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Anti-inflammatory Activity of *Bromelia hieronymi*: Comparison with Bromelain

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Key words
- *Bromelia hieronymi*
- Bromeliaceae
- bromelain
- anti-inflammatory
- plant proteases

Abstract

Some plant proteases (e.g., papain, bromelain, ficin) have been used as anti-inflammatory agents for some years, and especially bromelain is still being used as alternative and/or complementary therapy to glucocorticoids, nonsteroidal antirheumatics, and immunomodulators. Bromelain is an extract rich in cysteine endopeptidases obtained from *Ananas comosus*. In this study the anti-inflammatory action of a partially purified extract of *Bromelia hieronymi* fruits, whose main components are cysteine endopeptidases, is presented. Different doses of a partially purified extract of *B. hieronymi* were assayed on carrageenan-induced and serotonine-induced rat paw edema, as well as in cotton pellet granuloma model. Doses with equal proteolytic activity of the partially purified extract and bromelain showed significantly similar anti-inflammatory responses. Treatment of the partially purified extract and bromelain with E-64 provoked loss of anti-inflammatory activity on carrageenan-induced paw edema, a fact which is consistent with the hypothesis that the proteolytic activity would be responsible for the anti-inflammatory action.

Abbreviations

COX-2:	cyclooxygenase-2
E-64:	(trans-epoxysuccinyl-L-leucylamido (4-guanidino)butane)
IEF:	isoelectric focusing
NO:	nitric oxide
PGE_2:	prostaglandin E_2
PPE:	partially purified extract

Introduction

Numerous species of the family Bromeliaceae are characterized by the production of proteases in unusual amounts. The best known is pineapple (*Ananas comosus* Merr.), from whose stems bromelain is obtained, an extract rich in cysteine endopeptidases. Bromelain has been shown to have both *in vivo* and *in vitro* anti-edematous, anti-inflammatory, anti-thrombotic, and fibrinolytic effects. In USA and Europe bromelain and other proteases (e.g., trypsin and chymotrypsin) are employed as althernative and/or complementary therapy to glucocorticoids, nonsteroidal antirheumatics, and immunomodulators; its low toxicity makes it also useful in the treatment of chronic inflammation [1,2].

Anti-inflammatory activity of bromelain could be due to the increase of fibrinolytic activity [3], the enhancement of plasmin concentration [4] due to activation of plasminogen to plasmin [5], the decrease of plasma fibrinogen [6], bradykinin, and prekallikrein levels [7], decrease of PGE_2, substance P, and thromboxane A_2 levels [8,9], reduction of COX-2 expression by inhibiting the activation of NF-κB [10], modulation of cell surface molecules involved in migration [11] and cellular activation [12–14], as well as modulation of cytokine production by inhibition of signal transduction in T cells [13]. Even when the anti-inflammatory mechanism of bromelain has not yet been definitively elucidated, its proteolytic activity seems to be implicated [3,11–16].

For ficin and bromelain, Netti et al. [17] found percentages of inhibition of rat paw edema similar or better than those of indomethacin, acetylsalicylic acid, and phenylbutazone. In animal models, the route of administration of proteolytic preparations is mostly oral, although the anti-edematous efficacy of bromelain administered intraperitoneally has also been tested [18].

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Proteases from different plant species have been isolated and characterized in one of our laboratories, including three species belonging to the family Bromeliaceae: *Bromelia hieronymi* Mez [19], *B. balansae* Mez [20], and *Pseudoananas macrodontes* (Morr.) Harms [21], whose proteases were found to be cysteine endopeptidases, but up to date none of them had been tested as potential anti-inflammatory drugs. The main proteolytic components of *B. hieronymi* are hieronymain I, II, and III, which are present in the extract in a relation of 20.4 : 7.2 : 1.0, with molecular masses of about 24 kDa, optimum pH range 7.5–9.0, and pI values ranging from 5.9 to 10.4 [22–24]. In this study the anti-inflammatory action of a partially purified extract of *B. hieronymi* fruits and its comparison with that of bromelain are presented.

Materials and Methods

Chemicals

Casein (from bovine milk), carrageenan tipo IV, Coomassie brillant blue R-250, serotonin, indomethacin (> 99%), cysteine, E-64, and Tris were purchased from Sigma-Aldrich Co. pI markers kit was purchased from GE Amersham Bioscience. Ampholytes (Biolyte 3–10), acrylamide, and bisacrylamide were obtained from Bio-Rad. Ketamine chlorohydrate (50 mg/mL) was purchased from Holliday Scott S. A., and xylazine chlorohydrate (20 mg/mL) from Richmond Laboratories Vet Pharma. Dexamethasone sodium phosphate (4 mg/mL) was purchased from Norgreen SA.

Plant material and extraction

Bromelia hieronymi fruits were collected by Prof. Lucas Roic in Santiago del Estero, Argentina. A voucher specimen (Leg. Venturi, LP 7050) is deposited at the herbarium of the Vascular Plant Division, Faculty of Natural Sciences and Museum, La Plata National University, Argentina. Fruits were washed with distilled water, dried and stored at − 20 °C until extraction.

Plant extract was obtained by chopping and homogenizing frozen unripe fruits (50 g) with 250 mL of 0.1 M sodium phosphate buffer (pH 6.0) containing 5 mM EDTA and 5 mM cysteine as protective agents. The homogenate was filtered and centrifuged, and supernatants were collected and treated with four volumes of cold (− 20 °C) 95% ethanol [25]. The ethanol precipitate was redissolved with 0.1 M sodium phosphate buffer (pH 6.0). Finally, the PPE was lyophilized and stored at − 20 °C.

Extract composition

To determine the proteolytic activity of PPE, caseinolytic activity assays (casein 1%, 0.1 M Tris-HCl buffer pH 7.5, 37 °C, 2 min) were carried out as indicated in a previous work [23]. An arbitrary enzyme unit (caseinolytic units, CU), was used to express proteolytic activity [26]. The protein content was determined by Bradford's Coomassie blue dye-binding method [27], using bovine serum albumin as standard.

IEF was developed on 5% polyacrylamide gels containing broad pH range ampholytes in a Mini IEF Cell (Model 111; Bio-Rad). The lyophilized PPE were dissolved in deionized water, precipitated with 3 volumes of cold (− 20 °C) acetone, centrifuged, and the protein sediment redissolved in a volume of deionized water. Focusing was carried out under constant voltage conditions in a stepped procedure: 100 V for 15 min, 200 V for 15 min, and 450 V for 60 min. Gels were fixed and then stained with Coomassie brillant blue R-250.

Inhibition of cysteine endopeptidases

PPE and bromelain without proteolytic activity (PPE/E-64 and bromelain/E-64, respectively) were obtained by treatment with E-64, a specific inhibitor of cysteine proteases. PPE (60 mg/mL) and bromelain (20 mg/mL) were incubated with E-64 (90 µM) during 30 min at 37 °C. The residual proteolytic activity of PPE/E-64 and bromelain/E-64 were measured by caseinolytic activity assays (casein 1%, cysteine 15 mM, 0.1 M Tris-HCl buffer pH 7.5, 37 °C, 2 min).

Animals and experimental design

Animals: Wistar albino rats were used in the experiments. Animals were purchased, housed and cared for at the Animal Resource Facilities (Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis). The experimental protocols (F-61/09, F-66/09, and F-68/09) were approved by the Laboratory of Animal Care and Use, Institutional Committee (No. 342/10, March 2010) in compliance with argentine official resolutions for animal care guidelines (ANMAT No. 6344/96). Animals were randomly assigned to different groups (n = 6), provided with standard rodent chow diet (Cooperación) and water *ad libitum* and maintained at a constant temperature of 24 ± 1 °C and humidity of 55 ± 5% with 12-h light/dark cycle.

Drug administration: Doses of PPE were prepared by dissolving the lyophilized powder in sterile water. The dose of bromelain was prepared by dissolving the commercial powder (B4882; Sigma Aldrich) in 0.1 M sodium phosphate buffer (pH 6.0). All doses (0.5 mL) were administered i. p.

Carrageenan-induced paw edema in rats: Anti-inflammatory activity was assessed on the paw edema induced by carrageenan, following the method described by Winter et al. [28]. One hour after administration of drugs, animals were injected in the subplantar region of left hind paw with 0.1 mL/rat of carrageenan type IV 2% w/v suspended in saline. The volumes of both hind paws were measured in triplicate using a plethysmograph (Ugo Basile 7140) at intervals of 1, 3, 5, and 7 h after injection of carrageenan. The edema volume is expressed in each animal as the difference between the average volumes of both hind paws. The inhibition percentage of edema was calculated for each group in comparison with control group as $(Vc - Vt/Vc) \times 100$, where Vc and Vt are the edema average volumes of control and treated groups, respectively.

Two carrageenan-induced paw edema experiments were done. Firstly, in order to know an effective dose, male Wistar rats weighing 150–200 g were divided into six groups, of which three received PPE (45, 90, and 180 mg/kg body weight); the control group received 0.1 M sodium phosphate buffer (pH 6.0), one of the reference groups received indomethacin (10 mg/kg body weight) and the other one received bromelain (55 mg/kg body weight).

Then, to determine whether the anti-inflammatory activity of PPE and bromelain is due to the action of proteases, male Wistar rats weighing 200–250 g were divided into six groups, which received PPE (150 mg/kg body weight), PPE/E-64 (150 mg/kg body weight), bromelain (50 mg/kg body weight), bromelain/E-64 (50 mg/kg body weight), E-64 (0.25 µmol/kg body weight), and 0.1 M sodium phosphate buffer pH 6.0 (control group), respectively.

Serotonin-induced paw edema in rats: Male Wistar rats weighing 140–160 g were divided into four groups: two groups received PPE (90 and 180 mg/kg body weight), the control group received 0.1 M sodium phosphate buffer (pH 6.0), and the reference group

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received bromelain (55 mg/kg body weight). One hour after drug administration, all groups received a subplantar injection (0.1 mL) of a 0.01% serotonin solution in normal saline [29]. The edema volumes were measured at 30, 60, and 120 min after injection of serotonin following the same procedure used in the carrageenan-induced edema method. Percent inhibition of edema was calculated as mentioned.

Cotton pellet-induced granuloma in rats: This assay was carried out following the method of Meier et al. [30]. Female Wistar rats weighing 110–130 g were anaesthetized by i.p. injection of a mixture of ketamine (75 mg/kg) and xylazine (12 mg/kg). Granuloma was induced in all animals by subcutaneous implant of a sterile cotton pellet (50 mg) in the dorsal area. One day after the implant, animals were divided into four groups and i.p. inoculated once daily during 6 days as follows: two groups received PPE (90 and 180 mg/kg body weight), the control group received 0.1 M sodium phosphate buffer (pH 6.0), one reference group received bromelain (55 mg/kg body weight) and the other one dexamethasone (3 mg/kg body weight). On day 7, body weight was recorded and the animals were sacrificed by cervical decapitation. The pellets surrounded by granuloma tissue were removed and weighed. Spleen and thymus of animals were dissected out and weighed. The anti-inflammatory effect was assessed by determining the inhibition percentage of granuloma formation in the groups under study as compared with the control as follows: $(mc - mt/mc) \times 100$, where *mc* and *mt* are the average weights of control and treated groups, respectively. Reduction percentages of thymus and spleen weights were calculated with the same formula.

Statistical analysis

The GraphPadPrism software version 5.0 was used for statistical analysis. Data obtained are presented as mean ± SEM. The data were analyzed with One Way ANOVA followed by Tukey multiple comparison test. A probability of $p < 0.05$ was considered significant.

Results

The protein content of PPE was 20 ± 2 µg/mg, and its specific enzymatic activity was 10 ± 1 CU/mg of protein (37 °C, pH 7.5). After treatment with E-64, specific enzymatic activity of PPE decreased to 0.20 ± 0.04 CU/mg of protein (cysteine 15 mM, 37 °C, pH 7.5). IEF of PPE showed the six characteristics bands of *B. hieronymi* [22] with the following pI values: 5.9, 6.4, 7.6, 8.3, and two bands > 9.3 (**Fig. 1**). Specific enzymatic activity of bromelain and bromelain/E-64 were 3.2 ± 0.2 and 0.4 ± 0.2 CU/mg of protein (cysteine 15 mM, 37 °C, pH 7.5), respectively.

The effect of different doses of PPE (45, 90, and 180 mg/kg), bromelain (55 mg/kg), and indomethacin (10 mg/kg) on carrageenan-induced paw edema is shown in **Table 1**. All doses of PPE showed anti-inflammatory activity. Anti-inflammatory activity of PPE (180 mg/kg) was not significantly different to that of bromelain at 1, 3, 5, and 7 h. Indomethacin showed significant anti-inflammatory activity at 5 and 7 h. The maximum effect for all compounds was obtained at 5 h with the following inhibition percentages: 44, 75, and 78% for 45, 90, and 180 mg/kg of PPE, respectively, 76% for bromelain, and 66% for indomethacin. The anti-inflammatory effects of PPE (90 and 180 mg/kg) were not significantly different at 1, 3, and 5 h, but they were significantly different at 7 h.

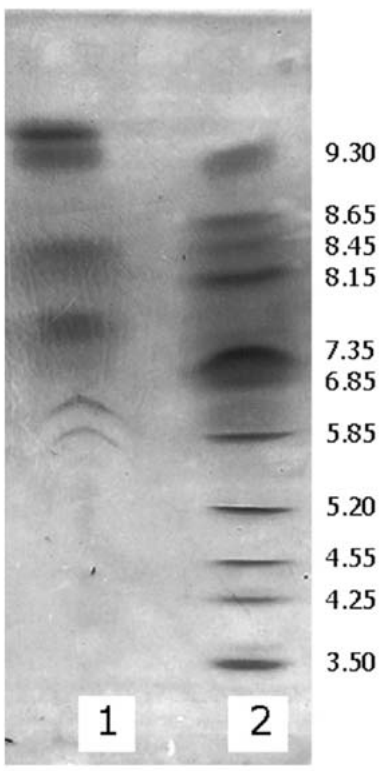

Fig. 1 Isoelectrofocusing. Lane 1, PPE; Lane 2, pI markers: amyloglucosidase (pI 3.50), glucose oxidase (pI 4.25), trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), carbonic anhidrase B (pI 5.85), myoglobin (pI 6.85 and 7.35), lectins (pI 8.15, 8.45, and 8.65), and trypsinogen (pI 9.30).

Table 2 shows the effect of PPE and PPE/E-64 (150 mg/kg), bromelain and bromelain/E-64 (50 mg/kg), and E-64 (0.25 µmol/kg) on carrageenan-induced paw edema. PPE and bromelain showed significant anti-inflammatory effect along the whole assay, while PPE/E-64 and bromelain/E-64 showed only significant anti-inflammatory effect at 1 h after carrageenan injection. Even when during the whole assay the inhibition percentages of PPE were higher than those of PPE/E-64, they were only at 5 h statistically significant (71% > 13%, $p < 0.05$). Inhibition percentages of bromelain were significantly higher than those of bromelain/E-64 along the whole assay. E-64 did not show anti-inflammatory effect.

Table 3 shows the effects of PPE (90 and 180 mg/kg) and bromelain (55 mg/kg) on serotonin-induced paw edema in rats. PPE (180 mg/kg) showed significant anti-inflammatory activity at 30 and 60 min after serotonin injection, with inhibition percentages of 37 and 28%, respectively. Bromelain exhibited significant anti-inflammatory activity at 30, 60, and 120 min (51, 42, and 31%, respectively), values higher but not significantly different from those of PPE (180 mg/kg). PPE (90 mg/kg) did not show anti-inflammatory action at any time.

The effect of PPE, bromelain, and dexamethasone on cotton pellet-induced granuloma and on thymus and spleen weights are shown in **Fig. 2**. All compounds reduced significantly granuloma formation relative to control at the 7th day. The percent inhibition for PPE (180 mg/kg), bromelain, and dexamethasone were 41, 34, and 55%, respectively, PPE and bromelain values being not significantly different. PPE (90 mg/kg) had inhibitory effect (13%), but it was not significant. All anti-inflammatory doses also significantly reduced thymus weight relative to controls. The reduction percentage of PPE (180 mg/kg) and bromelain was the same and notably lower (28%) than that of dexamethasone (74%), while PPE (90 mg/kg) reduced 18% but not significantly. Spleen weight

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Table 1 Effect of different doses of PPE, bromelain, and indomethacin on carrageenan-induced paw edema in rats.

Time (h)	Control	PPE (45 mg/kg)		PPE (90 mg/kg)		PPE (180 mg/kg)		Bromelain (55 mg/kg)		Indomethacin (10 mg/kg)	
	Edema volume (mL)	Edema volume (mL)	Inhibition (%)	Edema volume (mL)	Inhibition (%)	Edema volume (mL)	Inhibition (%)	Edema volume (mL)	Inhibition (%)	Edema volume (mL)	Inhibition (%)
1	0.34 ± 0.01	0.24 ± 0.02	29_{bc}	0.21 ± 0.03	38_{b}^{*}	0.22 ± 0.02	35_{b}^{*}	0.31 ± 0.05	9_{bc}	0.30 ± 0.01	12_{bc}
3	0.59 ± 0.06	0.52 ± 0.09	12_{c}	0.27 ± 0.03	54_{ba}^{**}	0.25 ± 0.02	58_{b}^{**}	0.27 ± 0.05	54_{ba}^{**}	0.50 ± 0.05	15_{ac}
5	1.17 ± 0.11	0.66 ± 0.10	44_{a}^{*}	0.29 ± 0.09	75_{b}^{***}	0.26 ± 0.01	78_{b}^{***}	0.28 ± 0.03	76_{b}^{***}	0.40 ± 0.04	66_{ba}^{***}
7	1.24 ± 0.09	0.91 ± 0.12	27_{ac}	0.75 ± 0.12	40_{ad}^{**}	0.38 ± 0.06	69_{b}^{***}	0.32 ± 0.06	74_{b}^{***}	0.53 ± 0.04	57_{bd}^{***}

Edema volumes are expressed as the mean ± SEM (n = 6). Inhibition (%) represents the mean percentage reduction in paw volume compared with the controls. Tukey's test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the control group; the means with one common subscript letter are not significantly different at $p < 0.05$

Table 2 Effect of E-64 (specific cysteine proteases inhibitor) on anti-inflammatory activity of PPE and bromelain in carrageenan-induced paw edema model.

Time (h)	Control	PPE (150 mg/kg 30 ± 3 CU/kg)		PPE/E-64 (150 mg/kg 0.90 ± 0.12 CU/kg)		Bromelain (50 mg/kg 32 ± 2 CU/kg)		Bromelain/E-64 (50 mg/kg 4 ± 2 U/kg)		E-64 (0.25 µmol/kg)	
	Edema volume (mL)	Edema volume (mL)	Inhibition (%)	Edema volume (mL)	Inhibition (%)	Edema volume (mL)	Inhibition (%)	Edema volume (mL)	Inhibition (%)	Edema volume (mL)	Inhibition (%)
1	0.42 ± 0.07	0.22 ± 0.02	48_{b}^{***}	0.26 ± 0.04	38_{b}^{**}	0.22 ± 0.03	48_{b}^{***}	0.28 ± 0.02	33_{ba}^{*}	0.40 ± 0.03	5_{ac}
3	0.67 ± 0.16	0.20 ± 0.03	70_{b}^{***}	0.45 ± 0.13	33_{cb}	0.18 ± 0.02	73_{b}^{***}	0.50 ± 0.14	25_{c}	0.62 ± 0.06	7.5_{c}
5	1.15 ± 0.20	0.33 ± 0.05	71_{b}^{***}	1.00 ± 0.16	13_{c}	0.20 ± 0.01	83_{b}^{***}	0.92 ± 0.20	20_{c}	1.03 ± 0.07	10_{c}
7	1.44 ± 0.22	0.95 ± 0.17	34_{a}^{*}	1.25 ± 0.06	13_{ac}	0.28 ± 0.07	80_{b}^{***}	1.31 ± 0.16	9_{c}	1.20 ± 0.07	17_{c}

Edema volumes are expressed as the mean ± SEM (n = 6). Inhibition (%) represents the mean percentage reduction in paw volume compared with the controls. Tukey's test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the control group; the means with one common subscript letter are not significantly different at $p < 0.05$

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Table 3 Effect of different doses of PPE and bromelain on serotonin-induced paw edema in rats.

Time (m)	Control	PPE (90 mg/kg)		PPE (180 mg/kg)		Bromelain (55 mg/Kg)	
	Edema volume (mL)	Edema volume (mL)	Inhibition (%)	Edema volume (mL)	Inhibition (%)	Edema volume (mL)	Inhibition (%)
30	1.22 ± 0.05	1.18 ± 0.05	3.3_c	0.77 ± 0.14	37_a**	0.60 ± 0.09	51_a***
60	1.09 ± 0.04	1.16 ± 0.05	-6_c	0.78 ± 0.10	28_a*	0.63 ± 0.08	42_a**
120	0.83 ± 0.04	0.70 ± 0.03	16_{ac}	0.66 ± 0.07	20_{ac}	0.57 ± 0.07	31_a*

Edema volumes are expressed as the mean ± SEM (n = 6). Inhibition (%) represents percentage mean reduction paw volume compared with the controls. Tukey's test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the control group; the means with one common subscript letter are not significantly different at $p < 0.05$

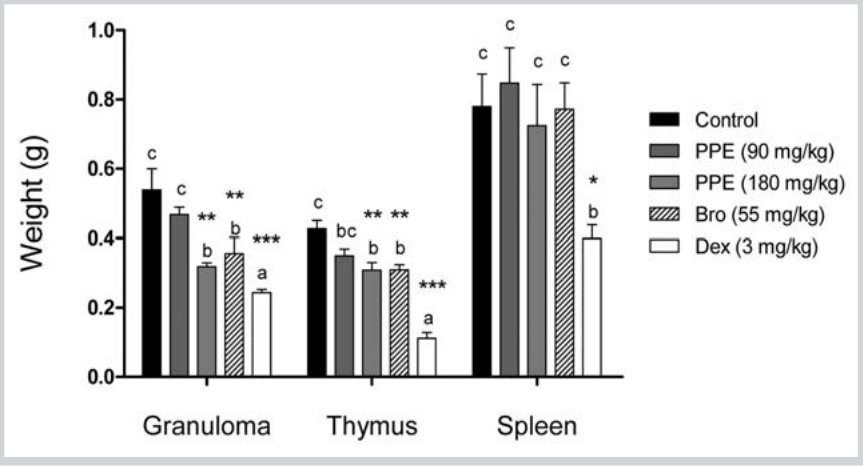

Fig. 2 Effect of different doses of PPE, bromelain (Bro), and dexamethasone (Dex) on cotton pellet granuloma formation and weights of thymus and spleen in rats. Values are expressed as the mean ± SEM (n = 6). Tukey's test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the control group; the groups with one common letter are not significantly different at $p < 0.05$.

was only significantly reduced by dexamethasone. **Fig. 3** shows the body weight changes during the experiment. Average body weight increments were 22 g for control group, 20 and 14 g for PPE-treated groups (90 and 180 mg/kg, respectively), and 13 g for bromelain. In contrast, dexamethasone produced significant body weight loss (− 14 g).

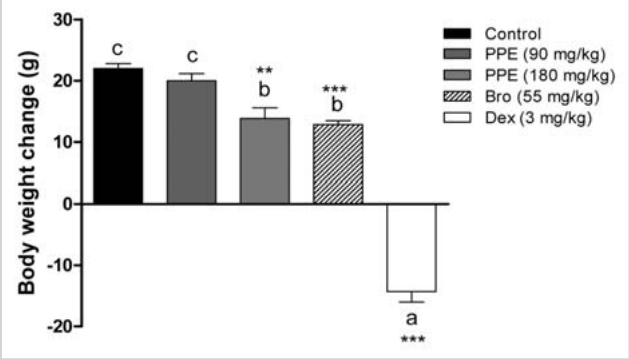

Fig. 3 Effect of PPE, bromelain (Bro), and dexamethasone (Dex) on body weight in rats after treatment. Body weight change was calculated as difference between final and initial body weight. Values are expressed as the mean ± SEM (n = 6). Tukey's test: ** $p < 0.01$, *** $p < 0.001$ compared to the control group; the groups with a common letter are not significantly different at $p < 0.05$.

Discussion

Acute inflammatory response is characterized by an increase in vascular permeability, extravasation of fluid and plasma proteins, and cellular infiltration from blood vessels to the inflamed area, leading to the edema formation. A number of chemical mediators have been identified in the inflammatory response: histamine, serotonin, NO, eicosanoids, cytokines, and products derived from plasma systems (coagulation, complement, and kinins). The effect of *B. hieronymi* extract (PPE) on acute inflammation was evaluated by the carrageenan- and serotonin-induced rat paw edema assays. Carrageenan-induced rat paw edema is a widely used test to determine the anti-inflammatory activity of drug, and doses of NSAIDs in this model correlate well with effective dose in patients [31].

The development of edema in this model has been described as a biphasic process [32]. The initial phase of edema (0–1 h), which is not inhibited by NSAIDs, has been attributed to the release of histamine, serotonin, and bradykinin [33]. The second phase (1–6 h) has been associated with a local infiltration and activation of neutrophils [34–36] and the elevated production of prostaglandins [33,37], which more recently has been attributed to the induction of COX-2 in the hindpaw [38,39]. There is a time-dependent increase in PGE_2 and NO production as well as in the neutrophils infiltration associated with the increase of paw volume [40]. PPE showed a significant anti-inflammatory effect from the first hour after injection of carrageenan with an efficacy of about 35%, which increased at 3 h, reaching maximum efficacy at 5 h with significant inhibition percentages similar to those of bromelain and indomethacin (about 75%). The lowest dose tested (45 mg/kg) was significantly inhibitory only at 5 h. While anti-inflammatory effects of 90 and 180 mg/kg of PPE were significantly equivalent to those of bromelain from the first hour until 5 h post carra-

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geenan injection, at 7 h only the 180 mg/kg PPE dose maintained a significant effect equivalent to that of bromelain (about 70 %). Because the proteolytic activity of PPE (180 mg/kg) and bromelain (55 mg/kg) are equivalent (35–36 CU/kg), and taking into account that the anti-inflammatory action of bromelain has been attributed to the presence of proteases, the effects of PPE and bromelain after being inhibited with E-64 was evaluated. Loss of proteolytic activity in PPE and bromelain by E-64 treatment provoked the consequent loss of anti-inflammatory effect from 3 h, being more evident at 5 h for PPE, while for bromelain at 5 and 7 h. Given the sequential action of inflammatory mediators described for this model, it could be speculated that PPE, like bromelain [10, 11, 15], predominantly acts by inhibiting the infiltration/activity of neutrophils or the release/actions of NO and prostaglandins, whose production peaks usually appear between 3 and 6 h after injection of carrageenan [40]. Furthermore, cysteine proteases would be involved in the process. At 1 h, the lower inhibition percents of PPE and bromelain (**Table 2**) could be due to low inhibitory effects on mediators of the first phase (0–1 h) and/or to still little concentration of active principles in action site. The latter hypothesis could also explain the different results obtained for bromelain at 1 h in experiments 1 and 2 (**Tables 1** and **2**). Whatever the correct explanation, effects at 1 h of PPE and bromelain would not depend on the proteolytic activity of cysteine proteases because anti-inflammatory action is not lost after E-64 treatment, which would indicate the presence of other components acting only on the first phase.

The effect of PPE in the first phase of edema development was evaluated using serotonin-induced edema paw model. Only PPE (180 mg/kg) inhibited edema formation 0.5 and 1 h after the injection of serotonin, showing percentages of inhibition significantly similar to those of bromelain. These results would indicate that part of the anti-inflammatory effect of PPE and bromelain at the first phase of carrageenan-induced edema could be due to inhibitory effect on serotonin.

When the acute inflammatory response fails to eliminate the causative agent or to restore the normal physiology of the injured tissue, a chronic state of inflammation occurs, which is characterized by infiltration of lymphocytes and macrophages and can lead to a proliferative phase accompanied with tissue changes (formation of new capillaries and proliferation of fibroblasts). A morphological type of this stage is granulomatous inflammation, characterized by an organized collection of macrophages. The cotton pellet-induced granuloma is a representative model for studying drugs against this inflammation phase: the granuloma formed by day 7 is characterized by the formation of a vascularized fibrous capsule containing fibroblasts and infiltrating mononuclear cells [41].

PPE showed anti-inflammatory effect on the chronic inflammatory process by inhibiting the cotton-induced granuloma formation. PPE (180 mg/kg) and bromelain (55 mg/kg) had significantly similar inhibition percentages, but they were lower to that of dexamethasone (3 mg/kg). Some of the adverse effects of dexamethasone were also evaluated. As an indication of the immunosuppressive action, thymuses and spleens were weighed [42, 43], and the catabolic effect was determined by measuring the loss of body mass. While dexamethasone caused significant atrophy of spleens and thymuses, bromelain and PPE (180 mg/kg) only reduced the thymuses weight and did it in equal percentage, which was significantly lower (almost the half) than that of dexamethasone.

Bromelain and PPE (180 mg/kg) did not provoke loss of body weight, even when the body weight gain was significantly lower than that of the control group. On the contrary, loss of body mass was significant for dexamethasone. PPE (90 mg/kg) did not significantly reduce the formation of granuloma but had no immunosuppressive effect nor resulted in body weight loss. Taking into account the predominant role of T-cell in the development of granulomatous inflammation [44] and the slight thymic atrophy that accompanies the inhibition of granuloma formation by PPE and bromelain, one might assume that part of the anti-inflammatory mechanism of action would involve the regulation of T cell activity.

Finally, comparing the anti-inflammatory effect of the assayed doses of the extract of *B. hieronymi* with bromelain, it can be seen that in all three inflammation models existed significantly similar responses between PPE (180 mg/kg) and bromelain (55 mg/kg) doses. Notably, the proteolytic activity of both doses are almost equivalent (average 35 CU/kg), a fact which is consistent with the hypothesis that the proteolytic activity would be responsible for the anti-inflammatory action [3, 11–16].

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Conflict of Interest

The authors report no conflict of interest.

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