukocyte migration. However, CA at concentration of 150 µg/mL was a potent chemoattractant agent, similar to fMLP. Some researchers reported that CA was able to induce, *in vitro*, migration of the human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC) in chemotactic migration assay[31]. Ours results could indicate that, in high concentration, the CA has an irritant potential and this effect could be related to the activation of intracellular signaling pathways responsible for cell migration and leukocyte activation.

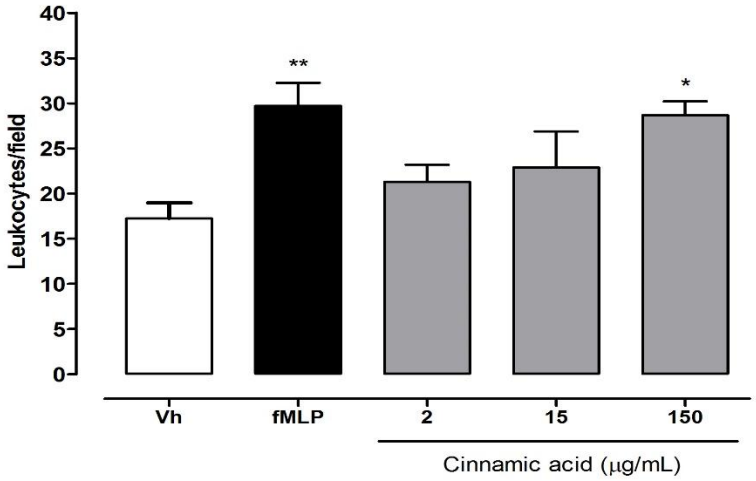

Fig. 1: CA used as a chemotactic agent in concentrations of 2, 15, and 150 µg/mL. Leukocytes were obtained from zymosan-induced peritonitis (1 mg/cavity). Values are mean ± SEM (n = 5) and are representative of three independent experiments. * $p < 0.05$; ** $p < 0,01$ compared to Vh (vehicle). (ANOVA, Tukey test).

The cytotoxic potential of CA was tested in leukocytes. CA at the concentrations 3, 10, 30, 60 and 90 µg/mL presented low cytotoxicity (83, 95, 76, 76 and 88 %, respectively). Some studies demonstrate that CA did not change the macrophages[19-20] and monocytes viability[32], although cytotoxic to tumor cells[33].

The effect of CA on cutaneous inflammatory response was evaluated in the model of ear edema induced by croton oil. This croton oil is an irritant agent that contain 12-0-Tetradecanoylphorbol-13-Acetate (TPA) and induces edema by stimulating of enzyme phospholipase A_2. This enzyme promotes increasing the formation of eicosanoids such as prostaglandin E2 (PGE2) and leukotriene B4 (LTB4), bradykinin and some cytokines such as Tumor Necrosis Factor alpha (TNF-α) and interleukin (IL)-1β. Prostaglandins and leukotrienes are inflammatory mediators involved in edema and leukocyte migration[34-36].

As observed in Figure 2A, the topical application of croton oil induced a cutaneous inflammatory response, characterized by an increase of the ear thickness. Dexamethasone (0.1 mg/ear) significantly inhibited ear edema. CA did not reduce ear edema at concentrations of 0,5, 1 and 2,5 mg/ear, but ear edema was reduced the highest concentration (5 mg/ear).

The possible involvement of leukocytes influx in response to croton oil application was assayed by the measurement of the MPO activity. MPO, a pro-inflammatory enzyme present in the intracellular granules of neutrophils, is frequently used as marker of polymorphonuclear leukocytes influx into inflamed tissues[37]. The croton oil induced an increase of MPO activity in the 6 hours after the application. The topic application of CA, at concentration of 5,0 mg/ear was able to reduce the MPO activity, similarly the dexamethasone.

In this study, our results showed that the cinnamic acid in concentrations of 1.0 and 2.5 mg/ear was effective in reduce the NO production (46 and 59 %, respectively) (figure 2C). These results suggest that the anti-inflammatory effect of CA can partially surround the inhibition of NO production. However, treatment with the higher concentration of CA (5.0 mg/ear) increased in the NO production, suggesting an involvement of this mediator in the activation of chemotaxis *in vitro*.

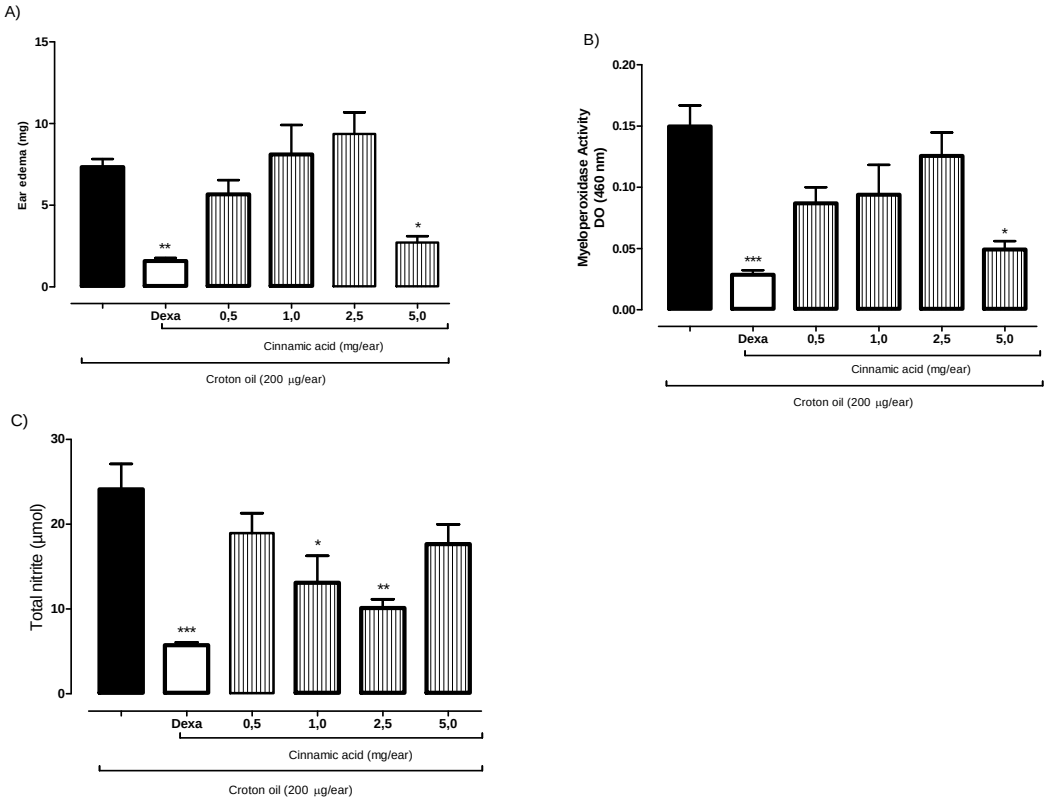

Fig. 2: Effect of topical treatment with CA on the formation of ear edema (A), activity of MPO (B) and concentration of NO (C) induced by croton oil. Data are presented as mean ± S.E.M., * $p < 0.05$; ** $p < 0.01$; *** p 0.001 compared to the control group (croton oil) (ANOVA, Tukey test).

To evaluate the systemic anti-inflammatory effect of CA was used carrageenan and dextran-induced paw edema model. Carrageenan-induced inflammatory response is characterized by an initial phase (1-2 hours) in which there is release of histamine, serotonin and bradykinin, followed by a late phase (3-4 hours), which is maintained mainly by liberation nitric oxide and cytokines (TNF-α and IL-1β). These cytokines in turn activate cyclooxygenase and prostanoid production, such as PGE2[38-42]. In this work, carrageenan promoted intense edema, which reached a maximum level at 6 h. CA, at doses 50, 125 and 250 mg/kg, v.o., significantly reduced the occurrence of edema at 2 h after the carrageenan stimulus (35, 30 and 29 %, respectively). Pretreatment of the animals with indomethacin (10 mg/kg) inhibited edema at all observed time points (Figure 3). Moreover, the CA did not inhibit MPO activity, but the treatment at the doses 50 and 250 mg/kg, was able significantly reduce the production of NO (46; 59 and 77%, respectively). Data from the literature report that CA inhibited the NO production reduced the inducible nitric oxide synthase (iNOS) protein expression and the levels of IL-6, TNF-α, and IL-1β in LPS-stimulated RAW 264.7 cells[20]. Our data suggest that the CA anti-inflammatory mechanism can partially surround the inhibition of NO production and the antiedematogenic effect could be due primarily by inhibition of autacoids released in the first hours after edema induction by carrageenan, such as histamine.

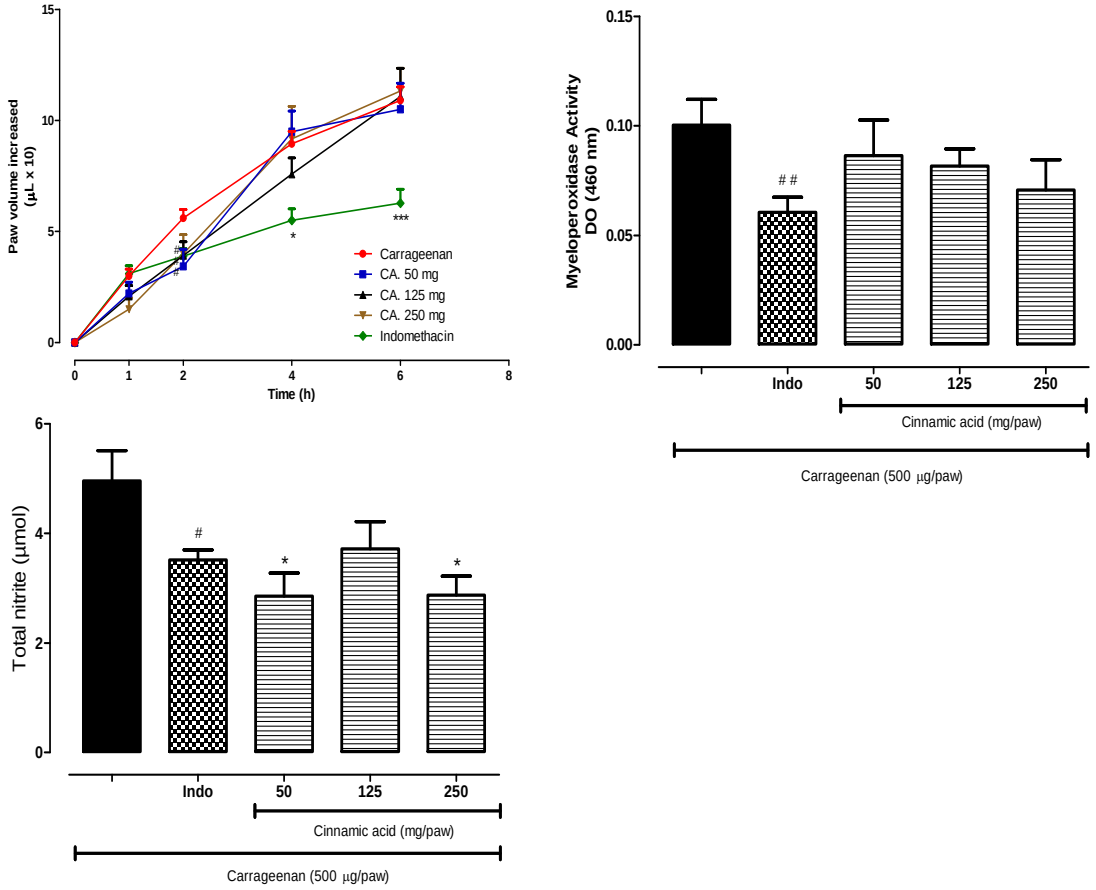

Fig. 3: Effect of topical treatment with CA on the paw edema (A), activity of MPO (B) and concentration of NO (C) induced by carrageenan. Data are presented as mean ± S.E.M., * $p < 0.05$; compared to the control group (carrageenan) (ANOVA, Tukey test).

Histamine is an autacoid involved in increased the vascular permeability and the edema formation. Dextran is a polysaccharide that triggers edema by release of histamine and serotonin from mast cells[43]. Our results demonstrate that CA was able to decrease the formation of paw edema induced by intraplantar injection of dextran in all doses tested (50, 125 and 250 mg/kg) (figure 4). However, the pretreatment with the CA did not inhibit MPO activity or NO production. This data suggest that CA possesses antiedematogenic activity possibly by reduce the release of histamine and serotonin from mast cell.

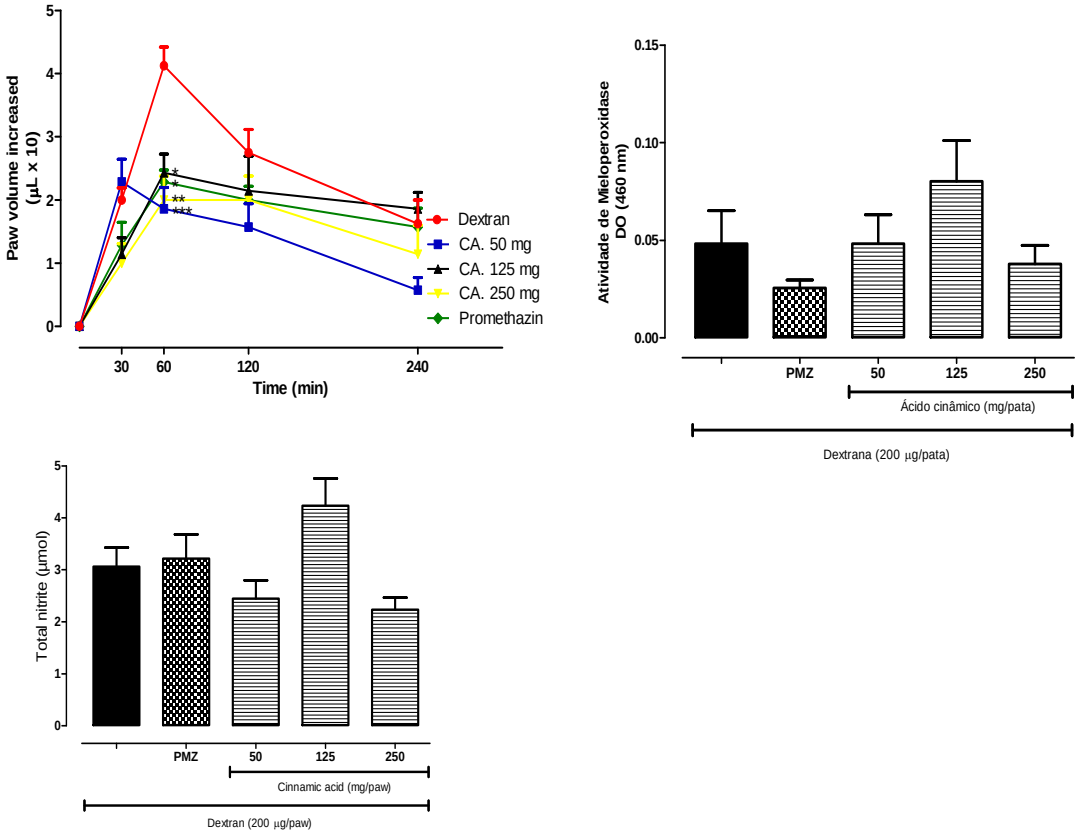

Fig. 4: Effect of topical treatment with CA on the paw edema (A), activity of MPO (B) and concentration of NO (C) induced by dextran. Data are presented as mean ± S.E.M., * $p < 0.05$; compared to the control group (dextran) (ANOVA, Tukey test)

CONCLUSION

In conclusion, these results suggested that CA possessed anti-inflammatory effects. The possible anti-inflammatory mechanism of CA be related to inhibition of the synthesis/release of inflammatory mediators such as NO and histamine.

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