ORIGINAL ARTICLE

Protein fraction of *Calotropis procera* latex protects against 5-fluorouracil-induced oral mucositis associated with downregulation of pivotal pro-inflammatory mediators

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Abstract Oral mucositis is an important dose-limiting and costly side effect of cancer chemotherapy. Soluble proteins obtained of the latex of *Calotropis procera* have been extensively characterized as anti-inflammatory in different experimentally induced inflammatory conditions, including arthritis and sepsis. In this study, the phytomodulatory laticifer proteins (LP) were challenged to regress the inflammatory events associated with 5-fluorouracil-induced oral mucositis. We also evaluated the expression of pro-inflammatory cytokines and inducible enzymes, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Oral mucositis was induced in hamsters by two injections of 5-fluorouracil (5-FU; 60 and 40 mg/kg, i.p., on experimental days 1 and 2, respectively). LP (5 mg/kg, i.p.) was injected 24 h before and 24 h after mechanical trauma of the cheek pouches. A normal control group received only saline. On day 10, the animals were sacrificed, and the cheek pouches were excised for macroscopic and histopathological analysis, myeloperoxidase activity measurement, and immunohistochemical assessment of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), iNOS, and COX-2. LP significantly inhibited macroscopic histopathological scores and myeloperoxidase activity compared with the 5-FU control group. 5-Fluorouracil also induced marked immunostaining of TNF-α, IL-1β, iNOS, and COX-2 on inflamed conjunctive and epithelial tissue compared with the normal control group. Such damage was significantly inhibited ($p<0.05$) by LP treatment compared with the 5-FU group. These findings demonstrate an anti-inflammatory effect of LP on 5-FU-induced oral mucositis. The protective mechanism appears to involve inhibition of the expression of iNOS, COX-2, TNF-α, and IL-1β.

Keywords Oral mucositis · 5-Fluorouracil · *Calotropis procera* · Inflammation · Chemotherapy toxicity

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Introduction

Mucositis is a clinical term that describes a syndrome characterized by ulceration of the mucosa of the entire digestive tract and associated symptoms (Sonis and Fey 2002). This side effect is common in cancer patients treated with various chemotherapeutic agents, particularly anti-metabolites (e.g., methotrexate and fluorouracil), and other agents, such as cisplatin, doxorubicin, and ifosfamide (Balis et al. 1985;

Bishop et al. 1986; Caballero et al. 1985; Roth et al. 1991) as well as in patients submitted to abdominal radiotherapy (Altmann 1974).

The oral form of mucositis is mainly characterized by the presence of ulcerative lesions (i.e., stomatitis) responsible for severe pain and discomfort. Patients complain mainly of pain that often keeps them from eating normally, which decreases their nutritional status.

Data relative to the risk of developing grade 3 or 4 oral mucositis have shown that the incidence of chemotherapy with 5-fluorouracil, capecitabine, or tegafur leads to a high rate (20–50 %) of alimentary tract mucositis. Chemotherapy with methotrexate and other anti-metabolites leads to a 20–60 % rate of alimentary tract mucositis according to the drug's given dose per cycle. In patients receiving high-dose head and neck radiation to the oral cavity and in hematopoietic stem-cell transplantation patients, the incidence is up to 85 and 75 %, respectively (Peterson et al. 2010).

Current research focuses on effective preventive and therapeutic options for oral mucositis that significantly improve patient quality of life. Preventive strategies are the usual approach to manage oral mucositis induced by chemotherapy. Since the guidelines for managing mucositis in cancer patients have been published, considerable progress in research and clinical application has been made (Rubenstein et al. 2004). Recently, these guidelines have been revised with changes of the original guidelines, including new therapeutic and preventive agents and protocols (Keefe et al. 2007). Despite advances, the available treatments are scarce, and none have proven to be totally effective. Oral mucositis is one of the leading causes for unplanned treatment interruptions which jeopardize the effectiveness of the treatment (Haagen et al. 2009).

Recently, our group began to study the pathogenesis of oral mucositis induced by 5-fluorouracil in hamsters. We demonstrated the role of nitric oxide and pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), in this model (Leitão et al. 2007; Leitão et al. 2008; Lima et al. 2005).

Calotropis procera is a laticiferous plant (Apocynaceae) locally known in Brazil as "algodão de seda," "leiteiro," "queimadeira," and "ciúme" (Kismann and Groth 1999; Lorenzi and Matos 2002). The latex components of *C. procera* appear to induce interesting biological effects, and the plant itself is known for its pharmacological potential. Previous studies that used the latex of *C. procera* reported anti-inflammatory (Kumar and Basu 1994; Sangraula et al. 2002), analgesic (Soares et al. 2005), anti-diarrheal (Kumar et al. 2001), and anti-oxidant (Kumar et al. 2011). Almost all of these studies investigated the properties and activity of the latex without previous fractionation of the material. More recent studies have also been conducted with the

protein fraction isolated from the whole latex (Ramos et al. 2007). This procedure has effectively shown that laticiferous proteins have many of the promising effects that have been demonstrated for the whole latex (Soares et al. 2005; Alencar et al. 2004; Ramos et al. 2009). Very recently, these researchers showed that laticifer proteins of *C. procera* protect animals against experimentally induced lethal sepsis with important effects on the homeostasis of blood coagulation (Oliveira et al. 2012; Ramos et al. 2012).

Since the demonstration of the anti-inflammatory effects of *C. procera* and the lack of effectiveness of existing mucositis therapeutics, we investigated the effect of *C. procera* latex proteins in 5-FU-induced oral mucositis.

Material and methods

Animals

Ninety male adult golden hamsters that weighed 120–150 g were obtained from the sector vivarium of the Federal University of Ceará. The surgical procedures and animal treatments were conducted in accordance with the Guidelines for Institutional Animal Care and were approved by our Institutional Animal Care and Use Committee of Federal University of Ceará (protocol no. 036/10). The animals were housed in temperature-controlled rooms and received water and food ad libitum.

Plant material and latex collection

The latex of *C. procera* (Ait.) R.Br. from the aerial parts of the plant was collected in plastic tubes that contained distilled water to give a dilution ratio of 1:2 (*v/v*). The native plants were located in the vicinity of Fortaleza, CE, Brazil. The plant voucher (sample specimen no. 32663) was deposited at the Prisco Bezerra Herbarium of the Universidade Federal do Ceará, Brazil, where the botanical material was identified by a local taxonomist. After the latex was collected, it was processed as described before (Alencar et al. 2004). This protocol consists of (1) centrifugation of the crude latex to partially precipitate rubber; (2) dialysis against distilled water using dialysis membranes with cutoff 8,000 Da to induce complete precipitation of the remaining rubber and all other hydrophobic materials. Even, small molecular water-soluble compounds are lost by dialysis. (3) New step of centrifugation at the same conditions to complete precipitation of rubber and water-insoluble compounds still present in the sample. This later step gives rise to a very clean and water-soluble fraction that is rich in proteins, named "laticifer proteins". This protocol is highly reproducible because it is easy to perform and absent of hard chemical treatments. Therefore, a homogeneous sample is

obtained. The chemical characterization of this fraction in terms of protein content and enzymatic activities has been extensively characterized by electrophoresis (1-D and 2-D), mass spectrometry, chromatography, proteomic approach, enzymatic assays based in colorimetric methods, and zymograms (Oliveira et al. 2007; Freitas et al. 2007; Ramos et al. 2009; Oliveira et al. 2010; Oliveira et al. 2012; Ramos et al. 2012).

Induction of experimental oral mucositis

The hamster oral mucositis model was modified and adapted from the original work described by Sonis et al. (1990) and later by Leitão et al. (2007). Oral mucositis was induced by two intraperitoneal (i.p.) injections of 5-fluorouracil (5-FU; Roche, Rio de Janeiro, Brazil) on days 1 and 2 of the experiment (60 and 40 mg/kg, respectively). To mimic the friction to which the oral mucosa is normally subjected, the animal cheek pouch mucosa was irritated by scratching with the tip of an 18-gauge needle on day 4 under tribromoethanol anesthesia (1 ml/100 g per animal, i.p.). The needle was linearly dragged twice across the averted cheek pouch until erythematous changes were noted. The animals were sacrificed on day 10 after the initial injection of 5-FU under tribromoethanol anesthesia. The cheek pouches were averted and photographed, and the hamster was then sacrificed. Samples of the cheek pouches were removed from six animals per group for histopathological analysis, immuno-histochemical assessment of TNF-α, IL-1β, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), and myeloperoxidase (MPO) determination as described below (Leitão et al. 2007).

Experimental groups

The hamsters were separated into seven groups: normal (N, animals not subjected to oral mucositis), mechanical trauma (MT, animals that received only mechanical trauma and treated with an i.p. saline injection), control (C, animals that were treated with saline and subjected to 5-FU-induced oral mucositis and mechanical trauma), and four groups that were treated with laticifer protein of *C. procera* (LP; 0.25, 1, 5, and 25 mg/kg, 24 h before and 24 h after mechanical trauma) and subjected to 5-FU-induced oral mucositis.

Macroscopic analysis of cheek pouch

At the time determined for each experiment, the animals were anesthetized with tribromoethanol (1 ml/100 g per animal, i.p.), and the cheek pouch was averted and photographed before the animal was sacrificed. The photographs were coded and used to score the lesions. For macroscopic analysis, signs of inflammation, such as erythema, hyperemia, hemorrhagic

areas, epithelial ulcerations, and abscesses, were evaluated in a single-blind fashion and graded as the following: score 0 (completely healthy cheek pouch with erosion or vasodilatation absent), score 1 (presence of erythema but no evidence of erosion in the cheek pouch), score 2 (severe erythema, vasodilation, and surface erosion), score 3 (formation of ulcers in one or more faces of the mucosa that do not affect more than 25 % of the surface area of the cheek pouch, severe erythema, and vasodilatation), score 4 (cumulative formation of ulcers of 50 % of the surface area of the cheek pouch), and score 5 (virtually complete ulceration of the cheek pouch mucosa; in this case, fibrosis makes oral mucosa exposure difficult) (Medeiros et al. 2011). The photographs were analyzed in a single-blind fashion by a trained evaluator.

Histopathological analysis

The specimens were fixed in 10 % neutral buffered formalin, dehydrated, and embedded in paraffin. Sections of 5-μm thickness were obtained for hematoxylin-eosin staining (H&E) and examined by light microscopy (×100 magnification). The parameters of inflammatory cell infiltration, vasodilatation, and the presence of hemorrhagic areas, edema, ulcerations, and abscesses were determined in a single-blind fashion and graded as the following: score 0 (normal epithelium and connective tissue without vasodilatation, absence of or discreet cellular infiltration, and absence of hemorrhagic areas, ulcerations, or abscesses), score 1 (discreet vasodilatation, reepithelization areas, discreet inflammatory infiltration with mononuclear prevalence, and absence of hemorrhagic areas, edema, ulcerations, or abscesses), score 2 (moderate vasodilatation, areas of hydropic epithelial degeneration, inflammatory infiltration with neutrophil prevalence, presence of hemorrhagic areas, edema, and eventual ulcerations, and absence of abscesses), and score 3 (severe vasodilatation, inflammatory infiltration with neutrophil prevalence, presence of hemorrhagic areas, edema, and extensive ulceration, and abscesses) (Leitão et al. 2007).

Myeloperoxidase assay

The extent of neutrophil accumulation in the cheek pouch samples was measured by assaying MPO activity. Briefly, the animals were sacrificed on day 10 after the initial injection of 5-FU, and the cheek pouch samples were harvested, weighed, and stored at −70 °C until required for the assay. For the MPO assay, the samples were defrosted, homogenized with a phosphate buffer (pH 6.0) that contained hexadecyl trimethylammonium bromide (Sigma), and centrifuged at 12,000×g for 2 min. Myeloperoxidase activity was measured in the supernatants according to the method previously described by Bradley et al. (1982). The data are expressed as

units of MPO per milligram of tissue, with 1 U MPO defined as the amount of enzyme responsible for the degradation of 1 μmol of hydrogen peroxide/min at 22 °C.

Immunohistochemistry

Immunohistochemistry for TNF-α, IL-1β, iNOS, and COX-2 on day 10 of oral mucositis was performed using the streptavidin-biotin-peroxidase method in formalin-waxed, paraffin-embedded tissue sections (4-μm thick) mounted on poly-L-lysine-coated microscope slides. The sections were deparaffinized and rehydrated through xylene and a graded series of alcohol. After antigen retrieval, endogenous peroxidase was blocked twice (10 min) with 3 % (v/v) hydrogen peroxide and washed in phosphate-buffered saline (PBS). The sections were incubated overnight (4 °C) with primary rabbit anti-iNOS, rabbit anti-IL-1β, goat anti-TNF-α, or goat anti-COX-2 antibody (Santa Cruz Biotechnology) diluted 1:200 in PBS plus bovine serum albumin (PBS-BSA 5 %). The slides were then incubated with biotinylated goat anti-rabbit (IgG) or biotinylated donkey anti-goat (IgG) diluted 1:400 in PBS-BSA. After washing, the slides were incubated with avidin-biotin-horseradish peroxidase conjugate (Strep ABC Complex; Santa Cruz Biotechnology) for 30 min according to the manufacturer's protocol. Immunostaining was visualized with the chromogen 3,3′-diaminobenzidine (DAB; liquid DAB+substrate chromogen system; Dako). Negative control sections were processed simultaneously as described above but with the first antibody replaced with PBS-BSA 5 %. None of the negative controls showed TNF-α, IL-1β, iNOS, or COX-2 immunoreactivity. The slides were counterstained with Harry's hematoxylin, dehydrated in a graded series of alcohol, cleared in xylene, and cover-slipped.

All of the slides were photographed, and the intensity of staining per stained cell population was determined in a single-blind fashion and graded with scores from 0 to 4.

Statistical analysis

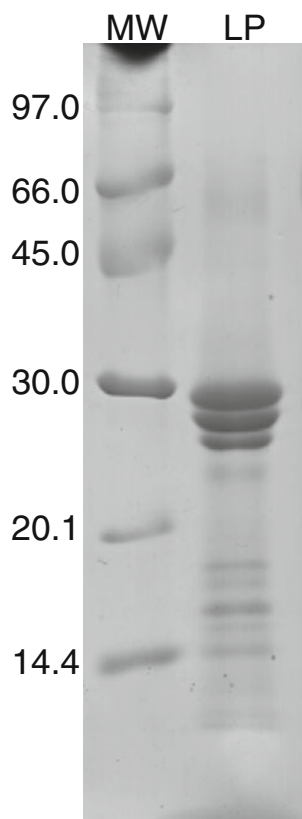The data are expressed as either mean ± SEM or median as appropriate. Analysis of variance, followed by

Fig. 1 Polyacrylamide gel (12.5 %) electrophoresis of LP (20 μg). Molecular weight markers (MW) were phosphorylase B, 97.0 kDa; bovine serum albumin, 66.0 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 KDa; trypsin inhibitor, 20.1 kDa; and alpha-lactalbumin, 14.4 kDa. The protein profile of LP was first shown by Ramos et al. (2006)
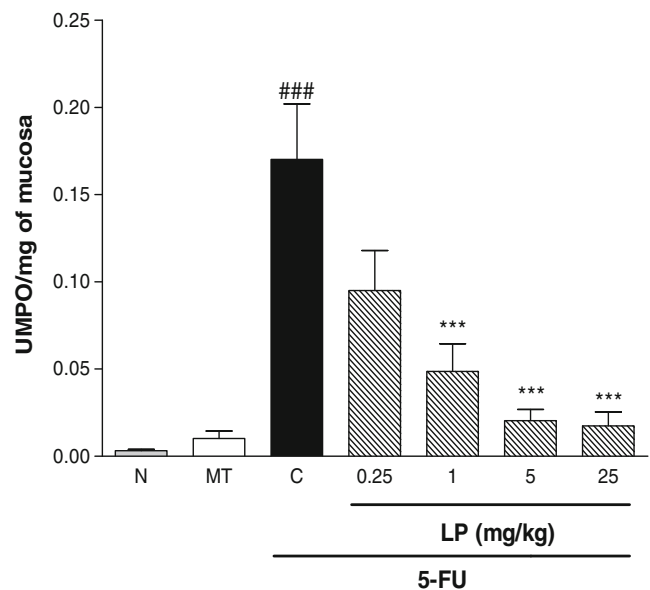

Fig. 2 Effect of C. procera on myeloperoxidase activity in the cheek pouch of hamsters subjected to the induction of oral mucositis. Saline (5-FU, control (C) group) or laticifer protein of C. procera (LP, 0.25–25 mg/kg) was injected 24 h before and 24 h after mechanical trauma in animals subjected to oral mucositis induced by 5-FU. The normal (N) group was not subjected to oral mucositis. The MT group was subjected only to mechanical trauma. After sacrifice, a sample of the cheek pouch was removed for the assessment of MPO activity. Bars represent the mean value ± standard error of the mean of the concentration of units of MPO per milligram of tissue. Triple asterisks indicate $p < 0.001$ significant difference compared with 5-FU-induced oral mucositis group; triple number signs indicate $p < 0.001$ compared with the N group. The number of animals in each group was at least five. The data were analyzed with analysis of variance and the Bonferroni test

Fig. 3 Macroscopic and microscopic aspects of normal hamster cheek pouches (**a** and **b**) and cheek pouches of animals subjected to 5-FU-induced oral mucositis that received saline, showing the presence of abscesses (**c**) and edema and inflammatory cell infiltration (**d**), and laticifer proteins of *C. procera* (LP, 5 mg/kg; **e** and **f**), showing the preservation of oral mucosa. Oral mucositis was induced by an i.p. injection of 5-FU followed by mechanical trauma of the cheek pouch. The animals received an i.p. injection of LP 24 h before and 24 h after mechanical trauma. Each cheek pouch was averted and photographed, and samples were removed and processed for hematoxylin and eosin staining after the animal was sacrificed. (×100)
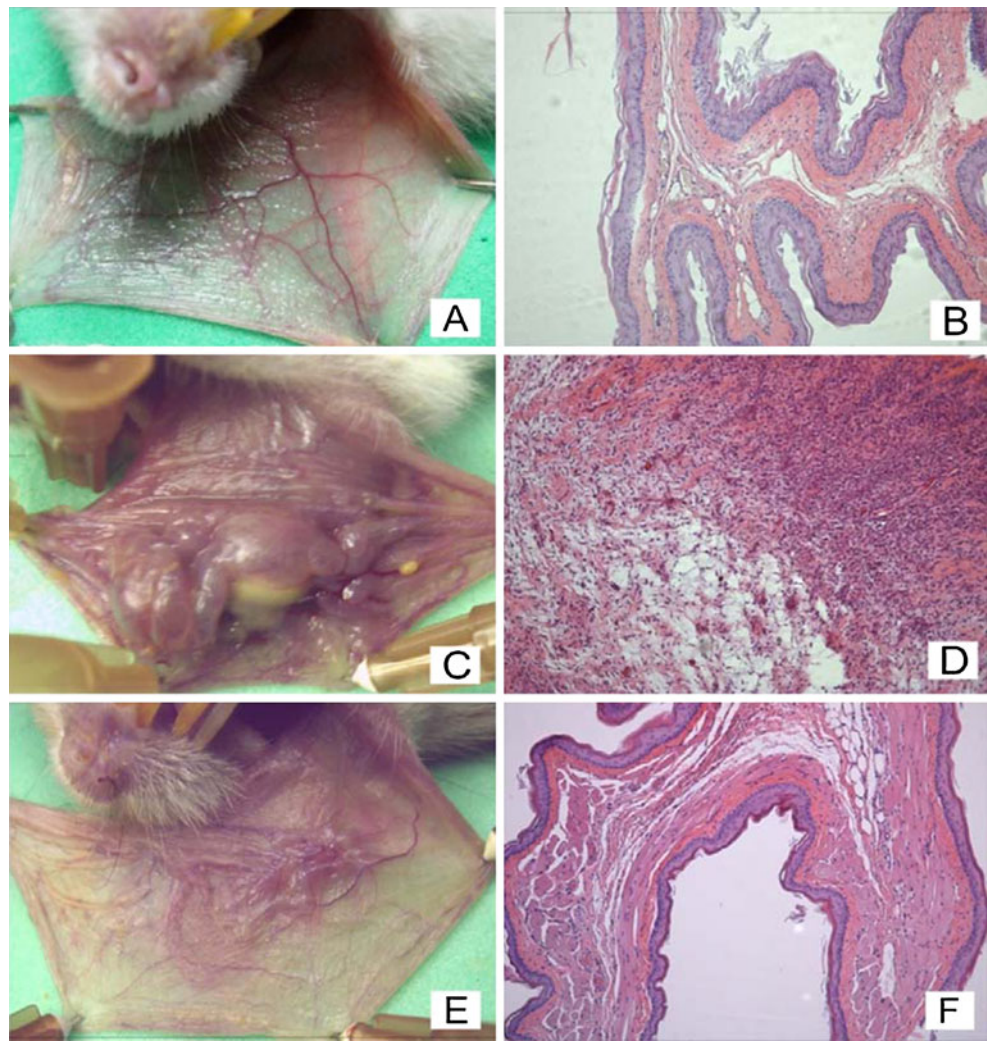
Bonferroni's test, was used to compare means, and the Kruskal–Wallis and Mann–Whitney tests were used to compare medians. Values of $p<0.05$ were considered statistically significant.

Results

This study was performed in an attempt to validate the anti-inflammatory activity of LP on mucositis. LP comprises the

Table 1 Macroscopic and microscopic analysis of hamster cheek pouch of animals submitted to experimental oral mucositis and treated laticifer proteins isolated from latex of *C. procera*

Experimental group	N	MT	5-FU				
			LP (mg/kg)				
			C	0.25	1	5	25
Macroscopic scores	(0–0) 0	(0–0) 0	(3–5) 4****	(2–5) 4***	(1–4) 3	(0–3) 1**	(0–4) 2.5
Microscopic scores	(0–0) 0	(0–2) 1	(3–3) 3	(2–3) 2	(0–3) 1.5*	(0–3) 1**	(0–3) 1.5

Saline (*C* control group) or Laticifer Proteins of *C. procera* (*LP* 0.25–25 mg/kg) was injected 24 h before and 24 h after mechanical trauma in animals submitted to oral mucositis induced by 5-FU. Normal group (*N*) composed of animals not submitted to oral mucositis, and *MT* group represent animals submitted only to mechanical trauma. Data represent the median values (and range) of macroscopic or microscopic scores in six animals per group. Data were analyzed by using Kruskal–Wallis followed by Dunn's

*$p<0.05$ and **$p<0.001$ (compared to saline-treated (C) animals); ***$p<0.05$ (compared to animals that received LP (1, 5, and 25 mg/kg)); ****$p<0.001$ (compared to MT and N groups)

Table 2 Effect of *C. procera* (LP) upon immunohistochemistry analysis for TNF-α, COX-2, IL-1β, and iNOS expression in the cheek pouches of hamsters subjected to 5-fluorouracil-induced oral mucositis

Tissue	TNF-α		COX-2		IL-1β		iNOS	
	Ep	Cj	Ep	Cj	Ep	Cj	Ep	Cj
N	(0–1) 0.5	(0–1) 0.5	(0–0) 0	(0–0) 0	(1–4) 1.5	(0–4) 2.5	(0–4) 2	(0–1) 0.5
MT	(1–2) 1.5	(1–1) 1	(0–2) 1	(1–2) 1	(1–2) 2	(1–3) 1	(2–3) 3	(1–3) 2
C	(1–3) 2	(3–4) 4****	(1–2) 2	(4–4) 4****	(0–4) 2	(3–4) 4***	(3–4) 4***	(3–4) 4***
LP	(0–1) 1	(1–2) 1*	(0–1) 0*	(1–2) 1**	(0–1) 0.5	(0–1) 0.5*	(0–3) 2*	(0–2) 0.5**

Saline (*C* control group) or laticifer proteins of *C. procera* (*LP* 5 mg/kg) was injected 24 h before and 24 h after mechanical trauma in animals submitted to oral mucositis induced by 5-FU. Normal group (*N*) composed of animals not submitted to oral mucositis, and *MT* group represent animals submitted only to mechanical trauma. The results are expressed the median values (and range) of scores of immunostaining intensity of the epithelial tissue (*Ep*) and conjunctive tissue (*Cj*) of six animals per group. Data were analyzed by using Kruskal–Wallis followed by Dunn's
*$p<0.05$ and **$p<0.001$ (compared to saline-treated animals); ***$p<0.05$ and ****$p <0.001$ (compared to N group)

major soluble protein fraction isolated from the latex of *C. procera*. The typical protein profile of LP has been previously shown by electrophoresis (1-D and 2-D) and mass spectrometry (Freitas et al. 2007; Oliveira et al. 2010). Here, Fig. 1 illustrates this profile. In order to estimate the anti-inflammatory activity of LP on mucositis, myeloperoxidase activity was measured in the cheek pouch as an indicator of neutrophil infiltration. Myeloperoxidase activity of the cheek pouch tissue of animals treated with MT and animals subjected to 5-FU-induced oral mucositis (5-FU/saline) was significantly increased ($p<0.001$) compared with the N group on day 10 of oral mucositis. LP significantly ($p<0.001$) reduced (71 %) the 5-FU-induced increase in MPO activity at a minimum dose of 1 mg/kg (Fig. 2). Even though statistically similar, LP at 5 mg/Kg was more effective reaching 88 % inhibition. Therefore, this dose was chosen for further analysis.

The i.p. administration of 5-FU, followed by mechanical trauma of the cheek pouch, caused significant lesions ($p<0.05$), reflected by accentuated erythema, hemorrhage, extensive ulcers, and abscesses (Fig. 3c and Table 1) compared with the N group (Fig. 3a and Table 1) and MT group (Table 1). The treatment of the animals with 5 mg/kg LP prevented 5-FU-induced oral damage, reflected by reduced erythema and the absence of ulcerations and abscesses on day 10 ($p<0.05$; Fig. 3e, g, respectively; Table 1).

The histopathology of the cheek pouch of the animals subjected to 5-FU-induced oral mucositis revealed ingurgitation and accentuated vascular dilatation, intense cellular infiltration with neutrophil prevalence, hemorrhagic areas, edema, abscesses, and extensive ulcers (Fig. 3d and Table 1) compared with the normal cheek pouches of hamsters not subjected to oral mucositis (Fig. 3b and Table 1) and the MT group (Table 1). The treatment with LP (Fig. 3f and Table 1) significantly ($p<0.05$) reduced 5-FU-induced inflammatory cell infiltration, edema, and hemorrhage and prevented the formation of ulcers and abscesses.

The cheek pouches of the hamsters subjected to 5-FU-induced oral mucositis (C group) showed marked and significant immunostaining of TNF-α ($p<0.001$), IL-1β ($p<0.001$), iNOS ($p<0.05$), and COX-2 ($p<0.05$) on inflamed conjunctive tissue (Table 2 and Figs. 4d, e and 5d, e) compared with the cheek pouches of the N group (Table 2 and Figs. 4b, c and 5b, c). In epithelial tissue, only iNOS immunostaining was significantly ($p<0.05$) different from the N group (Fig. 5e). LP (5 mg/kg) significantly decreased TNF-α ($p<0.05$), IL-1β ($p<0.01$), iNOS ($p<0.05$), and COX-2 ($p<0.01$) immunostaining in conjunctive tissue compared with the C group (Table 2 and Figs. 4d, e and 5d, e). In epithelial tissue, 5 mg/kg LP inhibited only COX-2 ($p<0.05$) and iNOS ($p<0.05$) immunostaining compared with the C group (Table 2 and Fig. 5f, g).

Discussion

The present study evaluated whether LP protects against 5-FU-induced oral mucositis. We also investigated the possible modulating effect on the expression of pro-inflammatory mediators. An important aspect of our study was the use of an animal model that mimics clinical oral mucositis. We demonstrated that LP significantly and dose-dependently reduced macroscopic and microscopic lesions induced by 5-FU in the oral mucosa. Oral mucositis is characterized by an intense inflammatory reaction caused by chemotherapeutic agents on mucosa lamina propria cells (Sonis et al. 2004a). We verified that LP treatment decreased inflammatory cell infiltration, edema, hemorrhage, ulcers, and abscesses. This effect of LP is consistent with several studies that reported an anti-inflammatory effect of this product of *C. procera* in different animal models (Ramos et al. 2009; Arya and Kumar 2005). Additionally, the pioneer study with the isolated latex protein fraction assessed the protective effect of LP against ifosfamide-induced hemorrhagic cystitis,

Fig. 4 Representative photomicrography of the effect of *C. procera* on the immunohistochemistry of *TNF-α* and *IL-1β* expression in the cheek pouches of hamsters subjected to 5-fluorouracil-induced oral mucositis. The cheek pouch tissue of the animals subjected to oral mucositis (**d** and **e**) presented intense immunostaining of TNF-α (**d**) and IL-1β (**e**) in inflammatory cells compared with the cheek pouch tissue of normal animals (**b** and **c**). The treatment with laticifer protein of *C. procera* (LP, 5 mg/kg; **f** and **g**) considerably reduced the immunostaining in conjunctive tissue compared with the untreated animals (**d** and **e**). The negative control represents a sample of the cheek pouch where the first antibody was replaced with PBS-BSA 5 %, and no immunostaining was detected (**a**). The *arrows* indicate examples of immunostained cells. ×400
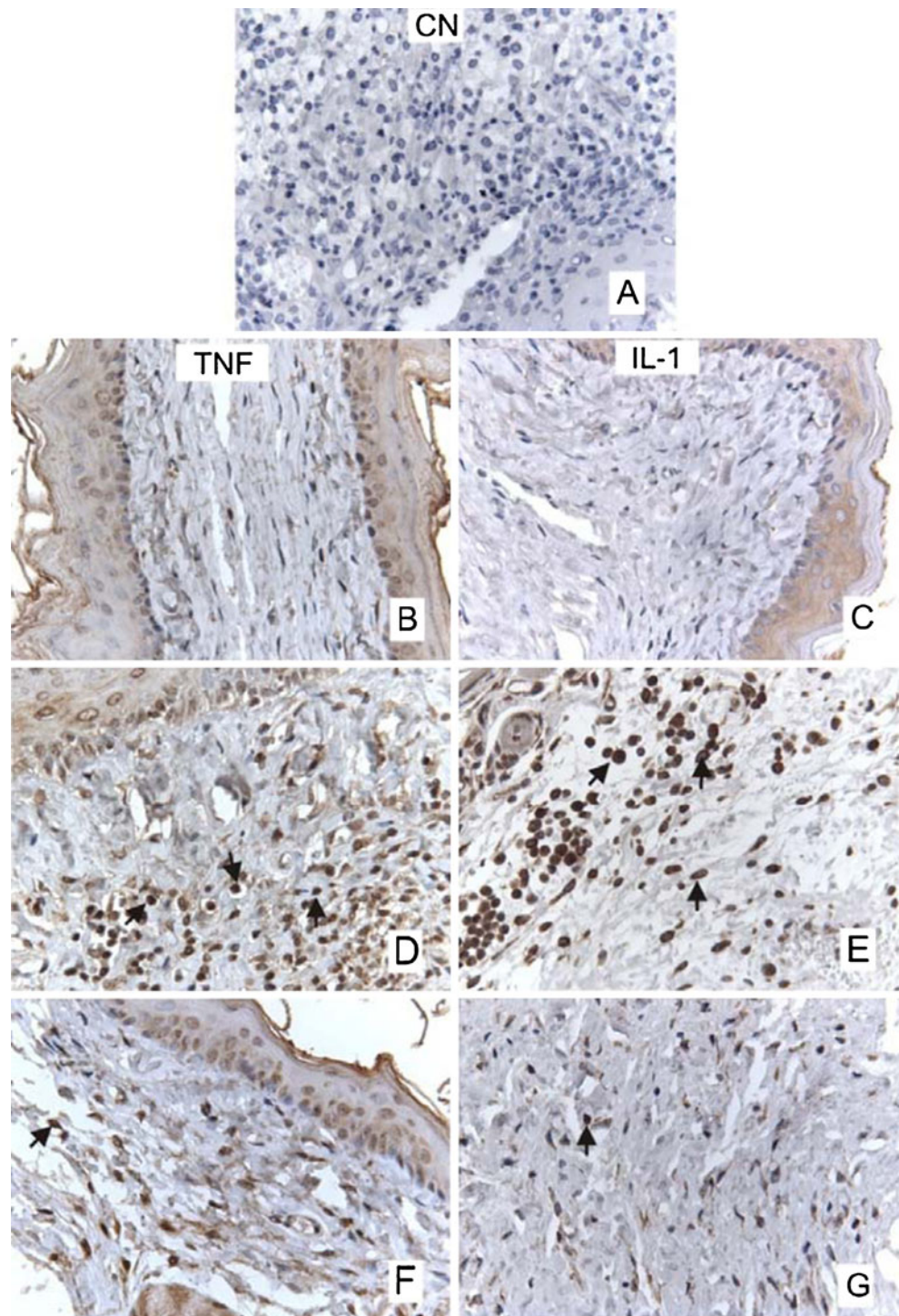

another clinically related animal model. In that animal model, the authors found a marked protective effect (Alencar et al. 2004).

In the inflammatory response, neutrophil migration is an important event that occurs at the microvascular level (Carlos and Harlan 1994). It is a consequence mainly of the release of neutrophil chemotactic factors by resident cells, inducing rolling and adhesion of neutrophils on endothelial cells, followed by their transmigration to the extravascular space (Guo and Ward 2002; Lindbom and Werr 2002). In the present study, we observed a dose-dependent inhibitory effect on MPO activity, indicating less neutrophil infiltration into the lesion site. Previous study reported that intravascular administration of LP and more purified LP-fractions significantly reduced neutrophil influx at the site of inflammation by inhibiting the rolling and adherence of neutrophils reflected by

Fig. 5 Representative photomicrography of the effect of *C. procera* on immunohistochemistry of *COX-2* and *iNOS* expressions in the cheek pouches of hamsters subjected to 5-fluorouracil-induced oral mucositis. The cheek pouch tissue of animals subjected to oral mucositis presented intense immunostaining of COX-2 (**d**) and iNOS (**e**) in inflammatory cells and epithelial tissue compared with the cheek pouch tissue of normal animals (**b** and **c**). The treatment with laticifer protein of *C. procera* (LP, 5 mg/kg; **f** and **g**) considerably reduced COX-2 (**d**) and iNOS (**e**) immunostaining in conjunctive tissue compared with the untreated animals. The negative control represents a sample of the cheek pouch where the first antibody was replaced by PBS-BSA 5 %, and no immunostaining was detected (**a**). *Arrows* indicate examples of immunostained cells. ×400
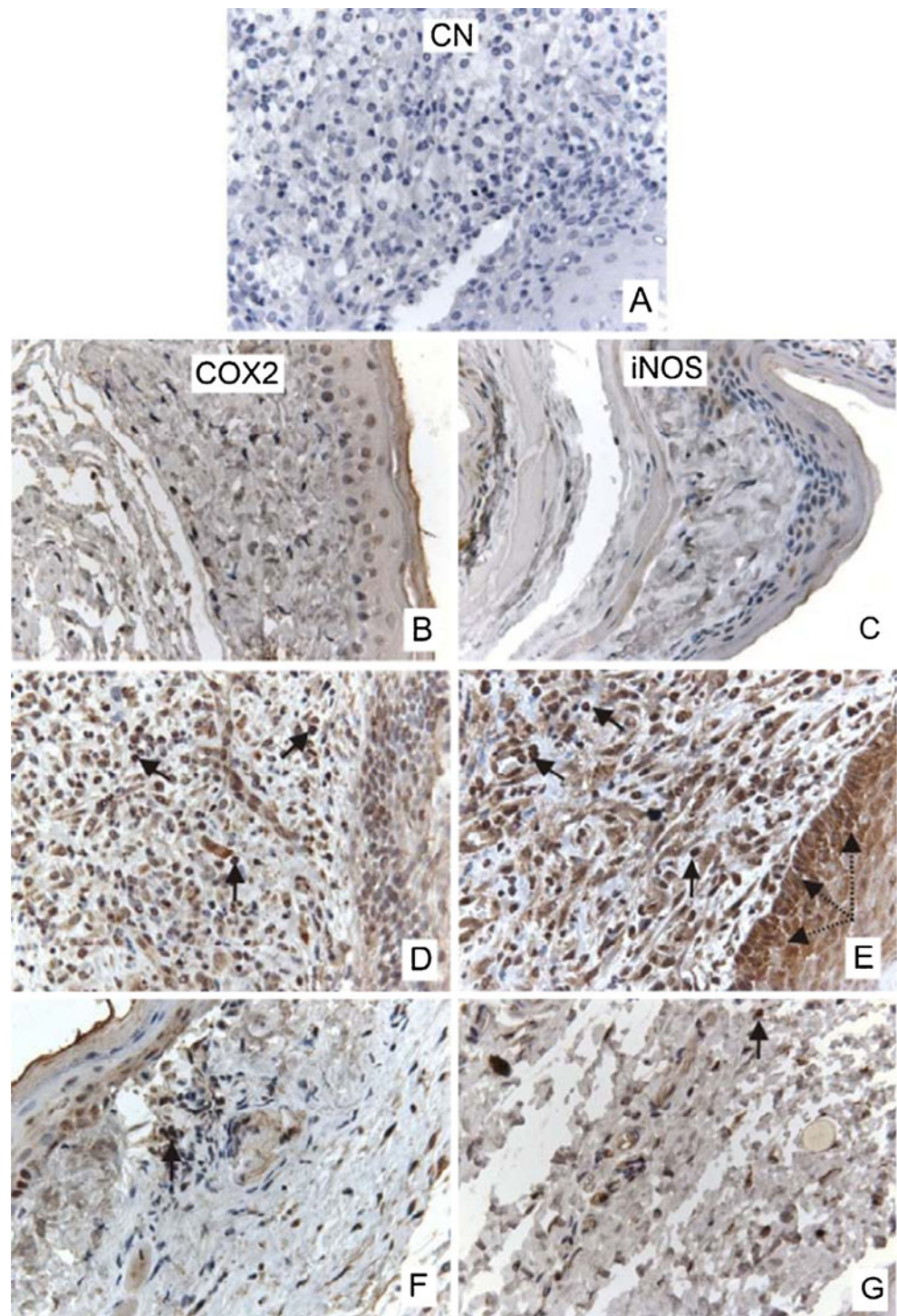
intravital microscopy (Ramos et al. 2009). Several mechanisms might be involved in this anti-inflammatory phenomenon. A possible mode of action involves the direct effect on selectins found on leukocyte or endothelial cell membranes (Assreuy et al. 1999). Another possibility is a direct effect on neutrophil migration through the downregulation of chemokine receptors (McColl and Clark-Lewis 1999). Some authors have suggested that neutrophil migration induced by IL-1β

and TNF-α is not caused by a direct effect on neutrophils but occurs via the release of chemotactic factors from resident macrophages (Faccioli et al. 1990). Despite the modulatory activity of LP on different pro-inflammatory mediators, as reported here and in preceding studies (Kumar et al. 2011; Lima-Filho et al. 2010; Alencar et al. 2004), the pathway underlying such an effect is still an aim of investigation. The ability of LP to inhibit inflammation even when

given by different route of administration is worth of note, and it is likely signalizing events involving LP recruit a complex web of chemokines.

In a model of intestinal mucositis, Tsuji et al. (2003) found that 5-FU delayed the elevation of acute inflammatory cytokines and increased portal endotoxin content, simultaneous gut mucosal injury, bacterial translocation to the mesenteric lymph nodes, and sepsis-like symptoms. Lima-Filho et al. (2010) reported a protective effect of LP in the control of systemic murine infection. This effect was associated with a modulating effect on pro-inflammatory cytokine expression rather than with increased bactericidal capacity. To test the hypothesis that LP modulates the inflammatory process by controlling pro-inflammatory mediators, we investigated the expression of TNF-α, IL-1β, COX-2, and iNOS. We found markedly reduced expression of these mediators after LP injection.

Agents known to attenuate the expression of cytokines have demonstrated the efficacy in the prevention of oral mucositis. Previous studies, including one of our own, demonstrated that inhibition of TNF-α production along with other cytokines is effective in preventing the development of mucositis (Lima et al. 2005). Pretreatment with the immunomodulating drugs thalidomide and pentoxifiline prevented mucositis in animals as well as atorvastatin, a statin that has many pleiotropic actions, including anti-inflammatory and anti-oxidant effects (Medeiros et al. 2011).

Previous studies found that COX-2 (Sonis 2002; Sonis et al. 2004b; Logan et al. 2007) and iNOS (Caballero et al. 1985) play important roles in mucosal injury and inflammatory events that lead to oral mucositis. In addition to its pro-inflammatory effects, COX-2 also plays an important role in angiogenesis (Vane et al. 1998). Furthermore, iNOS has been reported to be associated with several human malignant tumors, including breast, brain, lung, prostate, colorectal, and pancreatic carcinomas, Kaposi's sarcoma, and melanoma (reviewed by Singh and Gupta 2011). Proteins of *C. procera* latex were also reported to exhibit in vivo anti-cancer activity against sarcoma 180 (Oliveira et al. 2010). Combining pharmacotherapeutics with anti-inflammatory effects as an adjuvant to cancer treatment is desirable and might have important clinical implications for the management of mucositis. The use of *C. procera* latex proteins may be promising.

We demonstrated that the protein fraction of *Calotropis procera* latex protects against 5-FU-induced oral mucositis. We also verified an inhibitory effect on the expression of pro-inflammatory mediators. In conclusion, we showed that the protein fraction of the latex from *C. procera* was effective in the prevention of oral mucositis in a hamster model. However, well-designed clinical studies are needed to confirm the clinical efficacy of this treatment in humans. Despite that LP

represents a mix of latex proteins, a series of manuscripts has described different biochemical aspects and enzymatic profile of these samples, and no acute or chronic toxicity has been associated to this protein fraction (for review, see previous studies of Ramos et al.).

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