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Inflammation Research

ORIGINAL RESEARCH PAPER

Inflammation induced by phytomodulatory proteins from the latex of *Calotropis procera* (Asclepiadaceae) protects against *Salmonella* infection in a murine model of typhoid fever

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Abstract
Objective and design Laticifer proteins (LP) of *Calotropis procera* were fractionated by ion-exchange chromatography, and the influence of a sub-fraction (LP_{PI}) on the inflammatory response of Swiss mice challenged by *Salmonella enterica* Ser. Typhimurium was investigated.
Methods Mice ($n = 10$) received LP_{PI} (30 or 60 mg/kg) in a single inoculum by the intraperitoneal route 24 h before infection. To investigate the relevance of the proteolytic activity, three additional groups were included: the first one received heat-treated LP (30 mg/kg—30 min at 100 °C), the second received LP (30 mg/kg) inactivated by iodoacetamide, and a control group received only phosphate-buffered saline (PBS).
Results The survival rate reached 100 % in mice treated with LP_{PI} and was also observed with the other treatment, whereas the PBS group died 1–3 days after infection. The neutrophil infiltration into the peritoneal cavity of pretreated mice was enhanced and accompanied by high bacterial clearance from the bloodstream. Tumor necrosis factor-alpha mRNA transcripts, but not interferon-gamma, were detected early in spleen cells of pretreated mice after infection; however, the nitric oxide contents in the bloodstream were decreased in comparison to the PBS group.
Conclusions The inflammatory stimulus of *C. procera* proteins increased phagocytosis and balanced the nitric oxide release in the bloodstream, preventing septic shock induced by *Salmonella* infection.

Keywords Laticifer fluid · Experimental infections · Septic shock · Nitric oxide

Introduction

Natural plant products with anti-inflammatory properties are prospective sources of new drugs against infections and chronic inflammatory diseases [1]. For example, aqueous extracts of the stem bark of *Mangifera indica* and glucosylxanthone mangiferin were shown to suppress inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), and inhibit nitric oxide (NO) production by activated macrophages [2]. Catechins derived from green tea down-regulated the inducible gene expression and enzyme activity of nitric oxide synthase (iNOS) [3]. In the present study, we investigated the shrub *Calotropis procera* (Ait.) R. Br. (Asclepiadaceae), a widespread plant in Brazil and India commonly used in folk medicine. The plant's laticifer fluid is described as having healing, analgesic, antipyretic, anticancer, toxicological, inflammatory and anti-inflammatory properties [4–9]. Antibacterial activity against *Staphylococcus aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, *Klebsiella*

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pneumoniae, *Salmonella serovar* Paratyphi A and *S.* Typhi was also reported [10].

Although most of the medicinal properties reported for the latex of *C. procera* were obtained through use of aqueous and organic-solvent extracts, it is also rich in numerous proteins that can be separated by dialysis [11]. Non-dialyzable proteins (LP) have anti-cancer properties and can decrease neutrophil infiltration induced by carrageenan in the paw edema model [12, 13]. Freitas et al. [14] described proteins with molecular masses ranging from 5 to 95 kDa and at least four cysteine proteinases in the latex of *C. procera*. Proteinases have emerged as therapeutics against many medical disorders, including sepsis [15]. Furthermore, plant proteinases have been shown to influence immunity by acting to reverse the effects of circulating α_2-macroglobulin and α_1-protease inhibitor and also by interfering with the binding of cytokines such as transforming growth factor-beta and interleukin (IL)-1 beta [16].

The influence of the laticifer proteins (LP) of *C. procera* on the protection of Swiss mice against *S.* Typhimurium infection, the murine model of typhoid fever, was recently shown [17]. However, the immune mechanisms by which *C. procera* proteins prevented the bacterial septic shock are unclear. While these LP have been fractionated by ion-exchange chromatography and biochemically characterized, the aim of the present study was to investigate the effect of one sub-fraction, named LP_{PI}, on the protection rates and inflammatory response of Swiss mice infected by *S.* Typhimurium. This fraction was chosen because its administration was highly anti-inflammatory, decreasing the number of rolling and adherent leukocytes in the mesenteric microvasculature of animal models [18]. The influence of LP_{PI} on NO release, cytokine mRNA expression and neutrophil recruitment are discussed in light of previous biological activities reported for *C. procera* proteins.

Materials and methods

Plant collection and identification

Samples of *C. procera* (Ait.) R. Br. (Asclepiadaceae) latex were collected in the city of Fortaleza, in the state of Ceará in northeast Brazil. The voucher (sample specimen no. 32663) was deposited at the Prisco Bezerra Herbarium of the Ceará Federal University, and the plant was authenticated by a taxonomist from the school's biology department.

Procera proteins

Calotropis procera proteins were obtained as described by Alencar et al. [11]. Briefly, fresh *C. procera* latex was collected from healthy plants through small incisions directly into tubes containing distilled water to obtain a mixture (v/v). The samples were centrifuged (5,000×*g*) at 10 °C for 10 min. The precipitated material, showing a rubbery aspect, was discarded, while the suspended phase was submitted to exhaustive dialysis (cut-off 8,000 Da) against distilled water at 8 °C for 60 h and then centrifuged as previously mentioned. The new precipitate material was discarded, and the soluble phase devoid of rubber was freeze-dried. This fraction, containing almost all of the latex's soluble proteins, has been named laticifer proteins (LP). These proteins were fractionated by ion-exchange chromatography, as previously reported [12], through a CM-Sepharose fast-flow column (Amersham Biosciences, Brazil) previously equilibrated with 50 mM acetate buffer, pH 5.0. After washing the column with acetate buffer, the sample was sequentially eluted with buffer containing 0.2 and 0.3 M sodium chloride (NaCl). Fractions (4 ml) were collected at a flow rate of 1 ml/min, and the absorbance was read at a wavelength of 280 nm. Three distinct protein peaks—PI, PII and PIII (LP_{PI}, LPP_{II} and LP_{PIII})—were obtained (Fig. 1). The fractions corresponding to these peaks were dialyzed against distilled water and lyophilized. In the present study, LP_{PI} was used because previous data have shown that this fraction is highly anti-inflammatory [18].

Microorganisms

Salmonella enterica Subsp. *enterica serovar* Typhimurium was isolated from a human clinical case at the Ezequiel Dias Foundation (FUNED, Belo Horizonte, MG, Brazil), donated by Dr. Jacques Robert Nicoli (Minas Gerais Federal University). The bacteria were maintained at −18 °C in brain heart infusion (BHI) medium containing 50 % glycerol. During the experiments, bacteria were activated after culture in BHI broth at 37 °C for 24 h.

Animals

Adult male Swiss mice (30–35 g) and Wistar rats (180–200 g) were obtained from the Biotery of Ceará Federal University and were kept in an animal house with free access to water and a commercial feed (Purina, Paulínia, SP, Brazil). The animals were handled according to the standards set forth in the 'Guide for the Care and Use of Laboratory Animals' of the National Research Council, after approval by the university's animal ethics committee (protocol number 24/09).

Study design

Mice in the experimental groups were given a single inoculum of two doses of LP_{PI} (30 and 60 mg/kg) in 0.2 ml

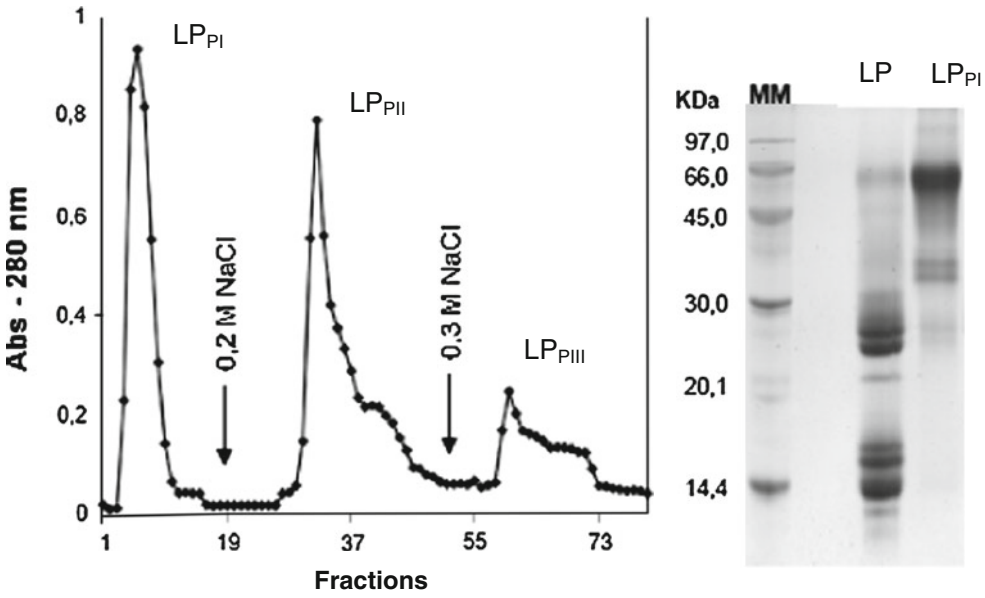

Fig. 1 Ion-exchange chromatography of LP on CM-Sepharose fast-flow column at pH 5.0. The *arrows* indicate the elution of the proteins with 50 mM acetate buffer, pH 5.0, and buffer containing 0.2 M and 0.3 M NaCl, respectively

of sterile phosphate-buffered saline (PBS), pH 7.2, by the intraperitoneal (i.p.) route. The protein dosages were based on previous data obtained with treatments with LP, which were protective against *Salmonella* infection. The control group received 0.2 ml of PBS devoid of *C. procera* proteins. Three additional experimental groups received LP, heat-treated LP (30 min at 100 °C) or LP submitted to inactivation by iodoacetamide (IAA). The experimental infection with *S.* Typhimurium was conducted following Lima-Filho et al. [19]. *Salmonella* Typhimurium was activated in BHI broth at 37 °C for 6 h and then cultured in MacConkey agar for another 24 h at 37 °C. Five to seven colony-forming units (CFU) were diluted in sterile PBS, pH 7.2, to attain a bacterial suspension containing 10^8 CFU/ml (according to the 0.5 tube of the MacFarland scale). The bacterial suspension was diluted 100-fold, and 0.2 ml (10^6 CFU) was administered to Swiss mice by the i.p. route 24 h after the inoculums of *C. procera* proteins.

Survival evaluation and bacterial clearance

Mice from all animal groups ($n = 10$) were observed for clinical signs and for mortality every day until 7 days post-infection. Additionally, mice treated with LP_{PI} or LP ($n = 5$) and the PBS group were sacrificed under anesthesia with halothane (Halocarbon Laboratories, USA) at 4 h and 24 h after infection. The liver, spleen and blood samples were aseptically extracted and submitted to serial decimal dilutions in PBS, and 0.1-ml aliquots were plated onto MacConkey agar (Oxoide). The enumeration of CFUs was carried out after incubation at 37 °C for 24 h, and the results were expressed as CFUs/g of organ or ml of blood.

Histological examination

The liver and spleen tissue samples were fixed in 10 % formaldehyde and submitted to histopathological examination. The samples were embedded in paraffin, and 5-μm sections were stained with hematoxylin-eosin (H&E). The slides were coded and examined by a single pathologist who was unaware of the experimental conditions. Histological examination was also conducted on the organs of survivors treated with LP_{PI} and LP at the end of the experiment, 7 days post-infection.

Influence of *C. procera* proteins on neutrophil infiltration into the peritoneal cavity

Initially, uninfected mice ($n = 5$) were given *C. procera* proteins by the i.p. route and neutrophil recruitment was evaluated at 4 h, 24 h and 7 days after the inoculums. The peritoneal cavity was washed with 3 ml of sterile saline (0.15 M NaCl) containing heparin (5 IU/ml). Leukocytes were counted in the recovered peritoneal fluid, following the method of Souza and Ferreira [20]. From these results, an additional animal group was composed to evaluate the influence of the administration route of LP and LP_{PI} on neutrophil infiltration into the peritoneal cavity. In this case, uninfected Wistar rats ($n = 5$) were treated by the endovenous (e.v.) or the i.p. route with LP (30 mg/kg), LP_{PI} (5 mg/kg) or PBS (control) 30 min before an i.p. injection of carrageenan (800 μg/cavity). An additional control group received PBS that was not injected with carrageenan. Four hours later, the peritoneal cavities of the rats were washed with 10 ml sterile saline containing

5 IU/ml heparin. The leukocyte count was determined as described above. Neutrophil migration to the peritoneal cavity was also evaluated in LP- and LP_{PI}-treated mice ($n = 5$) and the PBS group ($n = 5$) at 4 h, 24 h and 7 days post-infection, as described for the uninfected mice.

Analyses of cytokine mRNA expression in spleen cells

The spleens from the animals were removed aseptically and homogenized in 1 ml RNAzol solution (4 M guanidine isothiocyanate phenol + v/v) for extraction of total RNA. The homogenate was left on ice for 40 min–1 h. The samples were then submitted to chloroform–isopropanol extraction, as described in other studies [21], and quantified through spectrophotometer reading at 260 nm. The integrity of the extracted RNA was confirmed by electrophoresis in 1.5 % agarose gel. Reverse transcription was carried out with the use of murine leukemia virus reverse transcriptase at 37 °C for 60 min. Cytokines were amplified through polymerase chain reaction with the following primers: interferon-gamma (IFN-γ) (forward 5′ GGTGACATGAAAATCCTGCAG AGC 3′, reverse 5′ CGCTGGACCTGTGGGTTGTTGAC C 3′); tumor necrosis factor-alpha (TNF-α) (forward 5′ G ATCTCAAA GACAACCAACTAGTG 3′, reverse 5′ CTC CAGCTGGAAGACTCCTCCCAG 3′). The enzyme hypoxanthine ribosyltransferase was used as an internal control: forward 5′ GTTGGATACAGGC CAAGACTTTGTTG 3′, reverse 5′ GATTCAACTTGCG CTCATCTTAGGC 3′. Amplification was performed by 40 cycles consisting of denaturation at 94 °C for 90 s, primer annealing at 55 °C for 30 s, and primer extension at 72 °C for 60 s (Thermocycler PTC-100, MJ Research).

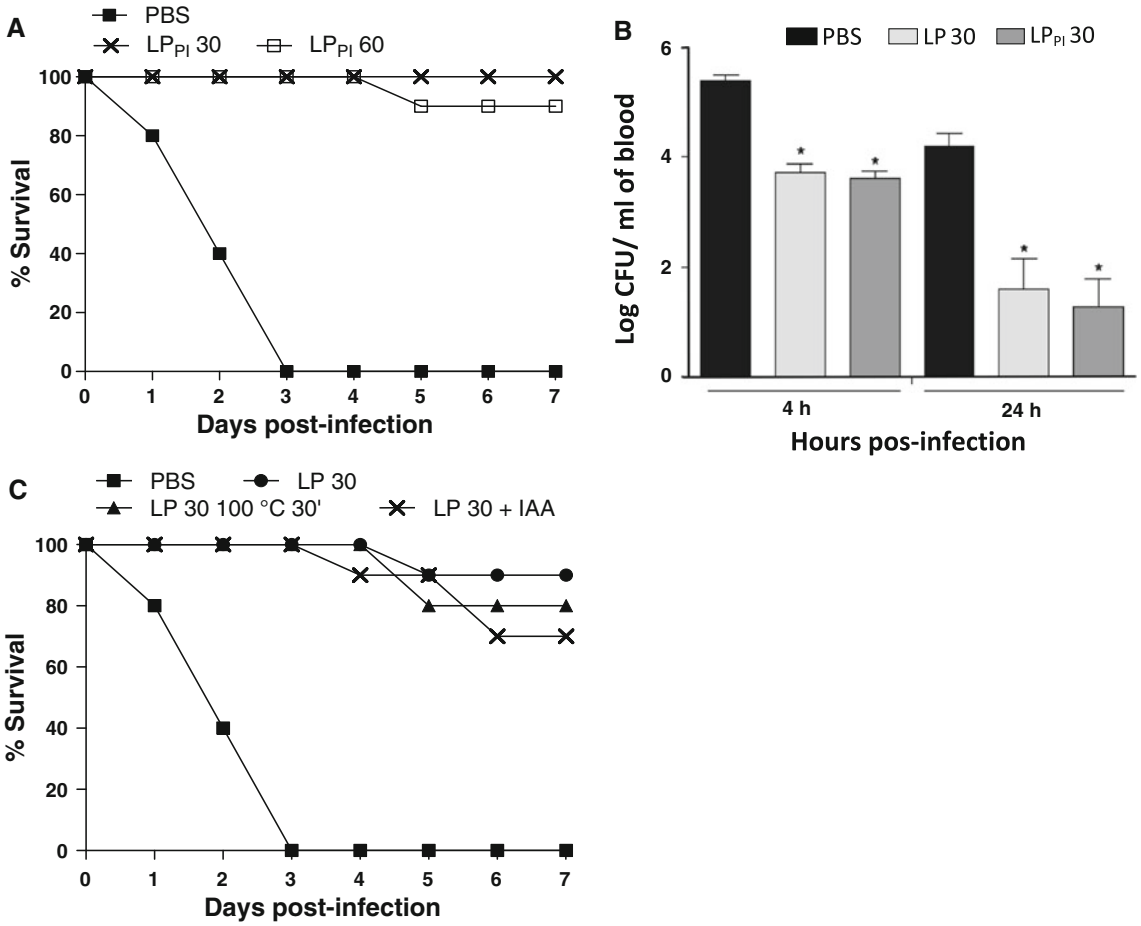

Fig. 2 The protective effect of LP_{PI} in Swiss mice subjected to lethal infection by *S.* Typhimurium. Mice were treated with 30 or 60 mg/kg of LP_{PI} (**a**). The *S.* Typhimurium CFU in the bloodstream at 4 h and 24 h after infection are shown (**b**). Survival was still observed in mice inoculated with heat-treated LP (30 mg/kg) or LP (30 mg/kg) submitted to proteolysis with IAA (**c**). Proteins were given by the i.p. route 24 h before bacterial challenge, and survival was monitored for 7 days. Survival rates of animals from all experimental groups were significantly different from the PBS group (untreated mice) at all tested doses ($n = 10$, $p < 0.05$; Mantel–Cox log rank test). *$p < 0.05$ indicates a significant difference between the CFU counts in the bloodstream compared to uninfected mice (PBS group) ($n = 5$; ANOVA–Bonferroni test)

Nitric oxide measurement

Blood aliquots (0.7–1.0 ml) were obtained through cardiac puncture at 4 h and 24 h post-infection and left to coagulate in sterile tubes at room temperature. Serum levels of NO (NO_3/NO_2) were measured following the method reported by Chen et al. [22]. A 0.1-ml aliquot of Griess reagent (1 % sulfanilamide and 0.1 % naphthalene diamine dihydrochloride in 1 % orthophosphoric acid) was added to 0.1 ml serum and incubated at room temperature for 10 min. Serum nitrite concentrations were then determined through reading the optical densities at 540 nm and comparison with a standard curve made from sodium nitrite.

Determination of adenosine deaminase activity (ADA)

The assay was carried out as described by Guisti [23]; the mice were pretreated with LP (30 mg/kg i.p.) or PI (5 mg/g i.p.) and were challenged with *Salmonella* 24 h later. Peritoneal fluids of healthy or infected mice were collected at 4 h and 24 h post-infection. After centrifugation, the supernatant (20 µl) was used as the enzyme source and incubated with 200 µl (22 mM) of adenosine at 37 °C for 1 h in 50 mM phosphate buffer, pH 7.2. The ammonium concentration after deamination of adenosine by the enzyme adenosine deaminase was measured by indophenol production (Berthelot reaction) and analyzed through spectrometry at 628 nm.

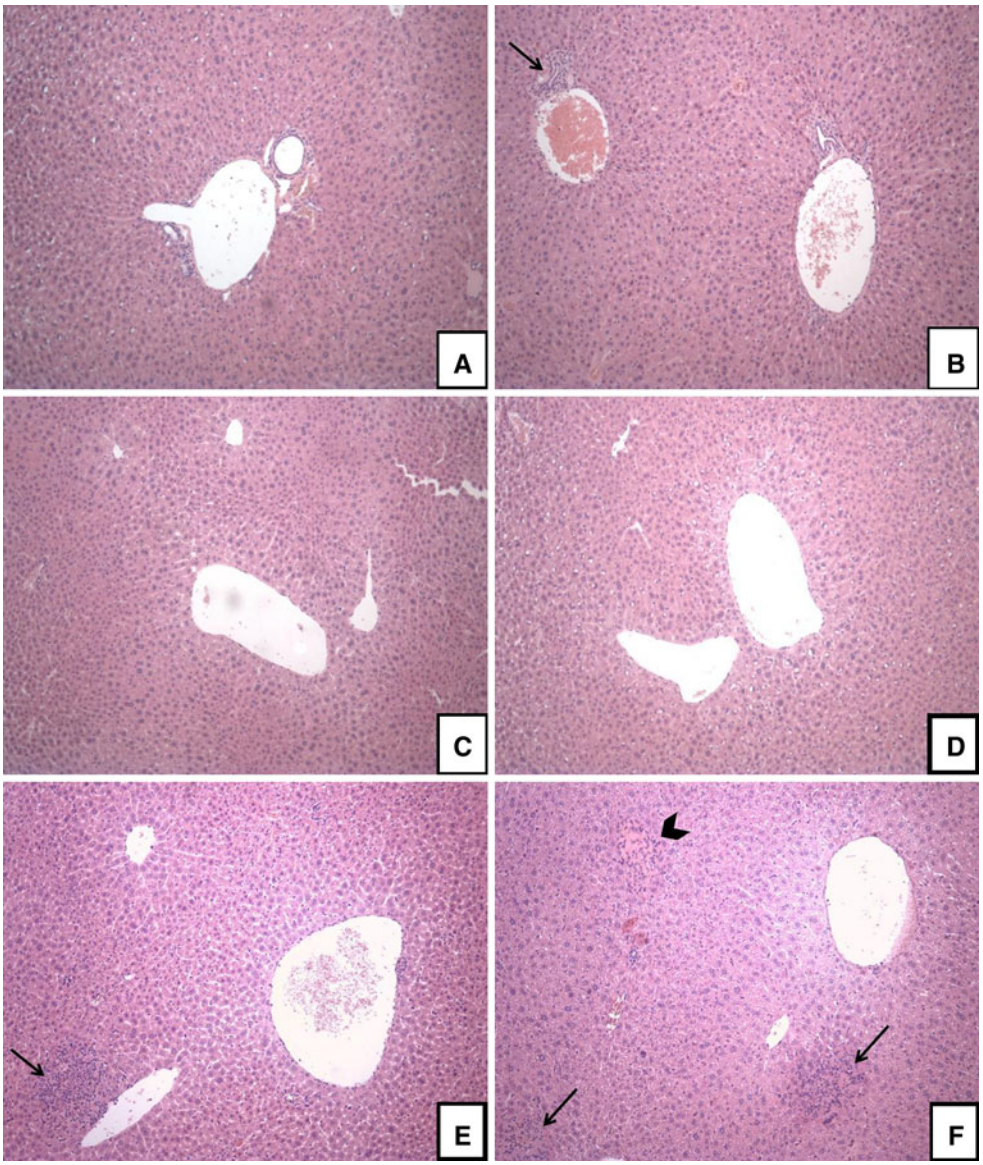

Fig. 3 Effect of LP (LP and LP_{PI}) from *C. procera* on the histopathological pattern of the liver in animals infected by *S.* Typhimurium. The figures show photomicrographs of histological sections from the liver of uninfected mice stained by the H&E method (**a**); PBS group—24 h (**b**); LP (30 mg/kg)—24 h (**c**); LP_{PI} (5 mg/kg)—24 h (D); LP (30 mg/kg)—7 days (**e**) and PI (5 mg/kg)—7 days (F). Magnification ×100. The *thin arrow* indicates inflammatory infiltrates, while *wide arrows* indicate necrosis foci

Enzymatic activities of transaminases and lactate dehydrogenase in serum

The glutamic oxaloacetic transaminase (TGO), glutamic pyruvic transaminase (TGP) and lactate dehydrogenase (LDH) enzyme levels were measured using commercial kits (Labtest). These methods were based on evaluating the enzymatic kinetics. The results are expressed in international units/ml.

Statistical analyses

The survival curves of the mice after bacterial inoculums represent two independent experiments and are expressed as percentages (%) of survival. The data were statistically evaluated by the Mantel–Cox log rank test ($p < 0.05$). The remaining data are reported as the mean ± SEM. Statistical significance was assessed by ANOVA followed by Bonferroni's test or the Student's t test. The level of significance was determined as $p < 0.05$.

Results

Intraperitoneal preinoculation with LP_{PI} latex fraction from *C. procera* induces protection against *Salmonella* infection, as previously shown for the entire LP fraction. Survival was measured 7 days post-infection and was 100 % in the mice treated with LP_{PI}, whereas the animals in the PBS group died 1–3 days after infection (Fig. 2a). The bacterial load in the bloodstream of the LP- and LP_{PI}-treated mice was reduced approximately 100-fold at 4 h and 24 h after infection ($p < 0.05$) (Fig. 2b). However, there was no significant difference between counts recorded in the

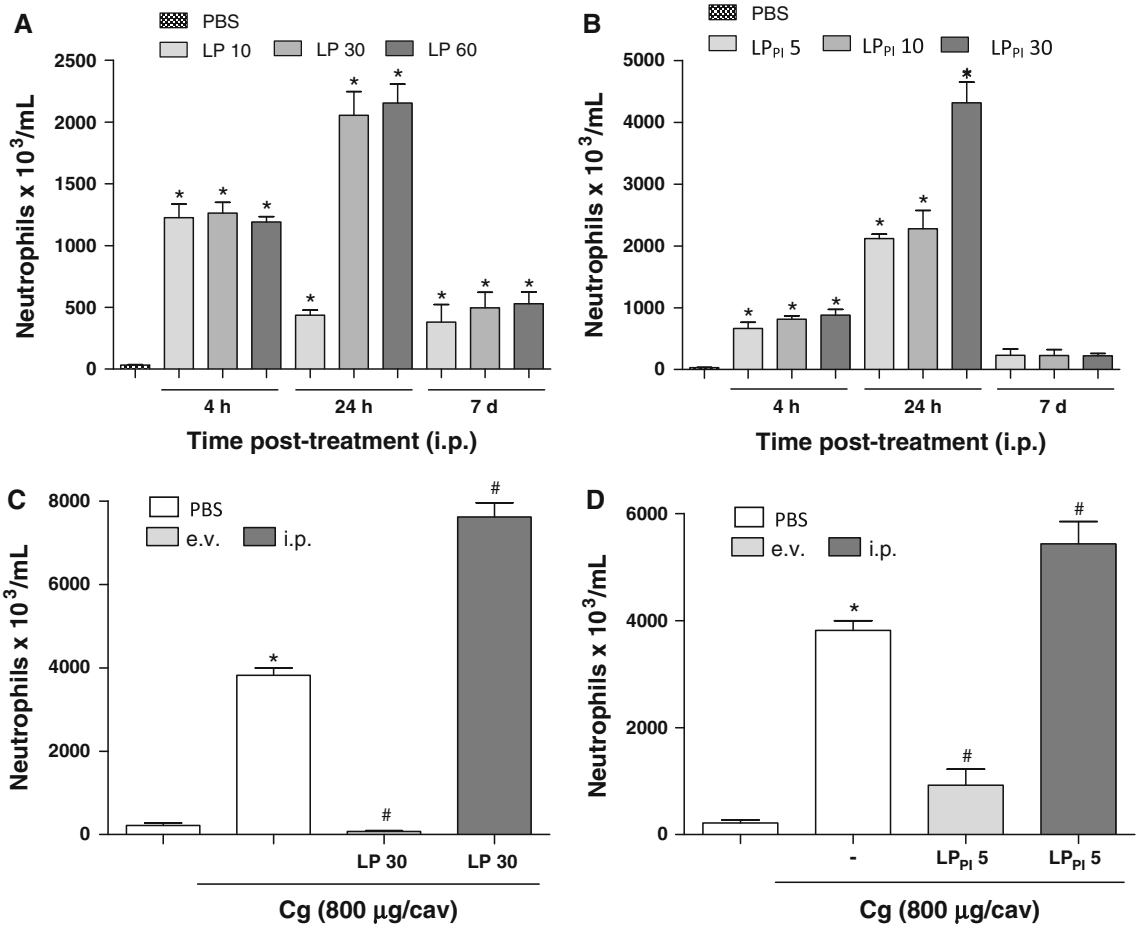

Fig. 4 Effect of LP of *C. procera* on neutrophil migration into the peritoneal cavity of healthy Swiss mice and Wistar rats. Mice were treated with LP (10, 30 or 60 mg/kg i.p.) (**a**) or LP_{PI} (5, 10 or 30 mg/kg i.p.) (**b**). Neutrophil migration was evaluated at 4 h, 24 h and 7 days after protein inoculums. To evaluate the influence of the administration route, rats were treated with LP (30 mg/kg) (**c**) or LP_{PI} (5 mg/kg) (**d**) by the e.v. or i.p routes 30 min before an i.p. injection of carrageenan (Cg–800 µg/cavity), whereas control mice received PBS. Neutrophil migration was evaluated 4 h later. The results are expressed as the mean ± SEM of neutrophils per cavity. $^{*}p < 0.05$ indicates a significant difference compared to the PBS group, and $^{\#}p < 0.05$ indicates a significant difference compared to the carrageenan group (PBS + Cg) ($n = 10$, ANOVA–Bonferroni test)

spleen and the liver compared to controls at these time points (data not shown). Survival was still evident with proteins submitted to heat treatment at 100 °C for 30 min or treated with IAA, and was at least 70 % by 7 days post-infection (Fig. 2c). Histological examination of liver in the PBS group revealed inflammatory filtrates other than apoptotic cells, as well as hypertrophy and congestion of the reticulum endothelial system 24 h after infection, while no significant alterations were seen in liver of mice submitted to pretreatment with LP or LP_{PI} during this interval (Fig. 3). However, there were moderate cell infiltrates, vacuolization and a few necrosis foci in the liver of these survivor mice 7 days after infection (Fig. 3). The presence of a few giant cells and inflammatory infiltrates was seen in the spleen of all animal groups 24 h post-infection regardless of the treatment.

The LP and LP_{PI} pre-inoculums delivered via the i.p. route induce strong neutrophil infiltration into the peritoneal cavity of uninfected mice stimulated by carrageenan ($p < 0.05$) (Fig. 4c, d). Conversely, the neutrophil migration into the peritoneal cavity was impaired when LP or LP_{PI} treatments were delivered by the e.v. route (Fig. 4). Significant recruitment of neutrophils into the peritoneal cavity was also observed in *Salmonella*-infected animals after i.p. inoculums with LP or LP_{PI} and remained strong 7 days post-infection (Fig. 5). Conversely, neutrophils failed to migrate into the peritoneal cavity in mice that were not treated with *C. procera* proteins (PBS group) (Fig. 5). The hematological profiles of healthy and infected mice confirm that treatment with LP or LP_{PI} did not hamper neutrophil migration into the peritoneal cavity and prevented the reduction of lymphocytes in the bloodstream commonly observed in septic animals (Table 1). No significant differences were observed among monocyte, eosinophil and basophil counts, regardless of the treatment (Table 1).

The level of NO in serum showed almost a three-fold increase in non-treated infected animals when compared to LP or LP_{PI} groups 24 h post-infection ($p < 0.05$) (Fig. 6a). Conversely, ADA activity was increased in the treated groups 24 h after infection ($p < 0.05$) (Fig. 6b). In addition, early TNF-α mRNA expression was observed at 4 h post-infection in the LP and LP_{PI} animal groups, but IFN-γ was not observed at this early time point (Fig. 6c). The TGO, TGP and LDH enzymatic levels were similar among all animal groups (data not shown).

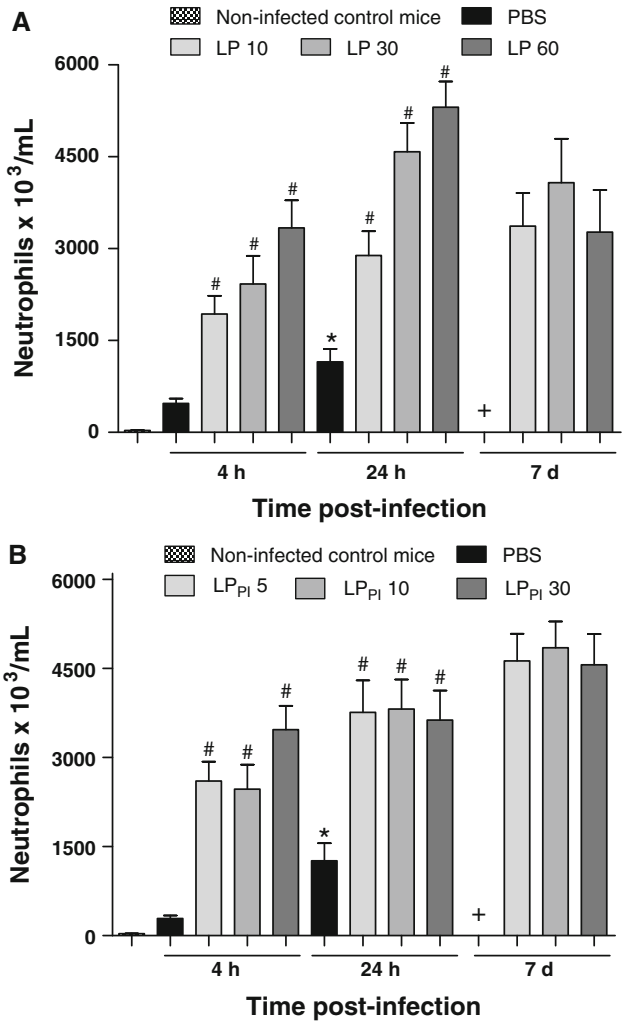

Fig. 5 Effect of LP from *C. procera* on neutrophil migration into the peritoneal cavity of mice challenged with *S.* Typhimurium. Swiss mice were treated with single inoculums of LP (10, 30 and 60 mg/kg) or LP_{PI} (5, 10 and 30 mg/kg) by the i.p. route 24 h before bacterial challenge. Neutrophil migration to the peritoneal cavity was evaluated at 4 h, 24 h and 7 days post-infection. The results are expressed as the mean ± SEM of neutrophils per cavity. + indicates that all mice from the PBS group died. $^{*}p < 0.05$ indicates a significant difference compared to uninfected mice, and $^{\#}p < 0.05$ indicates a significant difference compared to the PBS group ($n = 10$, ANOVA–Bonferroni test)

Discussion

The laticifer fluid of *C. procera* encompasses a large amount of proteins with anti-inflammatory properties and prospective pharmacological uses [11, 18]. We have shown that the enhanced protection by LP or LP_{PI} inoculums against *Salmonella* infection was not related to protease activity. However, although mice submitted to protein treatments did not show clinical symptoms of the infection, the bacterium was not cleared from the spleen and liver of the surviving animals 7 days after infection, causing moderate histological damage to these target organs. Furthermore, survival was accompanied by early bacterial clearance in the bloodstream, supporting the hypothesis that phagocytic activity stimulated by the protein inoculums facilitated bacterial removal. Afterwards, this hypothesis was confirmed because the protein inoculums caused a high local inflammatory stimulus after the bacterial challenge.

Table 1 Effect of LP from *C. procera* on the hematological profile of healthy mice or mice infected by *S.* Typhimurium

	Treatment	Dose (mg/kg)	Total leukocytes (10^3 cells/μl)	Differential leukocytes count (%)[a]				
				Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Healthy animals								
Control	PBS	–	2.96 ± 0.27	19.60 ± 3.04	72.50 ± 2.33	12.80 ± 3.54	0.40 ± 0.40	0.00 ± 0.00
24 h	LP	30	2.91 ± 0.21	21.00 ± 2.49	71.80 ± 3.33	9.60 ± 1.83	0.00 ± 0.00	0.00 ± 0.00
	LP_{PI}	5	3.71 ± 0.42	17.00 ± 2.71	78.33 ± 2.69	9.67 ± 0.21	0.00 ± 0.00	0.00 ± 0.00
7 days	LP	30	2.89 ± 0.47	16.67 ± 2.09	79.83 ± 2.30	10.20 ± 0.58	0.00 ± 0.00	0.00 ± 0.00
	LP_{PI}	5	2.32 ± 0.38	14.29 ± 3.57	74.43 ± 3.46	10.00 ± 0.84	0.29 ± 0.18	0.00 ± 0.00
Infected animals								
Control	PBS	–	1.30 ± 0.11*	40.50 ± 7.31*	54.80 ± 3.60*	9.20 ± 2.03	0.40 ± 0.24	0.40 ± 0.40
24 h	LP	30	4.08 ± 0.38* #	15.67 ± 3.02#	74.50 ± 4.29#	9.33 ± 3.31	0.17 ± 0.17	0.00 ± 0.00
	LP_{PI}	5	2.86 ± 0.43#	16.29 ± 2.77#	76.29 ± 3.35#	9.57 ± 0.95	0.62 ± 0.32	0.14 ± 0.14
Control	PBS	–	[b]	[b]	[b]	[b]	[b]	[b]
7 days	LP	30	3.71 ± 0.43	60.50 ± 3.39*	29.00 ± 2.71*	9.86 ± 2.18	0.14 ± 0.14	0.00 ± 0.00
	LP_{PI}	5	3.04 ± 0.36	47.0 ± 4.66*	36.57 ± 4.56*	11.13 ± 3.19	0.50 ± 0.38	0.25 ± 0.25

* $p < 0.05$ indicates statistical significance compared to animals from the control group and # $p < 0.05$ compared to animals from the *Salmonella* group ($n = 10$, ANOVA—Student's and Newman–Keuls test)

[a] Results are expressed as the mean ± SEM of leukocytes in the animals' blood

[b] Indicates that all mice from the *Salmonella* group died

Interestingly, the i.p. inoculum of *C. procera* proteins into uninfected rats also prompted inflammation, whereas an e.v. inoculum provoked anti-inflammation. The occurrence of antagonistic activities due to the inoculum routes of *C. procera* proteins remains to be elucidated.

Salmonella Typhimurium causes a strong inflammatory response in susceptible mice that results in sepsis and septic shock [24]. In contrast, resistance to disseminated infection results from the activation of CD4 + T cells as part of the adaptive immune response [24]. In the early stages of the disease, macrophage-derived IL-12 is important in stimulating IFN-γ release by natural killer cells, which in turn enhance the microbicidal capacity of macrophages through the production of NO and its derivatives from the nicotinamide adenine dinucleotide phosphate-oxidase cascade [25, 26]. However, the cytokine IFN-γ played a minor role in the protective effect induced by *C. procera* proteins because mRNA transcripts were not clearly evidenced after infection. Additionally, protection was enhanced during innate immunity due to a non-specific inflammatory stimulus by the *C. procera* proteins. The activation of the inflammatory cascade by LP and LP_{PI} before infection reduced the bacterial load in the bloodstream after infection, preventing extensive inflammation. TNF-α-derived macrophages are major mediators of acute inflammation against *Salmonella* circulating bacterial antigens [27]. Therefore, stimulation of TNF-α through pretreatment with LP of *C. procera* was shown to be highly beneficial by recruiting macrophages and neutrophils to the peritoneal cavity before *Salmonella* infection.

Large amounts of IL-1β or TNF-α are known to be involved in bacterial sepsis, but blocking these cytokines as therapy for septic patients has failed in clinical trials and can lead to immunocompromisation and infections [28]. Accordingly, high amounts of macrophage-derived NO in the bloodstream are related to hypotension, cardiodepression, vascular hyporeactivity and septic shock [29, 30]. It was clear that the NO levels in the serum of untreated mice were substantially higher and remarkably increased the inflammatory response, producing premature death. Previous studies have shown that immunosuppression following infection with *S.* Typhimurium is related to high serum NO content released by activated macrophages [31]. Paradoxically, inoculation of mice with an attenuated *Salmonella* strain decreased the NO levels in serum and evoked a high protection against challenge with virulent *Salmonella* [32]. Likewise, the inflammatory stimulus of LP_{PI} or LP balanced the NO content in serum after *Salmonella* inoculums, preventing immunosuppression and septic shock. This finding was corroborated by increased ADA activity, which is reported as a down-regulator of several pro-inflammatory cytokines involved in NO release [33, 34].

The enhancement of immunological function has been described for medicinal plants in the prevention and treatment of infectious diseases [35]. Additionally, the use of proteins as vaccines or immunological adjuvants represents an interesting tool because amino acid sequences could be reproduced by genetic engineering [36]. While *C. procera* proteins have been purified and biochemically characterized, their role in counterbalancing the effects of

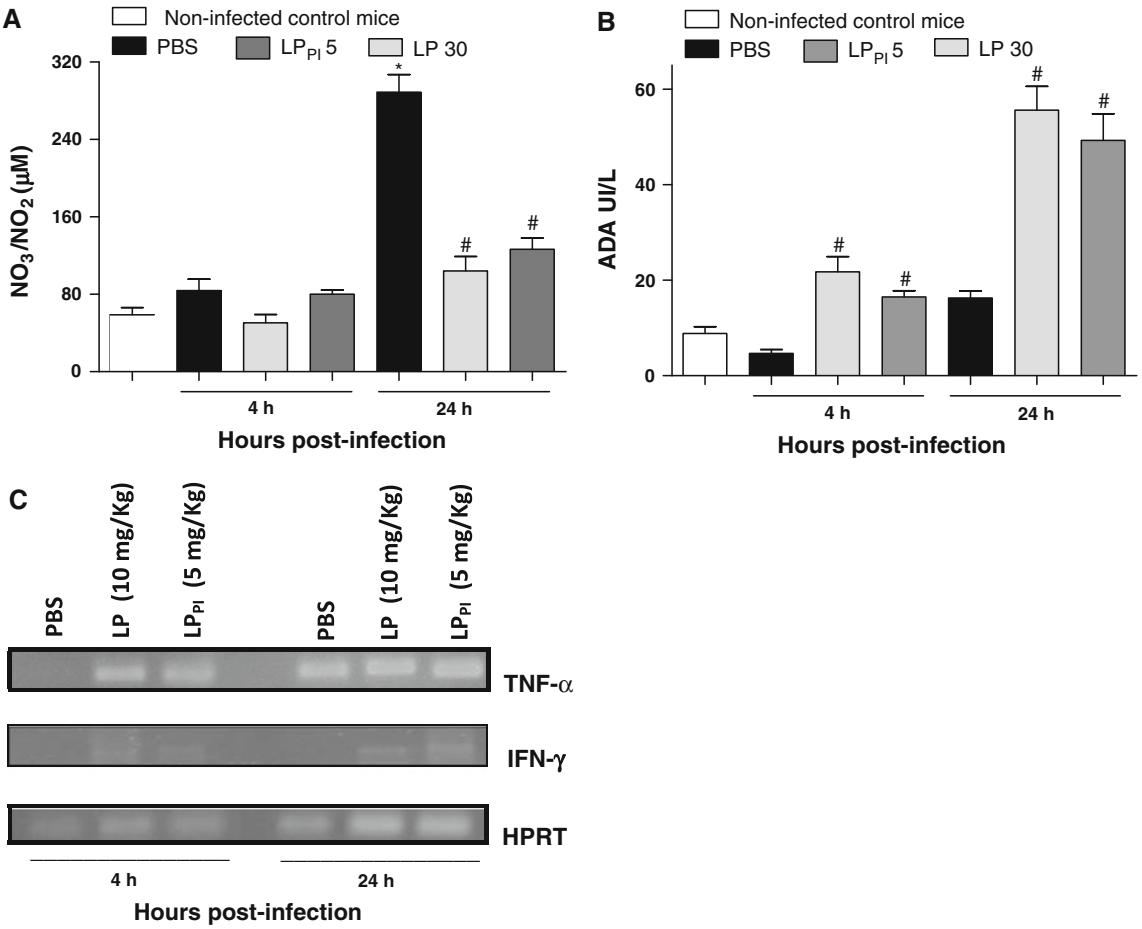

Fig. 6 NO concentrations, adenosine deaminase activity and TNF-α mRNA expression in mice treated with LP from *C. procera.* Swiss mice were treated with LP (30 mg/kg i.p.) or LP$_{PI}$ (5 mg/kg i.p.) 24 h before bacterial challenge. NO was titrated as nitrite and serum concentrations were determined by the Griess reaction (**a**), whereas adenosine deaminase activity was determined in the peritoneal fluid (**b**) of animals 4 h and 24 h after infection. The extraction of RNA by reverse transcription-polymerase chain reaction was conducted from spleen cells at the same intervals (**c**). The results are expressed as the mean ± SEM of the nitrite concentration in μM. $*p < 0.05$ indicates a significant difference compared to uninfected mice, and $\#p < 0.05$ indicates a significant difference compared to infected animals of the PBS group ($n = 10$, ANOVA–Bonferroni test). Hypoxanthine ribosyltransferase was used as an internal control of gene expression

NO in the host's defense against *Salmonella* is of prospective interest in the management of infections caused by Gram-negative bacteria.

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Conflict of interest The authors declare that there is no conflict of interests.

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