Evaluation of Anti-Inflammatory Activity of *Pseudananas macrodontes* (Morr.) Harms (Bromeliaceae) Fruit Extract in Rats

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Several species of the family Bromeliaceae are characterized by the production of proteases in unusual amounts, especially in fruits. Bromelain, an extract rich in cysteine endopeptidases obtained from *Ananas comosus* L., and a few other proteases have been used as anti-inflammatory agents for some years, but bromelain is still mainly being used as alternative and/or complementary therapy to the treatment with glucocorticoids, nonsteroidal antirheumatics, and immunomodulators. In this study, the anti-inflammatory action of a partially purified extract from *Pseudananas macrodontes* (Morr.) Harms fruits (PPE$_{Pm}$) is presented, whose main components are cysteine endopeptidases. The effect of PPE$_{Pm}$ was assessed in carrageenan-induced and serotonin-induced rat paw edema, as well as in the cotton pellet granuloma model. Doses with equal proteolytic activity of PPE$_{Pm}$ and bromelain produced significantly similar anti-inflammatory responses in the acute inflammatory models assayed, supporting the hypothesis that proteolytic activity could be responsible for the anti-inflammatory action. On the contrary, comparable anti-inflammatory effects of PPE$_{Pm}$ and bromelain in the chronic inflammatory assay required a much lower proteolytic activity content of PPE$_{Pm}$, which could be due to a differential affinity for the protein target involved in this process.

Key words: Anti-Inflammatory, *Pseudananas macrodontes*, Plant Proteases

Introduction

Many inflammatory diseases are becoming common in aging societies throughout the world. The clinically used anti-inflammatory drugs suffer from the disadvantage of side effects and high cost of treatment (in case of biologics). Alternatives to these drugs are traditional medicines and natural products, which offer a great hope in the identification of bioactive lead compounds and their development into drugs for treating inflammatory diseases (Gautam and Jachak, 2009). Many plant species and their products exhibiting experimental or clinical anti-inflammatory or analgesic activities have been recently reviewed (Anilkumar, 2010), one of the best examples being acetylsalicylic acid (aspirin) derived from the bark of *Salix alba* (Gómez Estrada *et al.*, 2011).

Several species of the family Bromeliaceae are characterized by the production of proteases in unusually high amounts, particularly in their fruits, probably with a role in defence responses (Boller, 1986; van der Hoorn and Jones, 2004). Bromelain,

an extract rich in cysteine endopeptidases obtained from *Ananas comosus* L. (Bromeliaceae) stems, has been shown to have both *in vivo* and *in vitro* antiedematous, anti-inflammatory, anticoagulant, and fibrinolytic effects and is employed in therapies alternative and/or complementary to treatments with glucocorticoids, nonsteroidal antirheumatics, and immunomodulators. Its low toxicity makes it also useful in the treatment of chronic inflammation (Maurer, 2001). In therapeutic use, bromelain is orally administered alone or together with rutin and/or other enzymes such as trypsin, chymotrypsin, pancreatin, papain, lipases, and amylases (Brien *et al.*, 2004).

The anti-inflammatory activity of bromelain involves an increase in the fibrinolytic activity, enhancement of plasmin concentration by activation of the conversion of plasminogen to plasmin, decrease of the levels of plasma fibrinogen, bradykinin, and prekallikrein (Maurer, 2001), decrease of prostaglandin E$_2$ (PGE$_2$), thromboxane A$_2$, and substance P levels (Vellini *et al.*, 1986; Gaspani *et*

al., 2002), as well as the modulation of cell surface molecules involved in migration (Fitzhugh *et al.*, 2008) and cellular activation (Hale and Haynes, 1992; Mynott *et al.*, 1999; Hale *et al.*, 2002), and reduction of cyclooxygenase-2 (COX-2) expression by inhibiting the activation of nuclear factor kappa-B (NF-κB) (Bhui *et al.*, 2011). Even though the mechanism of the anti-inflammatory action of bromelain has not yet been definitively elucidated, its proteolytic activity appears to be implicated (Fitzhugh *et al.*, 2008; Hale and Haynes, 1992; Mynott *et al.*, 1999; Hale *et al.*, 2002, 2005). For ficin and bromelain, Netti *et al.* (1972) found the degree of the inhibition of rat paw edema induced by different agents – carrageenan, serotonin, dextran, and brewer's yeast – similar or better than that achieved with indomethacin, acetylsalicylic acid, and phenylbutazone.

Two cysteine endopeptidases, *viz.* macrodontain I and II, have been isolated from *Pseudananas macrodontes* (Morr.) Harms fruits and characterized by us (López *et al.*, 2001). The N-terminal sequences of both proteases (including the Cys 26 of the active site) show a high degree of identity (92.6%), as differences in only two residues are observed. Macrodontain I possesses 85.7% identity with comosain, 85.2% with stem bromelain, and 77.8% identity with ananain, while macrodontain II possesses 88.9% identity with stem bromelain, 77.8% with ananain, and 76.2% with comosain (López *et al.*, 2001). The primary structure of macrodontain I is composed of 213 amino acids (UniProtKB/Swiss-Prot entry P83443; molecular mass, 23,486 Da), and the alignment with other plant cysteine peptidases revealed that the highest degree of identity (77%) was obtained with fruit bromelain, followed by ananain (71.9%), and stem bromelain (67.1%) (Brullo, 2003).

In this study, the anti-inflammatory action of a partially purified extract of *P. macrodontes* fruits (PPE$_{Pm}$) and its comparison with the action of bromelain in acute and chronic inflammation models was examined.

Material and Methods

Chemicals

Bromelain (B4882), carrageenan type IV, casein (from bovine milk), Coomassie brilliant blue G-250, cysteine, indomethacin (> 99%), serotonin, and Tris were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic

acid (EDTA) was purchased from Invitrogen (Carlsbad, CA, USA), sodium phosphate (98%) from Carlo Erba (Rodano, MI, Italy), ketamine chlorohydrate (50 mg/mL) from Holliday Scott (Beccar, BA, Argentina), xylazine chlorohydrate (10 mg/mL) from Richmond Laboratories Vet Pharma (Buenos Aires, Argentina), and dexamethasone sodium phosphate (4 mg/mL) from Laboratorios Klonal (Quilmes, BA, Argentina).

Plant material

Pseudananas macrodontes fruits were collected in Santa Ana, Department of Candelaria, Province of Misiones, Argentina (latitude, S 27°23'; longitude, W 55°33'), by Prof. Aníbal G. Amat. A voucher specimen (Leg. Amat, No. 1596) was deposited at the herbarium of the Faculty of Exact, Chemical and Natural Sciences of the National University of Misiones (MNES), Misiones, Argentina. Fruits were washed with distilled water, dried, and stored at –20 °C until extraction.

Preparation of plant extract

Crude and partially purified extracts were obtained according to López *et al.* (2000), with some modifications. Frozen unripe fruits (120 g) were chopped and homogenized with 600 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 at 5,000 x *g* for 30 min. The supernatant (crude extract) was collected, filtered, and stored at –20 °C until use. Then, to one volume of crude extract one volume of cold (–20 °C) 96% ethanol was added during 30 min at 0 °C and the mixture centrifuged at 12,000 x *g* for 30 min. The precipitate was discarded, and three volumes of cold ethanol were added to the supernatant during 2 h at 0 °C; the resulting mixture was centrifuged at 12,000 x *g* for 30 min. The ethanol precipitate was redissolved in 0.1 M sodium phosphate buffer (pH 6.0) after evaporation of the solvent *in vacuo*. Finally, the partially purified extract, named PPE$_{Pm}$, was lyophilized and stored at –20 °C.

Extract characterization

Assays on caseinolytic activity (1% casein, 0.1 M Tris-HCl buffer, pH 7.5, 37 °C, 2 min) were carried out to determine the proteolytic activity of PPE$_{Pm}$. An arbitrary enzyme unit (caseinolytic

unit, CU) was used to express the proteolytic activity (López *et al.*, 2000).The protein content was determined by the method of Bradford (1976), using bovine serum albumin as standard.

Animals

Wistar albino rats were purchased, housed, and cared for at the Animal Resource Facilities, Faculty of Chemistry, Biochemistry and Pharmacy, National University of San Luis, San Luis, Argentina. The experimental protocols were approved by the Laboratory Animal Care and Use Institutional Committee 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, Buenos Aires, Argentina) and water *ad libitum*, and maintained at a constant temperature of $(24 \pm 1)\,°C$ and a

humidity of $(55 \pm 5)\%$ under a 12-h light/12-h dark cycle.

Drug administration

PPE$_{Pm}$ doses were prepared by dissolving the lyophilized powder in sterile water. The dose of bromelain was prepared by dissolving the commercial material in 0.1 M sodium phosphate buffer (pH 6.0). All doses (0.5 mL) were administered intraperitoneally (i.p.).

Carrageenan-induced paw edema in rats

Anti-inflammatory activity was assessed on paw edema induced by carrageenan, following the method described by Winter *et al.* (1962). Male Wistar rats, weighing $170-220$ g, were divided into five groups ($n = 6$): two groups received PPE$_{Pm}$ [90 and 180 mg/kg body weight (BW)], the control group received 0.1 M sodium
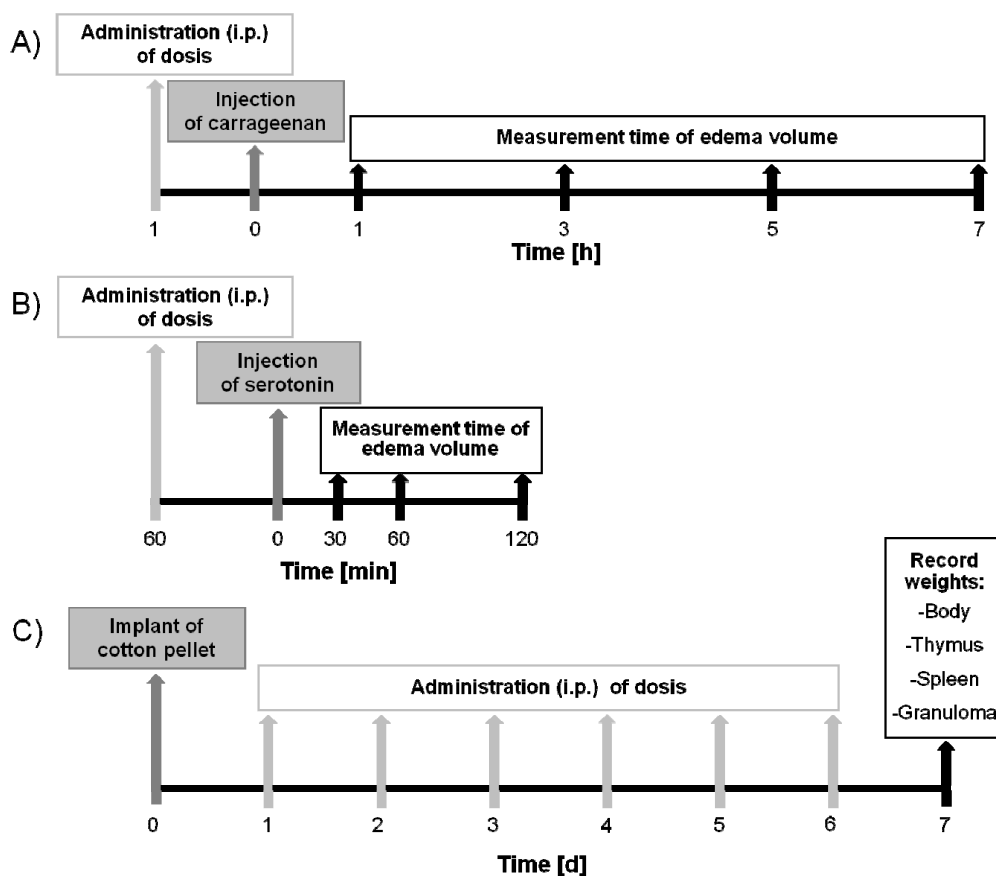

Fig. 1. Model design and dosing routines for rats in (A) carrageenan-induced paw edema, (B) serotonin-induced paw edema, and (C) cotton pellet-induced granuloma.

phosphate buffer (pH 6.0), one of the reference groups received indomethacin (10 mg/kg BW), and the other one received bromelain (50 mg/kg BW). One h later, 0.1 mL of 2% (w/v) carrageenan type IV suspended in saline was injected into the subplantar region of the left hind paw of each rat. Volumes of both hind paws were measured in triplicate using a plethysmograph (Ugo Basile, Varese, Italy) at 1, 3, 5, and 7 h after injection of carrageenan (Fig. 1A). The edema volume was expressed as the difference between the average volumes of the two hind paws. The inhibition percentage of edema was calculated for each group in comparison with the control group as $(1 - V_t/V_c) \cdot 100$, where V_c and V_t are the edema average volumes of control and treated groups, respectively.

Serotonin-induced paw edema in rats

Male Wistar rats, weighing 200–220 g, were divided into three groups ($n = 6$): one group received PPE$_{Pm}$ (180 mg/kg BW), the control group received 0.1 M sodium phosphate buffer (pH 6.0), and the reference group received bromelain (50 mg/kg BW). One h after drug administration, all groups received a subplantar injection of 0.1 mL of 0.01% serotonin solution in normal saline (Kalbhem and Smalla, 1977). Edema volume was measured at 30, 60, and 120 min after injection of serotonin following the same procedure used in the carrageenan-induced edema method (Fig. 1B). Percent inhibition of edema was calculated as mentioned above.

Cotton pellet-induced granuloma in rats

The assay was carried out following the method of Meier *et al.* (1950). Female Wistar rats, weighing 115–150 g, were anaesthetized by i.p. injection of a mixture of ketamine chlorohydrate (75 mg/kg BW) and xylazine chlorohydrate (12 mg/kg BW). Granuloma was induced in all animals by a subcutaneous implant of a sterile cotton pellet (50 mg) in the dorsal area. One d later, animals were divided into four groups and i.p. inoculated once daily during 6 d as follows: one group received PPE$_{Pm}$ (60 mg/kg BW), the control group received 0.1 M sodium phosphate buffer (pH 6.0), one reference group received bromelain (50 mg/kg BW), and the other one dexamethasone (4 mg/kg BW). On day 7, body weight was recorded, and the animals were sacrificed by cervical dislocation.

The pellets surrounded by granuloma tissue were removed and weighed. Spleen and thymus 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: $(1 - m_t/m_c) \cdot 100$, where m_c and m_t are the average weights of control and treated groups, respectively. Reduction percentage of thymus and spleen weights was calculated with the same formula. Body weight change was calculated as the difference between final and initial body weights. Fig. 1C shows model design and dosing routines.

Statistical analysis

The GraphPadPrism software version 5.0 was used for statistical analysis. Data obtained are presented as mean ± SEM. Raw data (edema volume and weights) were analysed with Oneway ANOVA followed by Tukey's Multiple Comparison test. A probability of $p < 0.05$ was considered significant.

Results and Discussion

The protein content of the partially purified extract obtained from *Pseudananas macrodontes* fruits (PPE$_{Pm}$) was (28 ± 2) µg/mg of lyophilized powder, and its specific enzymatic activity was (5.6 ± 0.4) CU/mg of protein (37 °C, pH 7.5). In the case of stem bromelain (B4882), the protein content was (200 ± 12) µg/mg of commercial powder, and specific enzymatic activity was (3.2 ± 0.2) CU/mg of protein (37 °C, pH 7.5).

Edema formation is one the events that happen during acute inflammatory response and is the result of changes in the microvasculature of the inflamed area. Carrageenan-induced rat paw edema is a model of acute inflammation widely used to determine the anti-inflammatory activity of drugs, and doses of nonsteroidal anti-inflammatory drugs (NSAIDs) in this test correlated well with effective doses in patients (Morris, 2003). Table I shows the effects of PPE$_{Pm}$ (90 and 180 mg/kg BW), bromelain (50 mg/kg BW), and indomethacin (10 mg/kg BW) administration on carrageenan-induced paw edema in rats. Both PPE$_{Pm}$ doses elicited an anti-inflammatory effect: the lower one (90 mg/kg BW) significantly inhibited edema formation after 3 h, reaching maximum relative inhibition (about 45%) between 3 and 5 h after carrageenan

Table I. Effect of PPE_{Pm}, bromelain, and indomethacin on carrageenan-induced paw edema in rats.

Time [h]	Control	PPE_{Pm} [90 mg/kg BW = (15 ± 1) CU/kg BW]		PPE_{Pm} [180 mg/kg BW = (31 ± 2) CU/kg BW]		Bromelain [50 mg/kg BW = (32 ± 2) CU/kg BW]		Indomethacin (10 mg/kg BW)	
	Edema volume [mL]	Edema volume [mL]	Inhibition (%)	Edema volume [mL]	Inhibition (%)	Edema volume [mL]	Inhibition (%)	Edema volume [mL]	Inhibition (%)
1	0.27 ± 0.03	0.19 ± 0.02	29_{bc}	0.12 ± 0.02	55_b***	0.18 ± 0.02	33_{bc}	0.24 ± 0.02	11_c
3	0.64 ± 0.07	0.35 ± 0.04	45_{ba}**	0.19 ± 0.02	70_b***	0.29 ± 0.03	55_b***	0.55 ± 0.06	14_{ca}
5	1.14 ± 0.08	0.6 ± 0.2	47_b**	0.23 ± 0.02	80_b***	0.28 ± 0.02	75_b***	0.38 ± 0.03	67_b***
7	1.3 ± 0.1	0.8 ± 0.2	38_a*	0.20 ± 0.02	85_b***	0.45 ± 0.04	65_d***	0.55 ± 0.04	58_{ad}***

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

administration, while the higher one (180 mg/kg BW) inhibited after 1 h, reaching the maximum effect (about 80%) between 5 and 7 h after carrageenan administration. Bromelain was significantly anti-inflammatory after 3 h and exhibited maximum inhibition (about 75%) after 5 h of carrageenan administration. Indomethacin showed significant anti-inflammatory activity 5 and 7 h after carrageeenan administration.

Edema development in this model has been described as a biphasic process (Vinegar *et al.*, 1969; Salvemini *et al.*, 1996). The initial phase of edema formation (0–1 h), which is not inhibited by NSAIDs, is attributed to the release of histamine and serotonin (Di Rosa *et al.*, 1971). The second phase (1–6 h) is associated with a local infiltration and activation of neutrophils (Boughton-Smith *et al.*, 1993) and the production of prostaglandins (Di Rosa *et al.*, 1971), which has been attributed to the induction of COX-2 (Nantel *et al.*, 1999). An increase in NO production is also associated with the increase of paw volume (Salvemini *et al.*, 1996).

Although PPE_{Pm} (180 mg/kg BW) had a significant anti-inflammatory effect from the first hour after injection of carrageenan, the maximum inhibition was reached 5 h after carrageenan administration, as in the case of bromelain and indomethacin. All doses tested produced significant inhibition after 7 h. It is known that indomethacin inhibits prostaglandins production (by inhibition of COX-1), while bromelain, in other experimental models, has been shown to reduce the production of NO (Wen *et al.*, 2006) and prostaglandins (Bhui *et al.*, 2011), as well as neutrophil migration (Fitzhugh *et al.*, 2008). These results allow us to conclude that PPE_{Pm} acts preferentially on the second rather than the first phase of carrageenan-induced edema, which could be explained by a better bioavailability and/or increased specificity against the release/action of mediators involved in the second phase (neutrophils, prostaglandins, and NO), whose production usually peaks between 3 and 6 h after injection of carrageenan (Salvemini *et al.*, 1996).

Table II. Effect of PPE_{Pm} and bromelain on serotonin-induced paw edema in rats.

Time [min]	Control	PPE_{Pm} [180 mg/kg BW = (31 ± 2) CU/kg BW]		Bromelain [50 mg/kg BW = (32 ± 2) CU/kg BW]	
	Edema volume [mL]	Edema volume [mL]	Inhibition (%)	Edema volume [mL]	Inhibition (%)
30	1.05 ± 0.07	0.75 ± 0.11	29_a*	0.52 ± 0.03	50_a***
60	0.87 ± 0.07	0.68 ± 0.12	22_{ca}	0.50 ± 0.04	42_a*
120	0.67 ± 0.06	0.56 ± 0.11	20_c	0.46 ± 0.04	31_c

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

To evaluate the action of PPE$_{Pm}$ on the early development of edema, the serotonin-induced edema assay was carried out. Table II shows the results of PPE$_{Pm}$ (180 mg/kg BW) and bromelain (50 mg/kg BW) administration on serotonin-induced paw edema in rats. PPE$_{Pm}$ significantly inhibited edema formation (29%) 30 min after serotonin injection, which was not significantly different from the action of bromelain (50%). Also, bromelain exhibited significant anti-inflammatory activity (42%) 60 min after injection.

Granulomatous inflammation is a morphological type of chronic state of inflammation, and the cotton pellet-induced granuloma in rats is a representative model for studying drugs against this inflammation phase (Bailey *et al.*, 1982). Anti-inflammatory action of PPE$_{Pm}$ was assayed in this model and compared with that of bromelain and dexamethasone. PPE$_{Pm}$ (60 mg/kg BW) and bromelain (50 mg/kg BW) were similarly effective in the cotton pellet-induced granuloma assay (Fig. 2). PPE$_{Pm}$, bromelain, and dexamethasone (4 mg/kg BW) significantly reduced granuloma formation relative to the control at day 7, inhibiting 26, 24, and 54%, respectively. Values for PPE$_{Pm}$ and bromelain were not significantly different from each other, but were significantly lower than that for dexamethasone. In addition, some side effects, common to long-term glucocorticoid therapy, were evaluated: the immunosuppressive action, by weighing thymus and spleen (Ben Rhouma and Sakly, 1994; Hori *et al.*, 1996), and the catabolic effect, by measuring the loss of body mass. While dexamethasone caused significant atrophy of spleen and thymus (50 and 86%, respectively), bromelain and PPE$_{Pm}$ only reduced thymus weight to the same low degree (26%). On the other hand, changes in body weight in the control group and those treated with PPE$_{Pm}$ and bromelain were not significantly different. Body weight increments at the end of the assay were 15 g for the control group, and 17 and 15 g for PPE$_{Pm}$- and bromelain-treated groups, respectively. In contrast, dexamethasone produced a significant body weight loss (–11 g).

These results are promising for the application of proteolytic preparations in the long-term treatment of chronic diseases; however, toxicological tests are required to confirm this view. Sixty mg PPE$_{Pm}$/kg BW was the dose of choice, because 33% mortality was observed with a dose of 180 mg/kg BW (data not shown). On the other hand, taking into account the predominant role of T-cells in the development of granulomatous inflammation (Co *et al.*, 2004) and the slight thymic atrophy that accompanies the inhibition of granuloma formation by PPE$_{Pm}$ and bromelain, also observed for *Bromelia hieronymi* extracts (Errasti *et al.*, 2013), one might assume that part of the anti-inflammatory action involves the regulation of T-cell activity.

Since the anti-inflammatory mechanism of bromelain has been attributed to its proteolytic activity (Fitzhugh *et al.*, 2008; Hale and Haynes, 1992; Mynott *et al.*, 1999; Hale *et al.*, 2000, 2005), the higher dose of PPE$_{Pm}$ (on weight basis) in the *acute* inflammatory assays was chosen to contain proteolytic activity equal to that of bromelain ($\approx$ 30 CU/kg BW). The observation that, on this basis, the effects of the two preparations were significantly similar, supports the notion that proteases are responsible for their anti-inflammatory activities. On the contrary, a much lower dose of PPE$_{Pm}$ [(10 $\pm$ 1) CU/kg BW] was sufficient to produce an anti-inflammatory effect comparable to that of bromelain (32 $\pm$ 2 CU/kg BW) in the *chronic* inflammatory assay, which could be due to differential affinities for the protein(s) critically involved in the latter process.
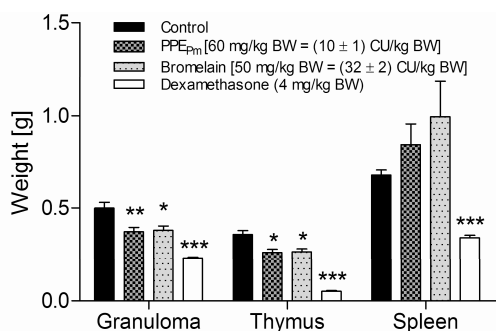

Fig. 2. Effect of PPE$_{Pm}$, bromelain, and dexamethasone on the formation of cotton pellet-induced granuloma and weights of thymus and spleen in rats. Values are expressed as the mean $\pm$ SEM ($n = 6$). Tukey's test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the control group.

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