

# Proteins derived from latex of *C. procera* maintain coagulation homeostasis in septic mice and exhibit thrombin- and plasmin-like activities

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**Abstract** The proteins derived from the latex (LP) of *Calotropis procera* are well known for their anti-inflammatory property. In view of their protective effect reported in the sepsis model, they were evaluated for their efficacy in maintaining coagulation homeostasis in sepsis. Intraperitoneal injection of LP markedly reduced the procoagulation and thrombocytopenia observed in mice infected

with Salmonella; while in normal mice, LP produced a procoagulant effect. In order to understand its mechanism of action, the LP was subjected to ion-exchange chromatography, and the three subfractions (LP<sub>PI</sub>, LP<sub>PII</sub>, and LP<sub>PIII</sub>) thus obtained were tested for their proteolytic effect and thrombin- and plasmin-like activities in vitro. Of the three subfractions tested, LP<sub>PII</sub> and LP<sub>PIII</sub> exhibited proteolytic effect on azocasein and exhibited procoagulant effect on human plasma in a concentration-dependent manner. Like trypsin and plasmin, these subfractions produced both fibrinogenolytic and fibrinolytic effects that were mediated through the hydrolysis of the A $\alpha$ , B $\beta$ , and  $\gamma$  chains of fibrinogen and  $\alpha$ -polymer and  $\gamma$ -dimer of fibrin clot, respectively. This study shows that the cysteine proteases present in the latex of *C. procera* exhibit thrombin- and plasmin-like activities and suggests that these proteins have therapeutic potential in various conditions associated with coagulation abnormalities.

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## Introduction

Plants belonging to several families are a rich source of latex that comprises various proteins, lipids, hydrolytic enzymes, and secondary metabolites. Latex is a complex emulsion that oozes out from various parts of the plant following injury and coagulates upon exposure to air and plays a vital role in plant defense against various pathogens such as insects and fungi (Morcelle et al. 2004; Hagel et al. 2008). The latex derived from different plants has also been

reported to exhibit bacteriolytic, larvicidal, and antihelminthic properties and has been used in the traditional medicinal system for the treatment of various diseases (Domsalla and Melziq 2008; Kumar and Arya 2006). Application of plant latex on fresh cuts to stop bleeding is a general practice followed by tribal and rural people (Richter et al. 2002; Thankamma 2003). The properties of latex in plant defense and in alleviating various diseases have been attributed to the presence of several hydrolytic enzymes of which proteases play a key role. Most of the proteases found in latices of different plants belong to cysteine and serine protease family and are structurally and functionally distinct (Morcelle et al. 2004; Domsalla and Melziq 2008).

*Calotropis procera* is a plant that grows in the wild. It belongs to family Apocynaceae and subfamily Asclepiadaceae and is commonly known as milkweed. The latex of this plant is well known for its toxic properties; nevertheless, it has been used for the treatment of various diseases (Kumar and Arya 2006). It has been demonstrated to exhibit potent anti-inflammatory property in various experimental models, and this activity is present both in the aqueous extract of the dry latex and the proteins isolated from the fresh latex (Kumar and Basu 1994; Alencar et al. 2004). The medicinal properties exhibited by this plant are very similar to that of *C. gigantea*, and the latex of both the species possesses proteolytic activities (Kumar and Arya 2006; Adak and Gupta 2006; Dubey and Jagannadham 2003; Abraham and Joshi 1979). The latex of *C. gigantea* has been shown to contain four cysteine proteases, namely Calotropin FI, FII, DI, and DII comprising different amino acid sequences, which exhibit different properties (Abraham and Joshi 1979; Pal and Sinha 1980). The latex of *C. procera* on the other hand contains distinct cysteine proteases called procerain and procerain B (Dubey and Jagannadham 2003; Singh and Dubey 2011). Like other members of Asclepiadaceae, the cysteine protease activity of the latex of *Calotropis* has been shown to exhibit both thrombin- and plasmin-like activities that result in blood coagulation and fibrin hydrolysis (Shivaprasad et al. 2009).

Sepsis is a systemic inflammatory condition associated with coagulation abnormalities and thrombocytopenia (Mavrommatis et al. 2000). Inflammatory activation in patients with severe infection is almost invariably related to activation of coagulation, and markers of inflammation, coagulation, and fibrinolysis are well known to predict an adverse outcome in sepsis (Levi and Schultz 2008). In a recent study, sepsis was induced in mice by intraperitoneal administration of *Salmonella enterica* serovar Typhimurium. In this model, the protective effect of proteins derived from the latex (LP) of *C. procera* has been demonstrated on the inflammatory response, and the lethal effect of bacterial infection (Lima-Filho et al. 2010). In view of this, the present study was carried out to evaluate the effect of LP on coagulation and

platelet count in septic mice and to study the proteolytic, thrombin- and plasmin-like properties of LP and its subfractions in vitro.

## Materials and methods

### Materials

Human (fibrinogen and thrombin), bovine trypsin, papain from *Carica papaya*, ethylenediamine tetraacetic acid (EDTA), azocasein, urea, and agarose were purchased from Sigma-Aldrich Co. (São Paulo, Brazil). Dithiothreitol (DTT) and molecular weight markers were obtained from GE Healthcare, Brazil. Other chemicals used in the study were of analytical grade.

### Latex protein isolation and ion-exchange chromatography

The latex of *C. procera* was collected from the terminal branches of the plant growing in Fortaleza, Ceara, Brazil and immediately mixed in distilled water (Soares et al. 2005). A voucher specimen of the plant (catalog no. 32663) was maintained at the Prisco Bezerra Herbarium of the Universidade Federal do Ceará, Brazil. Soluble proteins (latex proteins, LP) were recovered from the whole latex according to the method described by Lima-Filho et al. (2010). LP was further fractionated by ion-exchange chromatography on a CM-Sepharose fast flow column to obtain three subfractions named LP<sub>PI</sub>, LP<sub>PII</sub>, and LP<sub>PIII</sub> according to Ramos et al. (2009).

### *S. enterica* serovar Typhimurium

*S. enterica* serovar Typhimurium was isolated from a human clinical case at Fundação Ezequiel Dias (Belo Horizonte, Minas Gerais, Brazil) and was a kind gift from Dr. Jacques Robert Nicoli (Universidade Federal de Minas Gerais). The bacteria were maintained at  $-18^{\circ}\text{C}$  in brain heart infusion (BHI) medium containing 50% glycerol. For experimentation, the bacteria were activated by culture in BHI broth for 24 h at  $37^{\circ}\text{C}$  and serially diluted to attain a bacterial suspension containing  $10^7$  CFU/ml.

### Animals

The experiments were carried out on male Swiss mice weighing about 30 g, and each treatment group comprised of six mice. The animals were obtained from the Central Animal House of the Universidade Federal do Ceará and were kept in an animal house with controlled light and dark cycles (12 h), temperature ( $25^{\circ}\text{C}\pm 3$ ), and humidity (60–70%) and had free access to water and commercial sterile

diet (Purina, Paulínia, Sao Paulo, Brazil). Experimental procedures and animal handling were performed in accordance with the guidelines approved by the Institutional Animal Ethics Committee.

#### *Human plasma samples*

Samples of healthy human blood certified for transfusion were obtained from the Centre of Hematology and Hemotherapy of the State of Ceará (Ceará, Brazil). The blood was mixed with 0.11 M tri-sodium citrate in the ratio of 9:1 (v/v) and centrifuged at  $500\times g$  for 15 min at 20°C to obtain platelet poor plasma (PPP). Calcium is involved in the cascade of coagulation in a step anterior of that involving thrombin. Tri-sodium citrate, a chelating agent, makes the plasma poor in calcium. This effect drastically increases the time of coagulation. Thus, calcium was added in all determination in order to replace calcium in the reaction medium.

#### Methods

##### *Experimental design in sepsis model*

The animals were divided into four groups each comprising six mice. Group I and group II served as control where 0.2 ml of sterile saline was administered intraperitoneally, while sepsis was induced in group III and group IV by intraperitoneal administration of bacterial suspension ( $10^7$  CFU/ml) (Lima-Filho et al. 2010). The blood was collected 24 h after the bacterial inoculation from the retro-orbital plexus of the anesthetized mice, and the animals were sacrificed. Parameters like plasma clotting time and platelet count were measured. The effect of LP was studied in group II and group IV that was administered 24 h before bacterial inoculation.

##### *Platelet count*

The platelet count was determined in heparinized blood samples collected from the mice using a semiautomatic cellular analyzer Sysmex KX-21 N (Roche, USA).

##### *Proteolytic activity*

Proteolytic activity was assayed using azocasein as a substrate (Freitas et al. 2007). LP, papain (5–100 µg), and LP subfractions LP<sub>PI</sub>, LP<sub>PII</sub>, and LP<sub>PIII</sub> (60 µg) were incubated with 0.2 ml of 1% azocasein in a reaction volume of 1 ml in 10 mM Tris-HCl, pH 7.5 for 1 h at 37°C. The absorbance was read at 420 nm. One unit of activity was defined as the amount of enzyme required to increase the absorbance by 0.01, and the proteolytic activity was expressed as activity units.

##### *Plasma clotting activity*

The coagulant activity was determined by the method described by Condrea et al. (1983) where the formation of visible clot was taken as an end point. The clot formation was initiated by adding 30 µl of 0.25 M CaCl<sub>2</sub> to the mouse plasma. The time taken for the formation of clot from the time of addition of 20.8 mM CaCl<sub>2</sub> (final concentration) was recorded.

To evaluate the effect of LP on human plasma clotting time, the LP and its subfractions (10–80 µg) were reconstituted in 30 µl of buffer (10 mM Tris-HCl, pH 7.5 with 3 mM DTT) and incubated with 300 µl PPP for 1 min at 37°C. The clot formation was initiated by adding CaCl<sub>2</sub> as described before. The effect was compared with that of trypsin and papain. A control without the enzyme was included in the study.

##### *Activated partial thromboplastin time*

This coagulation assay was performed according to manufacturer specifications. First, human plasma samples (50 µl) were incubated for 3 min at 37°C with 10 µl of latex protein fractions (10–80 µg), prepared in buffer (10 mM Tris-HCl, pH 7.5 with 3 mM DTT), and 50 µl of activated partial thromboplastin time (APTT) kit reagent (CLOT, Bios Diagnóstica). After the incubation period, 50 µl of 25 mM CaCl<sub>2</sub> (final concentration 7.81 mM) (CLOT, Bios Diagnóstica) was added to the mixture, and the clotting time was recorded on a coagulometer (DRAKE, QUICK-TIMER model). Negative control was performed without the addition of enzyme source (LP), and papain was assayed in the same conditions as positive control.

##### *Prothrombin time*

This assay was performed in a similar manner to the APTT assay. First, 50 µl of human plasma samples were incubated with 10 µl of latex protein fractions (10–80 µg), prepared in buffer (10 mM Tris-HCl, pH 7.5 with 3 mM DTT) for 4 min at 37°C. After the incubation time, 100 µl of prothrombin time (PT) reagent (CLOT, Bios Diagnóstica) was added to the mixture, and the clotting time was recorded on a coagulometer (DRAKE, QUICK-TIMER model). Negative control was performed without the addition of enzyme source, and papain was assayed in the same conditions as positive control.

##### *Fibrinogen-agarose plate assay*

Fibrinogen polymerization assay was carried out by the method described by Joo et al. (2002). Fibrinogen-agarose mixture was prepared by mixing 3 ml of 1.2 % agarose

warmed to 50°C with 3 ml of 0.4% (w/v) human fibrinogen dissolved in 0.1 M Tris-HCl, pH 7.4 and warmed at 50°C. This mixture was poured into a Petri plate and allowed to solidify at room temperature for 1 h. Wells of 3 mm diameter were made on the plate, and 50 µg of protein fractions in 50-µl volume was applied into each well, and incubation was carried out at room temperature for 3, 6, and 24 h. The diameter of the turbid fibrin rings formed around the well indicated an extent of polymerization of fibrinogen. Buffer alone served as control, and 0.2 units of thrombin and 5 µg of papain served as positive control.

#### *Fibrinogen polymerization assay*

The kinetic analysis of fibrinogen polymerization activity of LP and its subfractions were carried out by spectrophotometric method (Shivprasad et al. 2010). Human fibrinogen (0.5 ml of 0.5% in 10 mM Tris-HCl, pH 7.4) was pre-warmed to 37°C, and fibrin clot formation was initiated by adding 50 µl of protein fractions (1 mg/ml in Tris buffer, pH 7.4). Increase in turbidity due to fibrin formation was recorded by measuring absorbance at 540 nm at different time intervals. The polymerizing activity of LP and its subfractions were compared with that of thrombin (0.2 units) and papain (5 µg).

#### *Fibrinogenolytic activity*

Fibrinogenolytic activity of LP and its fractions were determined by the method described by Ouyang and Teng (1976). Human fibrinogen (0.2 ml of 1 mg/ml solution in Tris buffer, pH 7.4) was incubated with different concentrations of papain and LP and its subfractions (5–80 µg) for 30 min at 37°C in the presence of 3 mM DTT. The reaction was terminated by adding 20 µl of stop buffer comprising 4% β-mercaptoethanol, 1 M urea, and 4% SDS. The hydrolyzed products were analyzed by 7.5% SDS-PAGE.

#### *Electrophoresis*

SDS-PAGE was performed by the method of Laemmli (1970), and the protein bands were visualized by staining with Coomassie Blue R-350.

#### *Human plasma clot hydrolyzing activity*

The plasma clot hydrolyzing activity of the LP and its fractions were determined by the method described by Rajesh et al. (2005). Plasma was obtained from the blood by adding 2 mg/ml of EDTA followed by centrifugation at 500×g for 15 min at 20°C. Equal volumes of plasma and 100 mM CaCl<sub>2</sub> were mixed to form fibrin clot. The clot was washed several times with phosphate buffer saline and

incubated with 10–80 µg of protein fraction in a 40 µl Tris buffer, pH 7.4, containing 3 mM DTT for 1 h at 37°C. The reaction was terminated by adding stop buffer as given above, boiled for 3 min, and centrifuged. A 20 µl aliquot of the supernatant was subjected to 7.5% SDS-PAGE.

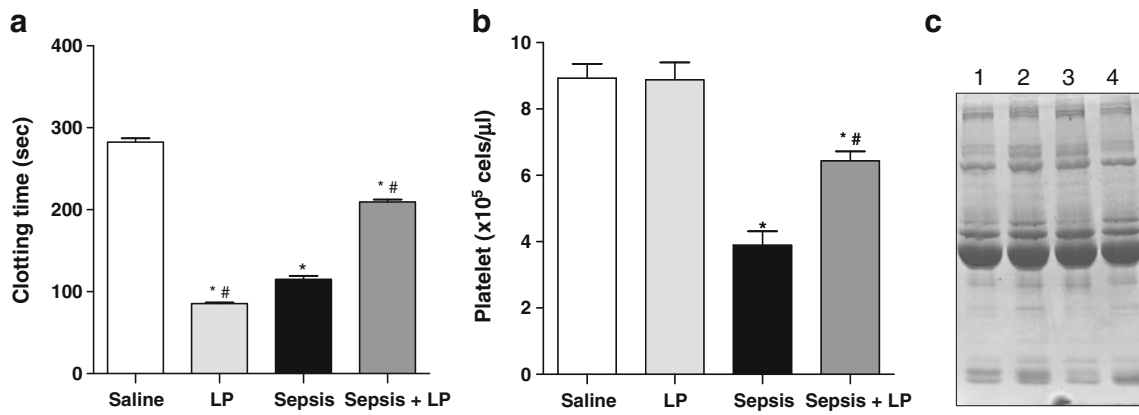
#### *Statistical analysis*

The results are expressed as mean ± SEM. Sepsis model included six animals per treatment group, and parameters like plasma clotting time and platelet count were measured in all the animals. The observations of other in vitro biochemical analysis were obtained from four independent experiments, and the tests were performed in duplicate. ANOVA was performed for multiple comparisons by Student Newman–Keuls test;  $p < 0.05$  was considered statistically significant.

## **Results**

The protein fraction isolated from the fresh latex of *C. procera* (LP) was evaluated for its effect on blood clotting and platelet count in a mouse model where sepsis was induced by intraperitoneal administration of *S. enterica* serovar Typhimurium. LP, when administered intraperitoneally, exhibited procoagulant properties, and the clotting time of the plasma was markedly reduced as compared to the saline-treated control group. The clotting time of the plasma was also markedly reduced in septic mice. Pretreatment of septic mice with LP afforded protection in this regard, and the clotting time in these mice was more than the septic control (Fig. 1a). The changes observed in the clotting time were related to the platelet count in these groups. Unlike clotting time, treatment of normal mice with LP did not alter the platelet count while the platelet count was significantly reduced in septic mice. Like clotting time, treatment of septic mice with LP produced an increase in platelet count in comparison to septic control (Fig. 1b). Besides the clotting time and platelet count, the effect of LP was also evaluated on the plasma protein profile in both control and septic mice. Figure 1c shows the SDS-PAGE analysis of plasma samples of these mice where no difference was observed among the four groups included in the study.

As plant lattices exhibiting cysteine protease activity have been shown to affect blood coagulation, the LP was first tested for its proteolytic activity using azocasein as a substrate. As shown in Fig. 2a, the proteolytic activity of LP increased with an increase in concentration. The proteolytic activity of papain, a member of cysteine proteinase family, was more pronounced than that of LP. The LP was further subjected to ion-exchange chromatography, and the three



**Fig. 1** Effect of LP on plasma coagulation time (a) and platelet count (b) in healthy and septic mice. Mice were treated with LP 24 h before infection with bacteria, and evaluations were done 24 h following the bacterial inoculation. The LP was administered at a dose of 30 mg/kg by intraperitoneal route. The serum samples of these mice were

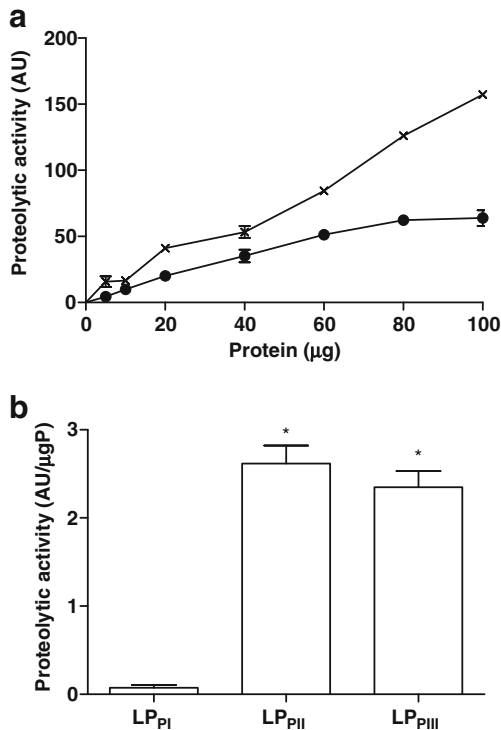
analyzed for protein profile by SDS-PAGE (c). Group 1: saline control; group 2: septic control; group 3: septic mice treated with LP; group 4: normal mice treated with LP. Asterisk indicates  $p < 0.05$  vs. saline-treated group, and number sign indicates  $p < 0.05$  vs. septic control

subfractions thus obtained were tested for their proteolytic activity. This activity was present in two subfractions namely LP<sub>P<sub>II</sub></sub> and LP<sub>P<sub>III</sub></sub>, and both were almost equieffective, while LP<sub>P<sub>I</sub></sub> did not exhibit any such activity (Fig. 2b).

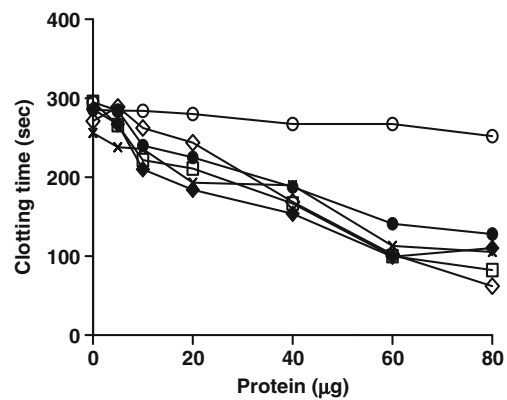
The LP and its subfractions were further tested for their effect on clotting time in vitro. It is interesting to note that LP

and its subfractions LP<sub>P<sub>II</sub></sub> and LP<sub>P<sub>III</sub></sub> produced a concentration-dependent decrease in clotting time as also observed for proteolytic enzymes trypsin and papain. The subfraction LP<sub>P<sub>I</sub></sub> did not produce any change in clotting time (Fig. 3). APTT and PT tests suggested that LP accelerates coagulation cascade by intrinsic pathway. LP ( $12.6 \pm 0.67$ ) and its proteolytic subfractions (LP<sub>P<sub>II</sub></sub> =  $8.8 \pm 1.37$  and LP<sub>P<sub>III</sub></sub> =  $16.9 \pm 1.96$ ) reduced at least by 50% the clotting time [mean  $\pm$  SEM (sec)] in APTT assay (control:  $30.50 \pm 0.88$ ), while this effect was not observed in PT assay (LP =  $12.6 \pm 0.35$ ; LP<sub>P<sub>II</sub></sub> =  $14 \pm 0.43$  and LP<sub>P<sub>III</sub></sub> =  $13.4 \pm 0.41$ ; control:  $15.00 \pm 0.44$ ).

The thrombin-like activity of LP and its subfractions were also evaluated by fibrinogen-agarose plate assay where both thrombin and papain brought about a polymerization of fibrinogen and a precipitation ring was formed. Similar



**Fig. 2** Proteolytic activity of LP (black circle), papain (5–100  $\mu$ g) (a) and LP subfractions LP<sub>P<sub>I</sub></sub>, LP<sub>P<sub>II</sub></sub>, and LP<sub>P<sub>III</sub></sub> (60  $\mu$ g) (b). Samples were incubated with azocasein (0.2 ml, 1 %) in a reaction volume of 1 ml in Tris buffer, pH 7.4 for 1 h at 37°C. Absorbance was read at 420 nm to determine the proteolytic activity. Values given are mean  $\pm$  SEM. Asterisk indicates  $p < 0.05$  vs. LP<sub>P<sub>I</sub></sub> group



**Fig. 3** Effect of different concentrations of LP (black circle), LP<sub>P<sub>I</sub></sub> (white circle), LP<sub>P<sub>II</sub></sub> (white square), and LP<sub>P<sub>III</sub></sub> (white diamond), and proteolytic enzymes trypsin (black diamond) and papain (multiplication sign) on plasma clotting time. Human plasma (300  $\mu$ l) was incubated with latex protein fractions, papain, and trypsin in Tris buffer, pH 7.4 for 1 min at 37°C. CaCl<sub>2</sub> (30  $\mu$ l, 0.25 M) was added, and the time taken for clot formation was recorded. The values represent the mean of observations of four experiments

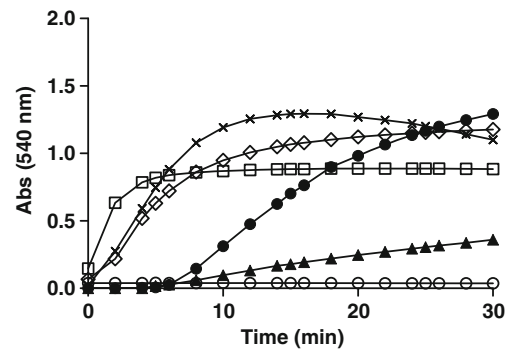


precipitation rings were formed by LP and its subfractions  $LP_{PII}$  and  $LP_{PIII}$ , and the ring size increased with the increase in incubation time.  $LP_{PI}$  was, however, ineffective in this regard (Fig. 4). The thrombin-like activity of LP and its subfractions were also substantiated by the spectrophotometric analysis. Like thrombin and papain, both LP and its subfractions  $LP_{PII}$  and  $LP_{PIII}$  produced an increase in absorbance of fibrinogen solution due to fibrin formation (Fig. 5). The formation of fibrin from fibrinogen by the action of LP and its subfractions resulted due to hydrolysis of  $A\alpha$ ,  $B\beta$ , and  $\gamma$  chains of fibrinogen, as also observed for papain. The hydrolysis of different units occurred in the order of  $A\alpha > B\beta > \gamma$ . The hydrolysis of  $A\alpha$  and  $B\beta$  chains resulted in a concentration-dependent increase in the intensity of hydrolyzed products, i.e., small molecular mass proteins. The  $\gamma$ -chain was resistant to hydrolysis at lower concentrations of proteins tested; however, it was hydrolyzed at higher protein concentrations (Fig. 6).

Proteases known to hydrolyze the  $\gamma$  subunit of fibrinogen are well known to hydrolyze fibrin clot. In view of this, the LP and its subfractions were tested for their plasma clot hydrolyzing activity by SDS-PAGE (Fig. 7). The electrophoresis of washed clot under reducing conditions produced protein bands like  $\alpha$ -polymer,  $\gamma$ - $\gamma$  dimer, and  $\alpha$  and  $\beta$  chains. Like papain, LP hydrolyzed the  $\alpha$ -polymer and  $\gamma$ -dimer, while  $LP_{PII}$  and  $LP_{PIII}$  completely hydrolyzed the  $\alpha$ -polymer and partially hydrolyzed  $\gamma$ -dimer. Unlike papain, the  $\alpha$  and  $\beta$  chains were resistant to the action of LP and its subfractions.  $LP_{PI}$  did not hydrolyze any of these proteins, and all the four bands were intact at all the concentrations.

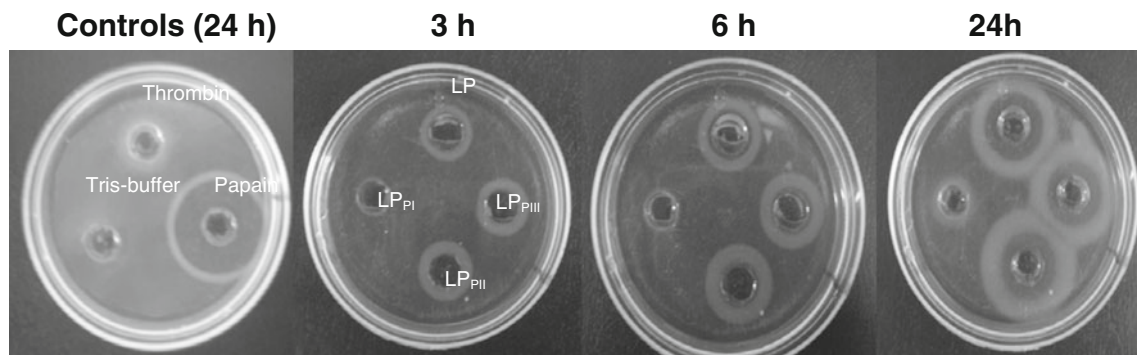
## Discussion

Sepsis, a state of systemic bacterial infection is associated with significant morbidity and mortality. Many of the ill



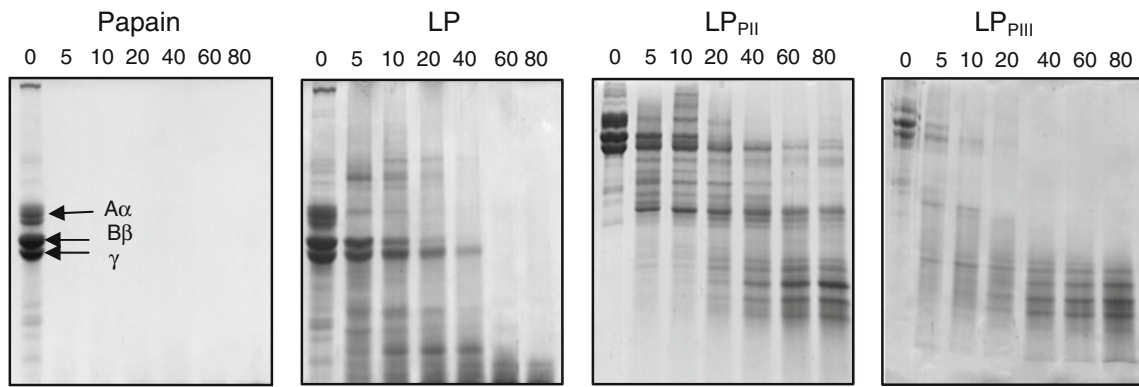
**Fig. 5** Spectrophotometric analysis of fibrin clot formation by LP (black circle) and its subfractions  $LP_{PI}$  (white circle),  $LP_{PII}$  (white square), and  $LP_{PIII}$  (diamond). Samples (50  $\mu$ l, 0.1 mg/ml) were added to 0.5 ml of 0.5% fibrinogen, and appearance of turbidity due to fibrin clot formation was recorded by measuring absorbance at 540 nm. Papain (multiplication sign, 5  $\mu$ g) and thrombin (black triangle, 0.1 U) were used as control. Values given are the mean of observations of four experiments

effects of this condition are attributed to enhanced host inflammatory response that is associated with coagulation abnormalities and thrombocytopenia (Mavrommatis et al. 2000). In a recent study, the proteins derived from the latex of the plant *C. procera* have been shown to afford protection in septic mice with regard to the inflammatory response and survival (Lima-Filho et al. 2010). The present study was carried out to evaluate the effect of these proteins on plasma coagulation and platelet count in the septic mice and to study the thrombin- and plasmin-like properties of the protein subfractions. In this study, intraperitoneal administration of *Salmonella* in mice produced a marked reduction in blood clotting time that could be attributed to the development of systemic inflammatory response following elevation of inflammatory cytokines like TNF- $\alpha$ , IL-1, IL-6, and complement activation (Pierrakos and Vincent 2010). Both inflammation and coagulation have been reported to be interrelated, and inflammatory mediators like TNF- $\alpha$  and IL-1 have been shown to promote coagulation (Faust et al.



**Fig. 4** Fibrinogen-agarose plate assay in determining thrombin-like activity of LP and its subfractions ( $LP_{PI}$ ,  $LP_{PII}$ , and  $LP_{PIII}$ ). The samples (50  $\mu$ g of protein) were prepared in 50  $\mu$ l of Tris buffer and poured into wells made on fibrinogen-agarose plate. Appearance of

turbid ring around the well indicates fibrinogen polymerization that was measured after 3, 6, and 24 h of incubation at 25°C. Thrombin (0.2 units) and papain (5  $\mu$ g) were used as positive control

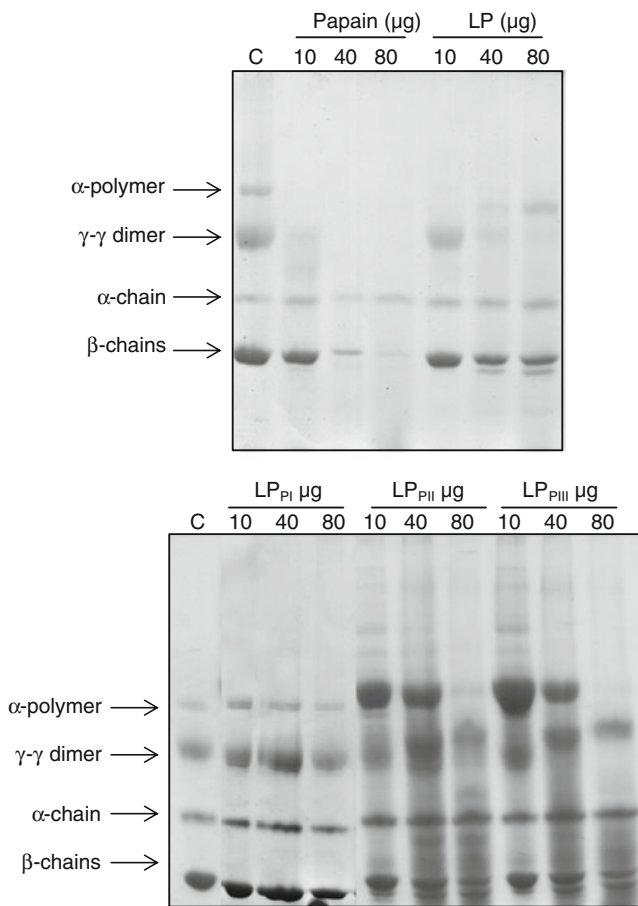


**Fig. 6** Fibrinogenolytic activity of LP and its subfractions LP<sub>PII</sub> and LP<sub>PIII</sub>. Fibrinogen (200 μg) was incubated with LP and its subfractions, ranging from 5 to 80 μg, for 30 min at 37°C in a reaction volume of 200 μl in Tris buffer, pH 7.4 with 3 mM DTT. Control is

nonhydrolyzed fibrinogen where no proteolytic enzyme has been added. Papain was used as positive control. The mixture was subjected to SDS-PAGE (7.5%) under reducing conditions

2001). Besides the release of inflammatory mediators, the septic condition is also associated with activation of

coagulation cascade, neutrophils, proteases, T cells, release of oxygen free radicals, aggregation of platelets, and endothelial damage (Anas et al. 2010). Low platelet count in septic mice as observed in the present study serves as a predictor of severity of disease that may lead to mortality (Baughman et al. 1993; Stephan et al. 1999). Treatment of septic mice with LP resulted in increase in clotting time and platelet count as compared to septic control. These effects could be attributed to the potent anti-inflammatory property of LP as reported earlier (Alencar et al. 2004). It is interesting to note that in the absence of an inflammatory response, the LP produced a procoagulant effect in the normal mice without affecting the platelet count. Also, the plasma protein profile of normal, septic, and LP-treated mice as revealed by SDS-PAGE did not show any difference.



**Fig. 7** Plasma clot hydrolyzing activity of LP and its subfractions LP<sub>PI</sub>, LP<sub>PII</sub>, and LP<sub>PIII</sub> at different protein concentrations. Human plasma clot formation was induced by adding 100 mM CaCl<sub>2</sub>, and the clot was further incubated with 10–80 μg of samples in Tris buffer, pH 7.4 for 60 min at 37°C. Papain was used as a positive control. The hydrolyzed products were analyzed by SDS-PAGE (7.5%). The inferior image is spliced of two distinct gels (LP<sub>PI</sub> + LP<sub>PII</sub>/LP<sub>PIII</sub>) that were performed concomitantly

Latex has been reported to produce a wide variety of pharmacological effects that have been attributed to the presence of hydrolytic enzymes like proteases in it. Interestingly, it has been used in the traditional system to stop bleeding and to promote wound healing. Both of these effects involve proteolytic events, namely, coagulation and fibrinolysis. Like papain, the proteolytic effect of LP on azocasein was dose-dependent and was associated with its subfractions LP<sub>PII</sub> and LP<sub>PIII</sub>. Both LP<sub>PII</sub> and LP<sub>PIII</sub> do not only produced a concentration-dependent decrease in clotting time but they also exhibited thrombin-like effect as revealed by fibrinogen assay and spectrophotometric analysis. The cysteine protease activity has earlier been reported in the heat stable protein fraction of the latex and the subfractions LP<sub>PII</sub> and LP<sub>PIII</sub> (Oliveira et al. 2007; Ramos et al. 2010). Like other members of Asclepiadaceae, the latex of *C. procera* exhibits thrombin-like activity due to the presence of cysteine protease activity in it (Freitas et al. 2007). The enzymes exhibiting thrombin-like property hydrolyze the Arg16–Gly and Arg14–Gly bonds in Aα and Bβ subunits of fibrinogen and release fibrinopeptide A and B, respectively, and results in the formation of insoluble fibrin

(Shivprasad et al. 2010). The thrombin-like property of proteins present in the latex subfractions LP<sub>PII</sub> and LP<sub>PIII</sub> could be responsible for the procoagulant effect of LP in normal mice as observed in the present study. However, APTT assays revealed that the proteolytic fractions accelerated plasma coagulation in a dose-dependent manner, while PT assays did not show changes in the time course of plasma coagulation when it was preincubated with the latex proteolytic fractions. It is therefore probable that in addition to the well-characterized thrombin-like activity, latex proteolytic fractions stimulate the earlier steps of the coagulation cascade including factors XII, XI, and IX, which specifically participate of the intrinsic pathway. It is not possible at this stage to determine the specific role of latex proteins in this process. However, the reduced time of plasma coagulation observed in APTT assays suggested the involvement of proteolytic enzymes since all the proteolytic fractions (LP, LP<sub>PII</sub>, and LP<sub>PIII</sub>) were active while LP<sub>PI</sub>, which is free of proteolytic activity, was the unique LP fraction lacking activity.

Inflammatory response associated with sepsis is well known to promote coagulation, and under these conditions, owing to the anti-inflammatory property of LP present in its subfraction LP<sub>PI</sub>, it produced a marked increase in the coagulation time as compared to septic control (Ramos et al. 2009).

Apart from its clot-forming property, the latex exhibiting protease activity has been reported to possess clot-dissolving property (Shivprasad et al. 2009; Rajesh et al. 2005). In our study, such a property was present in LP and its subfractions LP<sub>PII</sub> and LP<sub>PIII</sub>, while LP<sub>PI</sub> did not hydrolyze the clot. Demonstration of thrombin- and plasmin-like properties in LP, LP<sub>PII</sub>, and LP<sub>PIII</sub> provides scientific validation of the traditional use of latex of *C. procera* to stop bleeding and to promote wound healing as also suggested by Shivprasad et al. (2009) and Rajesh et al. (2005).

Thus, our study shows that protein fraction derived from latex of *C. procera* maintains coagulation homeostasis in septic mice. It remains unclear if this effect is associated to the anti-inflammatory property of LP and its subfraction LP<sub>PI</sub>. The subfractions LP<sub>PII</sub> and LP<sub>PIII</sub> possess proteolytic activity and exhibit thrombin- and plasmin-like effects and thus have a therapeutic potential in conditions associated with coagulation abnormalities.

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