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ORIGINAL ARTICLE

Cytotoxicity against tumor cell lines and anti-inflammatory properties of chitinases from *Calotropis procera* latex

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Abstract The role of chitinases from the latex of medicinal shrub *Calotropis procera* on viability of tumor cell lines and inflammation was investigated. Soluble latex proteins were fractionated in a CM Sepharose Fast-Flow Column and the major peak (LPp1) subjected to ion exchange chromatography using a Mono-Q column coupled to an FPLC system. In a first series of experiments, immortalized macrophages were cultured with LPp1 for 24 h. Then, cytotoxicity of chitinase isoforms (LPp1-P1 to P6) was evaluated against HCT-116 (colon carcinoma), OVCAR-8 (ovarian carcinoma), and SF-295 (glioblastoma) tumor cell lines in 96-well plates. Cytotoxic chitinases had its anti-inflammatory potential assessed through the mouse peritonitis model. We have shown that LPp1 was not toxic to macrophages at dosages lower than 125 μg/mL but induced high messenger RNA expression of IL-6, IL1-β, TNF-α, and iNOs. On the other hand, chitinase isoform LPp1-P4 retained all LPp1 cytotoxic activities against the tumor cell lines with IC_{50} ranging from 1.2 to 2.9 μg/mL. The intravenous administration of LPp1-P4 to mouse impaired neutrophil infiltration into the peritoneal cavity induced by carrageenan. Although the contents of pro-inflammatory cytokines IL-6, TNF-α, and IL1-β were high in the bloodstreams, such effect was reverted by administration of iNOs inhibitors NG-nitro-L-arginine methyl ester and aminoguanidine. We conclude that chitinase isoform LPp1-P4 was highly cytotoxic to tumor cell lines and capable to reduce inflammation by an iNOs-derived NO mechanism.

Keywords Anticancer activity · Folk medicine · Laticifer proteins · Nitric oxide

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Introduction

The medicinal properties and pharmacological potential of laticifer plant *Calotropis procera* (Aiton) Dryand. (Apocynaceae) have been broadly reported in literature (Dewan et al. 2000a, b; Arya and Kumar 2005; Hoedon et al. 2006; Chaudhary et al. 2015). In particular dosage oar, protein mixtures obtained from plant's latex have been investigated in infection plus inflammation models (Alencar et al. 2004; Lima-Filho et al. 2010). First studies have shown that three distinct protein fractions were obtained by ion exchange chromatography from the whole set of soluble latex proteins (LPs) (Alencar et al. 2004). Later, the proteins comprising the major peak (LPp1) were shown to be devoid of protease activity and enhance inflammation or anti-inflammation depending on administration route in the mouse peritonitis model (Alencar et al. 2006; Oliveira et al. 2012). Injection of LPp1 into mouse's peritoneal cavity enhances leukocyte recruitment and increases survival after challenge with *Salmonella enterica* ser. Typhimurium (Oliveira et al. 2012). LPp1 is not toxic to cultured macrophages and prompt messenger RNA (mRNA) expression of pro-inflammatory cytokines and the inducible nitric oxide synthase (iNOs) after infection with *Listeria monocytogenes* (Nascimento et al. 2016).

Recently, mass spectrometry analyses of individual proteins depicted from 2D electrophoresis indicated that LPp1 comprises a complex mixture of chitinases. However, although the protein profiles of LPp1 (P1 to P6) were not homogenous, only one peptide sequence of 19 amino acids (VPGYGVITNIINGGVECGK) with strong identity for a chitinase from *Pyrus pyrifolia* was recorded by mass spectrometry (Freitas et al. 2016). This data suggest the presence of different chitinase isoforms in *C. procera* latex being LPp1-P4 the most homogenous, with two protein bands ranging from 27 to 30 kDa (Freitas et al. 2016). Chitinases are widespread in nature and found, e.g., in virus, bacteria, fungi, plants, and mammals (Ali et al. 2007; Frederiksen et al. 2013; Lam and Ng 2010; Kesari et al. 2015; Elmonem et al. 2016). In plants, these proteins belong to glycosil hydrolase families 18 and 19 expressed during plant development and in response to abiotic stress or attack by chitin-containing pathogens (Kesari et al. 2015). Mammalian chitinase-like proteins (CLPs) such as YKL-39, YKL-40, SI-CLP, and YM1/YM2 contain the Glyco_18 domain but lack enzymatic activity (Lee et al. 2011). Although mammals also respond to antigens containing chitin-like structures, human CLPs are mainly induced during inflammatory disorders such as hepatitis, asthma, and inflammatory bowel disease (Di Rosa et al. 2015). However, its role in inflammation and cancer is not clear.

In this study, the effect of chitinases obtained from *C. procera* latex on viability of colon, brain, and ovary tumor cell lines was investigated. In addition, its effect on leukocyte recruitment and release of pro-inflammatory and anti-inflammatory cytokines plus nitric oxide was evaluated in vivo in a model of peritonitis induced by carrageenan. We confirmed that chitinase isoform LPp1-P4 was cytotoxic to all tumor cell lines and highly anti-inflammatory. These data are discussed in light of previous results and future prospects.

Material and methods

Calotropis procera chitinases

A plant specimen of *C. procera* (Voucher No. 32663) was deposited in the Prisco Bezerra Herbarium, Federal University of Ceará, Brazil. Latex proteins were obtained as described by Alencar et al. (2004). After dialyses and centrifugation, a clear supernatant rich in proteins and devoid of rubber was obtained (LP). These proteins were fractionated by ion exchange chromatography in a CM Sepharose Fast-Flow Column previously equilibrated with 50 mM of acetate buffer (pH 5.0) (Ramos et al. 2009). The major peak (LPp1) at 2 mg/mL was dissolved in 20 mM Tris-HCl buffer (pH 8.0), centrifuged at 20,000×*g* at 4 °C for 20 min, and filtered using a 0.22-μm filter (Millipore®). Then, it was subjected to ion exchange chromatography using a Mono-Q column coupled to an FPLC system, equilibrated previously with 20 mM Tris-HCl buffer (pH 8.0) (Freitas et al. 2016). Retained proteins were eluted using a linear gradient from 0 to 400 mM NaCl for 40 min at a 1 mL/min flow rate. Six peaks were obtained (LPp1-P1 to P6) and dialyzed against distilled water and lyophilized. Electrophoresis was performed in 12.5% polyacrylamide gels containing 0.1% SDS, as described by Laemmli (1970) (Fig. 1).

Effect of LPp1 on inflammatory cytokine network

Immortalized macrophages from C57BL/6 (Nagpal et al. 2009) mice were kindly provided by Dr. P. Tourlomousis of the Department of Veterinary Medicine, University of Cambridge. The macrophages were cultured in DMEM supplemented with 20% FCS, L-glutamine (2 mM), penicillin (100 μg/mL), and streptomycin (100 μg/mL) at 5% CO_2/37 °C for 3–4 days (20 mL). Then, macrophage suspensions were seeded in 96-well plates (0.2 mL, 2×10^5 cells per well). After overnight incubation at 37 °C in a 5% CO_2 atmosphere, the wells were washed with phosphate-buffered saline (PBS, pH 7.2) and 0.2 mL of LPp1 (at 62.2, 125, 250, or 500 μg/mL in DMEM) was added to the wells in order to determine non-toxic dosages. Wells containing medium only were used as controls. After incubation for 24 h at 37 °C, 20 μL of resazurin (0.15 mg/mL) was added to each well and the cells were cultured for further 4 h; metabolically active cells were detected by reduction of resazurin yielding the fluorescent compound resorufin, which was detected using a microplate

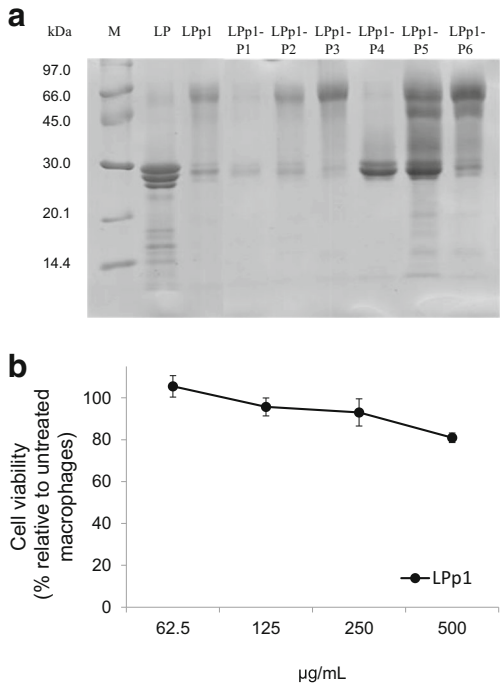

Fig. 1 Electrophoretic profile of *C. procera* latex proteins and cell viability of cultured macrophages after exposure to LPp1. **a** The protein samples were dissolved in 62.5 mM Tris-HCl buffer (pH 6.8) containing 1% SDS, in the presence or absence of 5% β-mercaptoethanol. Protein bands were visualized after staining with 0.1% Coomassie Brilliant Blue R-250. Phosphorylase B (97.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were used as molecular mass markers. **b** Cell viability was measured after 24-h exposure of macrophages to LPp1 through the resazurin assay

fluorometer (560-nm excitation/590-nm emission filter set). Results are expressed in terms of cell viability relative to untreated macrophages.

Real-time PCR was used in order to determine mRNA expression of inflammatory cytokines and iNOs. Macrophage suspensions were cultured overnight in 24-well plates in plain DMEM (2×10^5 cells per well), and LPp1 was added at 10 or 100 μg/mL to each well. LPS (2 μg/mL) was used as control. Following 24-h incubation, TRI reagent (Sigma) was added to each well for extraction of total RNA followed by chloroform-isopropanol treatment of the cell homogenate. RNA was quantified with a NanoDrop spectrophotometer at 260 nm. cDNA was synthesized using the Sigma SYBR Green Quantitative RT-PCR kit. Reverse transcription was carried out at 40 °C for 30 min followed by 94 °C for 10 min for MMLV inactivation. Amplification was carried out for 40 cycles with denaturation at 95 °C for 15 s and annealing plus extension at 60 °C for 60 s by real-time PCR (Rotor-Gene Q Qiagen). The following primers were used: TNF-α (forward 5′ CTGTAGCCCACG TCGTAGC 3′, reverse 5′ GGTTGTCTTTGAGATCCATGC 3′), IL-1β (forward 5′ TGAGCACCTTCTTTTCCTTCA 3′, reverse 5′ TTGTCTAATGGGAACGTCACAC 3′), IL-6 (forward 5′ TAATTCATATCTTCAACCAAGAGG 3′, reverse 5′ TGGTCC TTAGCCACTCCTTC 3′), and iNOs (forward 5′ TGTGGCTACCACATTGAAGAA 3′, reverse 5′ TCATGATAACGTT TCTGGCTCTT 3′). The enzyme hypoxanthine ribosyltransferase (HPRT) was used as internal control: forward 5′ GGGCTTACCTCACTGCTTTC 3′, reverse 5′ TCTCCACCAATAACTTTTATGTCC 3′. The results were analyzed according to Dussault and Pouliot [14]. For comparison of the levels of expression of genes of interest (GIs) between control and experimental groups, we used the following formula:

$$\Delta\Delta Ct = \left[\left(Ct\ GI_{Control} - Ct\ HPRT_{Control}\right) - \left(Ct\ GI_{Experimental} - Ct\ HPRT_{Experimental}\right)\right]$$

The results were expressed as fold variation using the formula $2^{\Delta\Delta CT}$.

Cytotoxicity of LPp1 chitinase isoforms against human tumor cell lines

HCT-116 (colon carcinoma), OVCAR-8 (ovarian carcinoma), and SF-295 (glioblastoma) tumor cell lines were obtained from the National Cancer Institute (Bethesda, MD, USA) kept in nitrogen until use. The cells were dissolved in RPMI medium plus 10% fetal bovine serum, 2 mM glutamine, 100 U/mLpenicillin, and 100 μL^{-1} streptomycin. For all experiments, cells were plated in 96-well plates (10^5 cells/well for OVCAR-8 and SF-295 cells or 0.7×10^5 cells/well for HCT-116 cells in 100 μL of medium) incubated for 24 h at 5% CO_2/37 °C. Then, LPp1-P1 to P6 (0.02 to 25 μg/mL) were dissolved in sterile PBS and added to each well. Doxorubicin and PBS were used as positive and negative controls, respectively. After new incubation for 72 h (5% CO_2/37 °C), the plates were centrifuged (1500 rpm/15 min) and the supernatant was replaced by 0.2 mL of RPMI containing 0.5 mg/mL MTT. After 3-h incubation (5% CO_2/37 °C), the plates were again centrifuged and the supernatant was discarded. The pellet was suspended in 150 μL DMSO, and the MTT formazan product was read at 595 nm (Spectra Count, Packard, Ontario, Canada). Experiments were conducted in duplicates and results expressed as percentage of the dye reduction compared to the control group PBS. The threshold for anticancer activity was set as $IC_{50} < 3$ μg/mL. The yield of active proteins was measured according to Bradford (1976).

Effect of LPp1-P4 on leukocyte recruitment

Experiments were conducted after approval by the institutional animal ethics committee of Ceará Federal University (CEUA No. 82/2014). The mouse peritonitis model was used to assess the anti-inflammatory potential of chitinases in LPp1-P4, which have shown cytotoxicity against tumor cell

lines. Groups of male mice (n = 6) were allocated at random and received an intravenous (i.v.) injection with LPp1-P4 (2 mg in 0.2 mL PBS/kg) 30 min prior intraperitoneal (i.p.) injection with carrageenan (500 μg per mouse). Control animals received PBS instead of plant's latex chitinases. After 4 h of the inflammatory stimulus, the animals were sacrificed by inhalation with excess Halotan®. Then, 3 mL of sterile PBS was injected into the mice's peritoneal cavity containing 5 U/mL of heparin. The abdomens were massaged for 1 min before to collect the peritoneal fluid for further analyses.

Leukocyte cell counts into the peritoneal fluid

Total and differential cell counts were conducted according to de Souza and Ferreira (1985). Samples of peritoneal fluid (20 μL) were diluted in 380 μL Turk's reagent, and total leukocyte cell counts performed with a Neubauer chamber by optical microscopy. For differential cell counts, 50 μL of peritoneal exudate was placed on slides and centrifuged (500× g at 25 °C/10 min). Then, the slides were stained with Fast Panoptic kit (Laborclin, Pinhais, Brazil), following the manufacturer's instruction, and visualized by optical microscopy.

Hematological profile

Blood samples were collected via orbital plexus into tubes containing heparin (5000 U). Hematologic determinations were performed in semi-automatic cell analyzer Sysmex KX-21N (Roche, USA). To determine the percentage of neutrophils, lymphocytes, eosinophils, basophils, and monocytes, blood smears were made on slides and stained as described by Horobin (2011).

Cytokine measurements in the serum of mice

The cytokine concentrations were determined in the serum by ELISA following the manufacturer's instructions (R&D Systems, Minneapolis, USA). Anti-mouse IL1-β, IL-6, IL-10, or TNF-α IgG antibodies were used to coat 96-well microplates, which were incubated at 4 °C for 12 h. Bovine serum albumin (1% in PBS, pH 7.4) was used as blockage solution. Samples of serum or peritoneal fluid were incubated for 1 h at 4 °C. Detection of cytokines was carried out with biotinylated monoclonal anti-IgG antibodies conjugated to peroxidase. Color was developed with 1:1 mixture of H_2O_2 plus tetramethylbezidine and stopped by adding 50 μL of H_2SO_4. Cytokine concentrations were calculated by comparison with standard curve values and absorbance determined at 450 nm. The results were expressed in picogram per milliliter.

Effect of LPp1-P4 on nitric oxide synthesis and neutrophil migration

Two inhibitors of the iNOs enzyme were used: NG-nitro-L-arginine methyl ester (L-NAME, unspecific inhibitor) and aminoguanidine (AG, selective inhibitor). Both inhibitors were administered to groups of male mice allocated at random (n = 6; 25 mg/kg), subcutaneously, 15 min prior to administration of LPp1-P4 (2 mg/kg) by i.v. route. Control animals received PBS instead of plant's latex chitinases. Additional controls included mice that received L-NAME or AG only. After 1 h, carrageenan (500 μg per mouse) was injected into the peritoneal cavity of all animal groups. After 4 h of the inflammatory stimulus, leukocyte cell counts were determined as described in "Leukocyte cell counts into the peritoneal fluid" section.

Statistical analysis

Statistical differences were calculated by analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test with confidence interval set as $P < 0.05$. These analyses were performed, and graphs obtained using the PRISM program version 5.0.

Results

Figure 1a depicts the progressive shift of protein profiles obtained when the total LPs from *C. procera* were fractionated following chromatography procedures. Since LPp1 is known to be implicated in cytotoxicity and NO inducing, LPp1-P1 to P6 were focused in the present study. Cytotoxicity assays have shown that LPp1 was not toxic to immortalized murine macrophages (Fig. 1b). Following 24-h exposure, cell viability was over 98% in macrophages treated with 62.2 or 125 μg/mL LPp1, whereas dosages of 250 and 500 μg/mL reduced viability by 13 and 18%, respectively, compared to untreated cells. However, the mRNA expression of pro-inflammatory cytokines IL-6, IL1-β, TNF-α, and the inducible enzyme iNOs increased significantly in macrophages treated with non-toxic dosages of LPp1 (10 and 100 μg/mL) ($P < 0.05$) (Fig. 2). In particular, production of mRNA transcripts for IL-6, IL1-β, and iNOs ranged from 40-fold to 300-fold at 100 μg/mL. Cytotoxic assays with colon and ovarian carcinoma besides glioblastoma tumor cell lines have shown that only LPp1-P4 was active with IC_{50} ranging from 1.2 to 2.9 μg/mL (Table 1). LPp1-P4 was therefore used in further assays. In vivo assays have shown that intravenous injection of LPp1-P4 impaired neutrophil infiltration into mouse's peritoneal cavity induced by carrageenan (Fig. 3). The hematological profile of these animals showed that protein treatments did not change the red series in comparison to the PBS group (Table 2). LPp1-

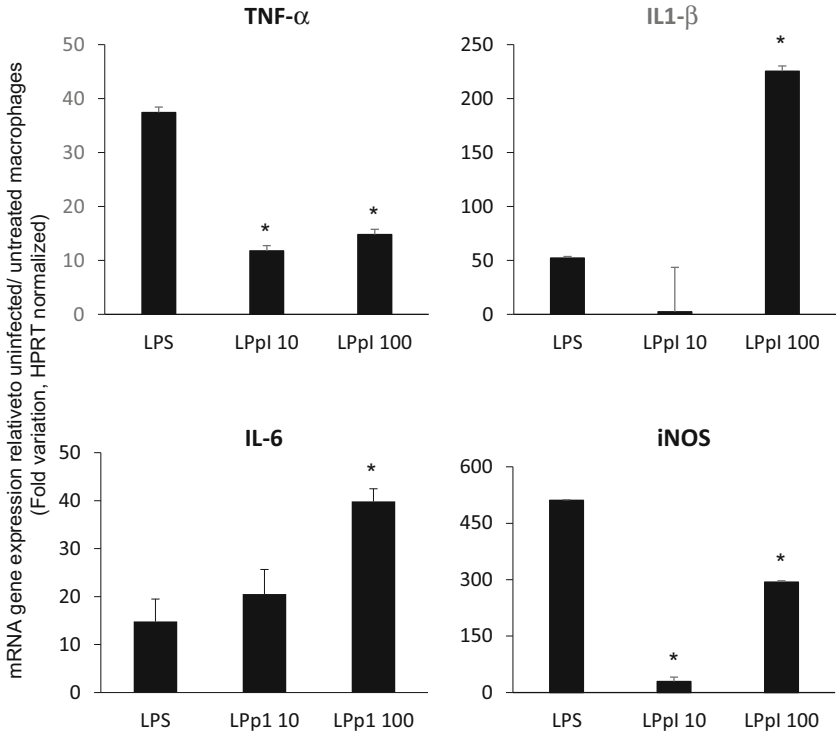

Fig. 2 mRNA expression of inflammatory cytokines and iNOs enzyme in macrophage cell cultures treated with LPp1. Naïve macrophages were cultured in 96-well plates (2×10^5 per well) and treated with LPp1 (10 or 100 μg/mL) for 24 h or LPS (2 μg/mL). *$P < 0.05$, comparison between LPp1-treated groups and those stimulated with LPS (Student's *t* test)

P4 chitinases increased the number of circulating neutrophils in control mice, but it was not significant after administration of the phlogistic agent ($P > 0.05$). In addition, the lymphocyte counts were decreased in comparison to the carrageenan group ($P < 0.05$), while the number of monocytes, basophils, eosinophils, and platelets has not changed in comparison to all animal groups. Intravenous treatments with LPp1-P4 increased the release of the pro-inflammatory cytokine IL-6 in the bloodstreams and peritoneal fluid in comparison to the carrageenan group ($P < 0.05$) (Fig. 4). Conversely, while TNF-α and IL1-β were significantly decreased in mice's peritoneal fluid, their contents in serum were increased ($P < 0.05$) (Fig. 4). The anti-inflammatory cytokine IL-10 was not detected in any of the animal groups neither in serum nor in the peritoneal fluid (data not shown). An assay was carried out to determine whether the inducible enzyme iNOs play a role on impairment of neutrophil recruitment in mice administered with LPp1-P4 (i.v.) plus carrageenan (i.p.). We confirmed that iNOs inhibitors L-NAME and AG reverted the anti-inflammatory effect of LPp1-P4, which significantly

Table 1 Cytotoxic activity of chitinase-like proteins from *C. procera* on tumor cell lines

	Cell lines		
Samples	HCT-116 (colon) IC_{50} (μg/mL)	Ovcar-8 (ovary) IC_{50} (μg/mL)	SF-295 (glioblastoma) IC_{50} (μg/mL)
P1	>25*	>25*	>25*
P2	>25*	>25*	>25*
P3	>25*	>25*	>25*
P4	2.2 (1.4–3.5)	1.2 (1.0–1.4)	2.9 (2.2–3.9)
P5	>25*	16* (11.3–22.5)	>25*
P6	>25*	>25*	>25*
Doxorubicin	0.01 (0.01–0.02)	0.26 (0.17–0.31)	0.25 (0.15–0.34)

Data obtained using the MTT method after 72-h incubation. Doxorubicin was used as positive control. The values showed in parentheses indicate the maximum and minimum values observed in two independent experiments performed in duplicate

*$P < 0.05$ indicates statistical difference (ANOVA followed by Student's Newman Keuls test)

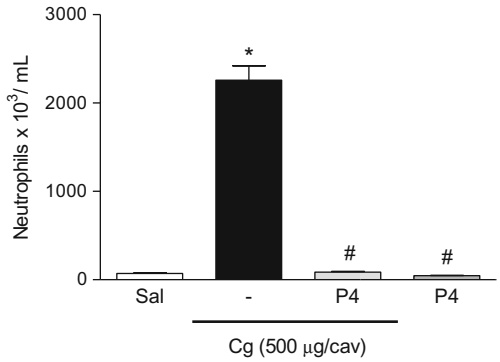

Fig. 3 Effect of P4 chitinases from *C. procera* latex on neutrophil recruitment. Groups of mice (n = 6) were injected intravenously with P4 (2 mg/kg) 30 min prior to the intraperitoneal injection with carrageenan (500 μg *per* mouse). Migration of neutrophils into the peritoneal cavity was evaluated 4 h after the inflammatory stimulus. Results are expressed as mean ± SEM of the number of neutrophils per milliliter of peritoneal fluid. *$P < 0.05$ indicates significant difference compared with the saline group (Sal), and #$P < 0.05$ indicates significant difference compared to the carrageenan group (Cg) (ANOVA followed by Bonferroni's test)

increased the infiltration of neutrophils into mouse's peritoneal cavity ($P < 0.05$) (Fig. 5).

Discussion

The potential of plant chitinases to mimic human CLPs on inflammatory processes and cancer immunotherapy is unknown. Human YKL-40 expressed by macrophages or colonic epithelial cells can be upregulated after stimulus by pro-inflammatory cytokines (Kawada et al. 2007). This CLP has been associated with poor prognosis in breast, lung, prostate, liver, bladder, colon, and other types of cancer, and its suppression reduces glioma cell invasion and increased cell death triggered by anticancer drugs (Ku et al. 2011; Kzhyshkowska et al. 2016). On the other hand, Oliveira et al. (2007) have shown that soluble proteins from *C. procera* latex were cytotoxic to human neoplastic cells such as SF-295 (brain), MDA-MB-435 (melanoma), HL-60 (promyeltocytic leukemia), and HCT-8 (colon). Toxicity was primarily concentrated in LPp1 and not evidenced against healthy peripheral blood mononuclear cells. Indeed, we have shown that LPp1 was not toxic to primary (Nascimento et al. 2016) or immortalized macrophage cell cultures from mice, that suggests a selective action against specific tumorigenic cancer cell lineages.

It has been shown that chitinases from different sources appear to exert anticancer properties. For example, bacterial chitinases produced structural damage to MCF-7 breast cancer cells in vitro and selectively lysed tumor cells in mice (Pan et al. 2005). However, the biological activity of human chitinases relies on interactions mediated by the Glyco_18 domain (Riabov et al. 2014). Accordingly, humanized family 19 plant chitinases with changes in the *N*-glycosylation pattern have been tested as anticancer agents (Schähs et al. 2007). Here, LPp1-P4 chitinase isoforms retained all anticancer activity of LPp1 against human tumor cell lines from the colon, brain, and ovary. Although anticancer properties of plant

Table 2 Hematological profile in Swiss mice treated with LPp1-P4 and then injected with carrageenan

Hematological parameters	Groups			
	PBS	Carrageenan	Carrageenan + P4	P4
Total leukocytes (10^3/μL)	1.66 ± 1.02	3.02 ± 0.89	2.16 ± 0.2	3.21 ± 1.33
RBC (10^6/μL)	7.92 ± 1.32	7.82 ± 0.52	8.21 ± 0.61	7.37 ± 1.14
Hemoglobin (g/dL)	12.45 ± 2.22	12.16 ± 0.59	13.71 ± 1.09	11.66 ± 1.8
Hematocrit (%)	34.16 ± 6.47	33.33 ± 1.83	36.38 ± 1.73	31.75 ± 4.9
MCV (fL)	43.01 ± 1.73	42.63 ± 1.24	42.26 ± 1.3	43.06 ± 0.92
MCH (pg)	15.7 ± 0.57	15.58 ± 0.76	15.66 ± 0.53	15.83 ± 0.39
MCHC (g/dL)	36.48 ± 0.54	36.51 ± 0.92	37.06 ± 1.36	36.76 ± 0.56
Platelets (10^3/μL)	699 ± 130	617.6 ± 134	540.3 ± 65.7	559.3 ± 71.9
Neutrophils (10^3/μL)	0.17 ± 0.08	1.13 ± 0.27*	1.02 ± 0.23*	2.18 ± 0.1*,***
Lymphocytes (10^3/μL)	2.02 ± 0.37	2.23 ± 0.29	1.63 ± 0.14**	2.15 ± 0.38
Monocytes (10^3/μL)	0.09 ± 0.03	0.06 ± 0.02	0.11 ± 0.05	0.1 ± 0.03
Basophils (10^3/μL)	0.02 ± 0.03	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0.03
Eosinophils (10^3/μL)	0.06 ± 0.007	0.07 ± 0.009	0.07 ± 0.02	0.07 ± 0.03

The animals received P4 (2 mg/kg, i.v.) or sterile PBS 30 min prior to the inflammatory stimulus (500 μg carrageenan/cav, i.p.). Blood analyses were performed 4 h after injection of carrageenan. Values are expressed as mean ± SEM (n = 6)

*$P < 0.05$ indicates significant difference compared to the PBS group; **$P < 0.05$ indicates significant difference compared to carrageenan group (ANOVA followed by Bonferroni's test)

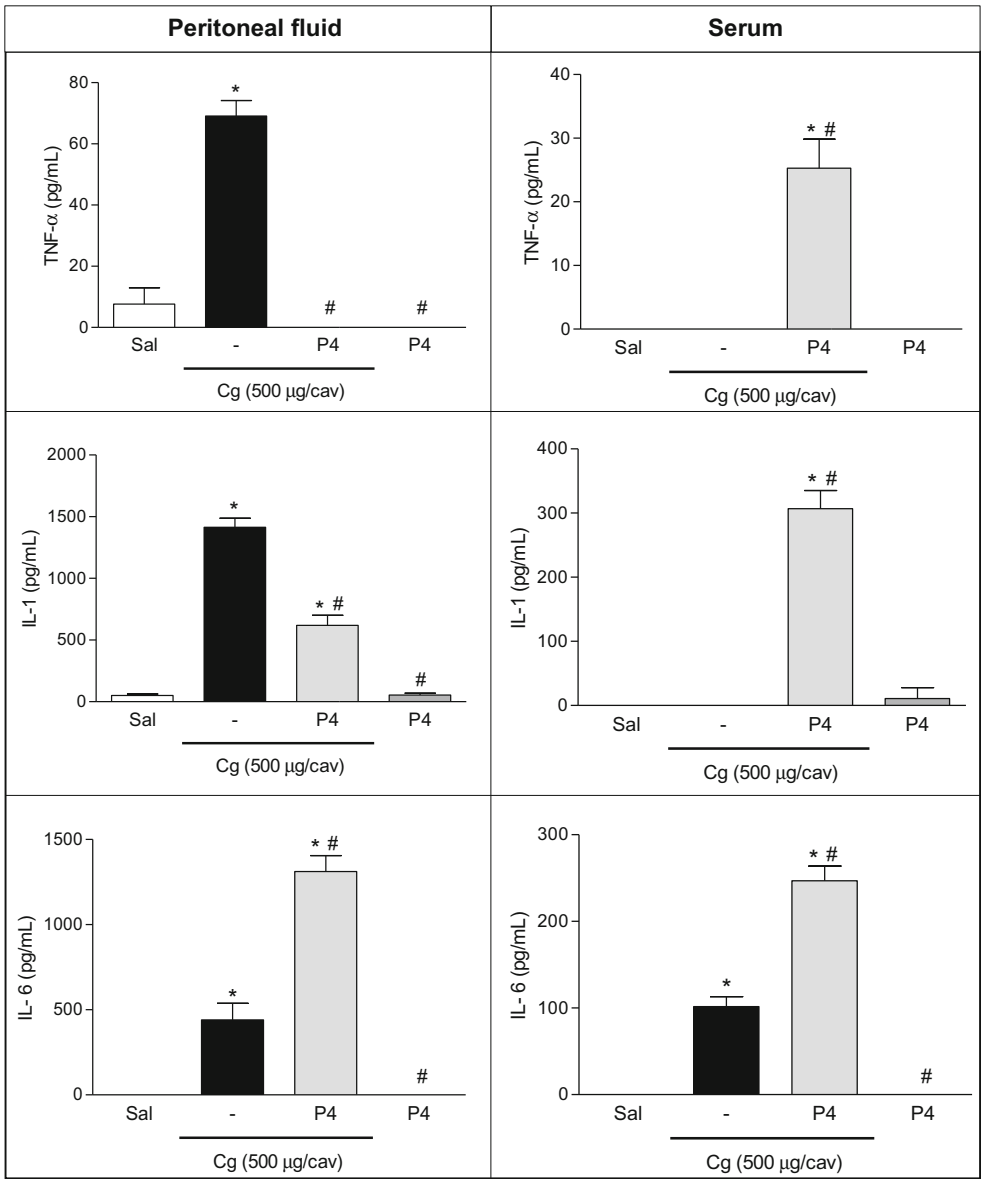

Fig. 4 Effect of P4 chitinases on the release of pro-inflammatory cytokines. Groups of mice ($n = 6$) were injected intravenously with P4 (2 mg/kg) 30 min prior to the intraperitoneal injection with carrageenan (500 µg *per* mouse). Cytokine measurements were carried out on the recovered peritoneal fluid and serum 4 h after injection of carrageenan. Values are expressed as mean ± SEM. *$P < 0.05$ indicates significant difference compared to saline group (Sal). #$P < 0.05$ indicates significant difference compared to the group carrageenan (Cg) (ANOVA followed by Bonferroni's test). IL-10 was not detected in any of the animal groups

chitinases appear to require participation of proteolytic enzymes, Xu et al. (2008) have shown that a recombinant chitinase from plant family 19 does not require proteases to kill cancer cells. Since *C. procera* LPp1 is naturally devoid of proteases or proteolytic enzymes, it is reasonable to assume that LPp1-P4 chitinases affect viability of tumor cells directly. This is corroborated by Oliveira et al. (2007) whom have shown that following treatment with reducing agents, such as DTT or 2-mercaptoethanol, LPp1 drastically lost its cytotoxicity over tumorigenic cells.

Although LPp1 enhanced in vitro activation pro-inflammatory cytokines plus the enzyme iNOs, LPp1-P4 was highly anti-inflammatory and capable to counterbalance the inflammatory stimulus of carrageenan impairing infiltration of leukocytes into mouse's peritoneal cavity. This effect was not due to IL-10, which is known to inhibit pro-inflammatory cytokine production by macrophages and dendritic cells (Geginat et al. 2016). Conversely, the serum levels of inflammatory cytokines were high in the bloodstreams after injection of the phlogistic agent in LPp1-P4-treated mice. Activated macrophages are main sources of TNF-α, IL1-β, and IL-6 which play a key role in leukocyte recruitment and microbial clearance through production of intracellular nitric oxide (NO) (Rivera et al. 2016). Exogenous NO derived from activation of the inducible enzyme iNOs protect macrophages from apoptosis induced by lipopolysaccharide (Malysheva et al. 2006). However, high NO contents can cause immunosuppression by reducing transmigration of activated leukocytes through vascular endothelium to the site of inflammation (Benjamim et al. 2002; Secco et al. 2003). Thus, the involvement of NO on anti-inflammatory effect enhanced by LPp1-P4 was investigated.

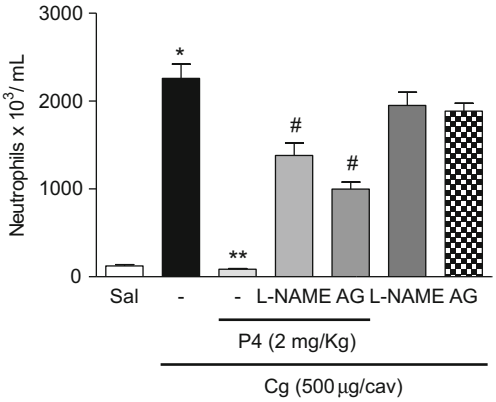

Fig. 5 Effect of nitric oxide synthase inhibitor (L-NAME and AG) on anti-inflammatory activity of *C. procera* P4 chitinases. The animals (n = 6) were administered with P4 (2 mg/kg, i.v.) 15 min after the injection of L-NAME or AG (25 mg/kg, s.c.). Carrageenan (500 µg/cav, i.p.) was administered 30 min after administration of P4. Neutrophil recruitment into the peritoneal cavity was evaluated 4 h after treatment. Results are expressed as mean ± SEM of neutrophils per cavity. *$P < 0.05$ indicates significant difference compared with saline group (Sal); **$P < 0.05$ indicates significant difference compared with carrageenan group; #$P < 0.05$ indicates significant difference compared to P4 group (ANOVA followed by Bonferroni's test)

As part of innate immunity, acute infections have been shown to upregulate chitinases and influence on spontaneous cancer regression (Lin et al. 2004; van Eijk et al. 2005). Accordingly, M1-type macrophages have shown to induce lyses in several types of cancer cells (Pan 2012), and therefore, its activation by LPp1 or LPp1-P4 could potentially enhance innate anticancer functions. In a previous study, we have shown that LPp1 enhanced the mRNA expression of TNF-α, IL1-β, and IL-6 besides iNOs in uninfected plus infected primary macrophage cell cultures (Nascimento et al. 2016). However, in vivo administration of LPp1 had an inhibitory effect on leukocyte rolling and adhesion, monitored by intravital microscopy, which was associated with high NO content in the bloodstreams (Ramos et al. 2009). Here, we corroborate these findings since LPp1-P4 is capable to prompt the NO release through activation of the inducible enzyme iNOs, probably due to macrophage-derived pro-inflammatory cytokines.

Though new studies are necessary to confirm the potential of *C. procera* latex proteins in cancer immunotherapy, we have shown that chitinase isoform LPp1-P4 with anti-inflammatory properties was highly cytotoxic to tumor cell lines. Since inflammation in the cancer microenvironment can lead to tumor growth and spread throughout the body, bioactive compounds with anti-inflammatory properties can represent a useful tool with potential clinical relevance.

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