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Involvement of NO in the inhibitory effect of *Calotropis procera* latex protein fractions on leukocyte rolling, adhesion and infiltration in rat peritonitis model

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ABSTRACT

Aim of the study: The latex of *Calotropis procera* has been used in the traditional medicinal system for the treatment of leprosy, ulcers, tumors, piles and diseases of liver, spleen, abdomen and toothache. It comprises of a non-dialyzable protein fraction (LP) that exhibits anti-inflammatory properties and a dialyzable fraction (DF) exhibiting pro-inflammatory properties. The present study was carried out to evaluate the effect of LP sub-fractions on neutrophil functions and nociception in rodent models and to elucidate the mediatory role of nitric oxide (NO).

Material and methods: The LP was subjected to ion exchange chromatography and the effect of its three sub-fractions (LP_{PI}, LP_{PII} and LP_{PIII}) thus obtained was evaluated on leukocyte functions in the rat peritonitis model and on nociception in the mouse model.

Results: LP sub-fractions exhibit distinct protein profile and produce a significant decrease in the carrageenan and DF induced neutrophil influx and exhibit anti-nociceptive property. The LP and its sub-fractions produced a marked reduction in the number of rolling and adherent leukocytes in the mesenteric microvasculature as revealed by intravital microscopy. The anti-inflammatory effect of LP_{PI}, the most potent anti-inflammatory fraction of LP, was accompanied by an increase in the serum levels of NO. Further, our study shows that NO is also involved in the inhibitory effect of LP_{PI} on neutrophil influx.

Conclusions: Our study shows that LP fraction of *Calotropis procera* comprises of three distinct sets of proteins exhibiting anti-inflammatory and anti-nociceptive properties of which LP_{PI} was most potent in inhibiting neutrophil functions and its effects are mediated through NO production.

1. Introduction

Calotropis procera Ait. R.Br. (Apocynaceae) is a tropical shrub found worldwide. The plant is well known for its poisonous properties that have been attributed to the presence of toxic substances in its latex. Nevertheless, it has been used in the traditional medicinal system for the treatment of leprosy, ulcers, tumors, piles and diseases of liver, spleen and abdomen (Kirtikar and Basu, 1935). Different parts of the plant have been shown to possess various biological activities namely, proteolytic, antimicrobial, larvicidal, anthelmintic, and insect repellant (Jain et al., 1996; Al-Qarawi et al., 2001; Dubey and Jagannadham, 2003; Singhi et al., 2004; Ramos et al., 2006). Latex of the plant that occurs in abundance in its aerial parts has been reported to exhibit medicinal properties like anti-diarrhoeal, anticancer, hepatoprotective, antiarthritic, antioxidant and antidiabetic (Kumar et al., 2001; Roy et al., 2005; Choedon et al., 2006; Kumar and Roy, 2007; Oliveira et al., 2007; Padhy et al., 2007). Interestingly, the latex produces both pro-inflammatory and anti-inflammatory properties in various experimental models depending upon the route of administration or fractions used (Singh et al., 2000; Sangraula et al., 2001; Alencar et al., 2004). The anti-inflammatory activity is present both in the aqueous and organic extract of its dried latex and in the non-dialyzable protein fraction of the latex while its dialyzable fraction is pro-inflammatory (Arya and Kumar, 2005; Alencar et al., 2006). The anti-inflammatory extract/fractions of the latex have also been

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shown to exhibit anti-nociceptive property (Dewan et al., 2000; Soares et al., 2005). Recently, the protein fraction of latex has been fractionated, characterized and demonstrated to possess various enzymatic properties (Freitas et al., 2007).

Inflammation is the response of the tissue to injury or insult and is associated with edema formation, fluid exudation and cellular influx. Neutrophils are the first and the most abundant leukocytes to be delivered to the site of inflammation after they are primed and activated by various inflammatory mediators (Malech and Gallin, 1987; Munder and Colditz, 1993). Both rolling and adhesion of neutrophils to endothelial cells are important components of the adhesion cascade that regulates neutrophil delivery (Mantovani et al., 1997). The adhesive interactions between neutrophils and endothelium are known to be modulated by nitric oxide (NO) (Kubes et al., 1991). The present study was carried out to evaluate the effect of anti-inflammatory latex protein sub-fractions on neutrophil function and nociception in rodent models and to elucidate the mediatory role of NO.

2. Materials and methods

2.1. Latex protein isolation

Latex was collected from the plant, *Calotropis procera* growing in the vicinity of Fortaleza, Brazil. The plant was identified by a taxonomist and a voucher specimen (No. 32663) was deposited at Prisco Bezerra Herbarium of the Universidade Federal do Ceará, Brazil. The latex was collected from aerial parts of the plant in distilled water to give a dilution of 1:2 (v/v). It was centrifuged at 5000 × *g* for 10 min at 10 °C and the rubber rich pellet was discarded. The supernatant was dialyzed against distilled water using a membrane with cut off of 8000 Da. Following 1 h of dialysis, the dialyzable fraction (DF) was collected and lyophilized. The non-dialyzable material obtained after exhaustive dialysis was again centrifuged and the supernatant thus obtained was lyophilized (LP) and used for further experiments.

2.2. Treatment of LP with pronase

LP was digested with pronase (*Streptomyces griseus*, SIGMA P-5147) at 37 °C in 100 mM phosphate buffer saline, pH 7.4. To 1 mg of LP, 0.098 U of enzyme were added three times i.e., at 0, 12 and 18 h and the reaction was terminated at 24 h by inactivating the enzyme at 60 °C for 10 min. The samples were lyophilized ($LP_{pronase}$) and the proteolysis was checked by electrophoresis.

2.3. Ion exchange chromatography of LP

LP was subjected to ion exchange chromatography on 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 200 and 300 mM sodium chloride. Fractions (4 ml) were collected at a flow rate of 1 ml/min and the absorbance was read at 280 nm. Three distinct protein peaks PI, PII and PIII (LP_{PI}, LP_{PII} and LP_{PIII}) were obtained and the fractions corresponding to these peaks were dialyzed against distilled water and lyophilized. These fractions were analyzed by SDS-PAGE and tested for biological activity.

2.4. SDS-PAGE of latex proteins

LP, $LP_{pronase}$, LP_{PI}, LP_{PII} and LP_{PIII} were reconstituted in 0.0625 M Tris buffer, pH 6.8 containing 2% SDS (sodium dodecyl sulphate) and subjected to electrophoresis on 12.5% polyacrylamide gel (PAGE) in the presence of SDS at 25 °C. Gels were stained with 0.1% Coomassie brilliant blue dye (R-350) in water:acetic acid:methanol (8:1:3.5) followed by de-staining with water:acetic acid:methanol solution.

2.5. Animals

The experiments were carried out on male Swiss mice (*Mus musculus*, 20–30 g) and male Wistar rats (*Rattus norvegicus*, 180–200 g) that were obtained from the Animal Breeding Facility of Universidade Federal do Ceará. The animals were maintained under standard conditions with a 12 h light-dark cycle at 27 °C and had free access to water and food. All experimental procedures were carried out in accordance with the guidelines of Institutional Animal Ethics Committee following approval.

2.6. Peritonitis model

Peritonitis was induced in rats by the intraperitoneal administration of 1% solution of carrageenan (500 μg/rat) or DF of latex (5 mg/rat). The rats were sacrificed 4 h later and the peritoneal cavity was washed with 10 ml sterile saline containing 5 IU/ml of heparin. The leukocyte count was determined in the fluid recovered from the peritoneal cavity (Souza and Ferreira, 1985).

The latex protein fractions (LP, $LP_{pronase}$, $LP_{PI–PIII}$) were administered intravenously (i.v.) at a dose of 10 mg/kg in 0.1 ml volume, 30 min before injecting carrageenan and their effect was evaluated on the leukocyte count. The comparison was made with the control group that received sterile saline intravenously. Parameters like rolling and adherent leukocytes and serum levels of nitric oxide were also measured in this model. To elucidate the role of NO in the anti-inflammatory action of LP_{PI}, animals were pre-treated subcutaneously with non-specific nitric oxide synthase (NOS) inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME, 50 mg/kg) or with a selective inhibitor of inducible NOS (iNOS) aminoguanidine (AG, 50 mg/kg) 15 min before administering LP_{PI} (10 mg/kg given i.v.). Thirty minutes later peritonitis was induced in these rats as described above and the leukocytes were counted.

2.7. Real-time measurement of leukocyte rolling and adhesion

The real-time measurement of leukocyte rolling and adhesion was carried out in mice by intravital microscopy (Baez, 1969; Rhodin, 1986; Fortes et al., 1991). Inflammatory response was induced in mice by an intraperitoneal injection of carrageenan (500 μg/mice). Two hours later the mice were anaesthetized and the mesenteric tissue was exteriorized for intravital microscopic examination *in situ*. The mice were kept in a thermostatically controlled environment at 37 °C and the preparation was kept moist and warm by irrigating with warm (37 °C) Ringer Locke's solution, pH 7.2–7.4 containing 1% gelatin. The images were viewed through 40× objective and projected by a video camera onto a monitor and recorded. Third order venuoles corresponding to post-capillary venuoles with a diameter of 10–18 μm were selected for the study with the help of an image splitter. Both rolling and adherent leukocytes were counted in a length spanning 10 μm in a 10 min interval. The cells were counted in five different fields of each recorded image.

2.8. Measurement of serum NO concentration

Serum levels of nitric oxide (NO_3/NO_2) were measured according to Feng et al. (2001). Briefly, 100 μl Griess reagent (1% sulfanilamide and 0.1% naphthalene diamine dihydrochloride in 1% orthophosphoric acid) was added to 100 μl serum, incubated at room temperature (25 °C) for 10 min and the optical density was read at 540 nm. Sodium nitrite was used as a standard for determining the serum concentrations of nitrite.