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In vitro tissue culture of the medicinal shrub *Calotropis procera* to produce pharmacologically active proteins from plant latex

Fabiano M. Teixeira[a], Márcio V. Ramos[a,*], Arlete A. Soares[b], Raquel S.B. Oliveira[a], Luiz Carlos P. Almeida-Filho[a], Jefferson S. Oliveira[d], José D.B. Marinho-Filho[c], Cristina Paiva S. Carvalho[a,*]

[a] *Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Campus do Pici, Caixa Postal 6033, Fortaleza, Ceará, Brazil*
[b] *Departamento de Biologia, Universidade Federal do Ceará, Campus do Pici, Bloco 906, Fortaleza, Ceará, Brazil*
[c] *Departamento de Fisiologia e Farmacologia, Faculdade de Medicina, Universidade Federal do Ceará, Caixa Postal 3157, 60430-270 Fortaleza, Ceará, Brazil*
[d] *Departamento de Ciências da Saúde, Universidade Federal do Piauí, Parnaíba, Piauí, Brazil*

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ABSTRACT

The latex of *Calotropis procera* is a rich source of proteins that have anti-inflammatory, anti-nociceptive and selective cytotoxic and anti-tumorigenic properties. In this study, two distinct protocols were developed to culture *C. procera in vitro* in order to obtain active molecules. Soluble proteins of both callus and root cultures were extracted and tested for various activities found in the latex. Anti-inflammatory and anti-nociceptive proteins were present in callus and root extracts; however, these proteins did not possess cytotoxic or anti-tumorigenic activity. Larvicidal proteins were present, but they were not related to others that have been reported previously in latex. This study confirms that tissue culture of *C. procera* is able to produce therapeutic-grade proteins that have the potential to relieve inflammation and pain associated with inflammatory disorders.

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1. Introduction

Plants are the basis of sophisticated traditional medicine approaches that have been used for thousands of years and continue to provide mankind with new remedies. Although some of the therapeutic properties attributed to plants have been proven to be erroneous, medicinal plant therapy is still supported by empirical discoveries [1]. For example, the non-cultivated shrub *Calotropis procera* is a reliable source of useful, albeit toxic, molecules [2,3]. This plant is geographically distributed throughout southern Asia, northern Africa and northeast South America. Despite the known toxicological properties of *C. procera*, which are primarily attributed to cardenolides [4], the root, bark, leaves and its milky latex are used for medicinal purpose [2] in the poorest regions of India and Africa, as well as Brazil to a lesser extent. Many of the curative properties attributed to the plant have been scientifically investigated [5,6], and latex-based extracts have been shown to modulate different inflammatory disorders [7–9]. In addition, latex-derived extracts induce selective cytotoxicity and anti-tumor activity [10,11]. The latex of *C. procera* has been shown to protect against gastric ulcers in rats, and latex extracts protected against inflammation and oxidative stress in arthritic rats [5,6]. In addition, UNBS1450, a cardiotonic steroid from *C. procera*, exerts anti-cancer activity. UNBS1450 01, a compound derivative, is a potent sodium pump inhibitor, inducing anti-proliferative activity and cell death [12].

The latex of *C. procera* is composed of different active compounds, including glycosides, tannins, proteins and isoprene (rubber), among others [12,13]. Despite the elucidation of numerous pharmacological properties of latex, information concerning the identification of active molecules is lacking. However, studies with the soluble protein fraction of latex have provided some insight into the possible identities of its active components. Anti-inflammation and anti-nociception activities derived from the latex protein fraction have been reported [14,15]. Recently, this fraction has been shown to confer selective cytotoxic activity and the induction of apoptosis in cancer cells lines *in vitro* [11]. In light of these properties of *C. procera* molecules, it would be not only of botanical interest, but also of biotechnological relevance to establish an *in vitro* protocol to produce these molecules. Therefore, an alternative protocol to obtain active molecules implicated in such important activities would be useful as an alternative to save natural resource and avoid extensive collect of latex of native specimens. In the present study, callus and root tissues of *C. procera* were

* Corresponding authors. Tel.: +55 85 3366 9403; fax: +55 85 3366 9789.
E-mail addresses: vramos@ufc.br (M.V. Ramos), cpaiva@ufc.br (C.P.S. Carvalho).

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prepared and the soluble proteins were characterized in terms of pharmacological properties related to latex.

2. Materials and methods

2.1. Plant material

Healthy *C. procera* plants growing around the beaches of Fortaleza (northeast of Brazil) were used as a seed source. A voucher of seeds was deposited at the Prisco Bezerra herbarium of the Universidade Federal do Ceará under code N. 32663 and certified by a local taxonomist. Fruits were kept in glass flasks (25 ± 3 °C) until they had ripened to open and released their seeds. Non-damaged seeds were selected and cleaned of silk fiber prior to use.

2.2. Seed germination and explant production

Healthy seeds of *C. procera* were treated with 70% (v/v) ethanol for 2 min before surface sterilization by dipping into 33% (v/v) sodium hypochlorite solution (v/v) for 15 min, followed by five rinses in sterile distilled water. Thereafter, five seeds were germinated in each glass flask containing 30 ml of 1/2 MS medium salts [16], supplemented with 100 mg/L myo-inositol, and 3% (w/v) sucrose (pH 5.8) that was solidified with 0.7% (w/v) agar (Sigma Chemical Co.).

Cultures were maintained at a photoperiod of 12 h and illuminated with cool white fluorescent lamps at a light intensity of 10–15 μmol/m^2/s. The culture room temperature was kept at 28 ± 2 °C. After 30 days under these culture conditions, plantlets reached ca. 5 cm in height and were used as an explant source.

2.3. Callus induction and root differentiation

In vitro-grown plantlets (30 days after germination) were used as the source of hypocotyl and cotyledon explants. Hypocotyls were aseptically removed and cut longitudinally and then cut into slices approximately 10 mm in length. Callus induction medium consisted of complete MS basal formulation as prepared for seed germination added of 3 μM 1-naphthylacetic acid (NAA) and 4.6 μM kinetin (KIN).

Callus induction was based on protocol described by Suri and Ramawat [17]. Each flask with five explants was considered to be one replicate, and for each experiment six replicates were used. Calli were sub-cultured every 4–6 weeks and then collected, freeze-dried and stored at 25 °C until use.

For root induction, cotyledons, instead of hypocotyls, were cultivated under the same experimental conditions. Roots were also collected 30 days after induction and freeze-dried as described above.

2.4. Histology

Cultured samples of calli and roots of *C. procera* were taken and fixed with Karnovsky solution [18]. After fixation, samples were dehydrated through an increasing ethanol series and infiltrated with glycol methacrylate resin (Leica Historesin Embedding Kit). Transverse and longitudinal sections were cut (5.0 μm) using a microtome Leica 2065. Slices were stained with 0.12% toluidine blue for 10 min followed by treatment with 0.03% basic fuccine for 30 s [19]. All samples were observed with an Olympus microscope with special attention paid to the occurrence of laticifer formation.

2.5. Protein extraction and analysis

To obtain soluble proteins from the calli and roots, the freeze-dried materials were homogenized in 50 mM Tris–HCl, pH 7.5 containing 100 mM NaCl to a proportion of 1:5 (w/v). The mixtures were stirred for 2 h, and the homogenates were centrifuged at 10,000 × *g* for 30 min at 10 °C. Soluble fractions were recovered, dialyzed in distilled water, freeze-dried and stored in a vial until use. Both callus proteins (CP) and root proteins (RP) were examined by electrophoresis and assayed in each experiment using the appropriate solution.

2.6. Protein profile

The protein profiles of CP and RP were evaluated by two-dimensional electrophoresis. Soluble proteins were obtained as reported before [13]. Samples were first resolved in a pH gradient ranging to 3–10 by isoelectric focalization and thus separated by SDS-PAGE on a 12.5% (w/v) polyacrylamide gel according to Laemmli [20].

2.7. Animals

Wistar male rats (190–200 g) (anti-inflammation) and Swiss mice (20–30 g) (all other determinations) were obtained from the Central Animal House of the Universidade Federal do Ceará, Brazil. Animals were maintained in plastic cages, kept in a controlled 12/12 h light/dark cycle room at a temperature of 25 °C and had free access to food and water. Experimental handling of animals was performed according to the current guide for care and use of laboratory animals as approved by the Institutional Ethical Committee. Prior to sample administration or sacrifice, animals were given halothane anesthesia.

2.8. Cytotoxicity and anti-tumor activity

The *in vitro* cytotoxicity and *in vivo* anti-tumor activity of CP and RP were investigated as described above for the latex proteins. The cytotoxicity of CP and RP were tested against HL-60 (leukemia), MDA-MB-435 (melanoma), SF-295 (brain) and HCT-8 (colon) human cancer cell lines, all of which were obtained from the National Cancer Institute (Bethesda, MD, USA) [11]. Anti-tumor activity was investigated in mice transplanted with sarcoma 180 cells [21].

2.9. Anti-inflammatory assays

To determine the anti-inflammatory activities of CP and RP, carrageenan-induced peritonitis and paw edema assays were performed. For peritonitis, samples were dissolved in 1 ml of sterile saline (1, 5 and 25 mg/kg) and injected intravenously (i.v.) into male rats. An inflammatory stimulus (carrageenan 500 μg/cav.) was given intraperitoneally (i.p.) 30 min later. Four hours later, the animals were sacrificed, and the peritoneal cells were recovered by washing each peritoneal cavity with 10 ml of sterile saline containing 5 UI/ml heparin. Total and differential cell counts were performed. Data are shown as the mean number (±SEM) of cells per milliliter of peritoneal solution. In another set of experiments, EDTA was used instead of heparin, and the nitric oxide (NO) content was determined in the peritoneal fluid and serum following the protocol of Feng et al. [22] using Griess reagent (1% sulfanilamide and 0.1% naphthalene diamine dihydrochloride in 1% orthophosphoric acid).

Paw edema was performed in male rats by injecting carrageenan (500 μg/paw) into the right hind paw 30 min after an i.v. injection of CP or RP (1 mg/kg). Animals were sacrificed 4 h after receiving the inflammatory stimulus, and samples of paw tissue were collected. Paw tissue myeloperoxidase activity was determined according to Alencar et al. [23] and Bradley et al. [24].

2.10. Anti-nociceptive assays

The anti-nociceptive activities of CP and RP were investigated in mice using two protocols. Acetic acid-induced writhing was performed following the method of Koster et al. [25]. Mice (n = 10) were injected i.p. with 0.6% acetic acid (10 ml/kg), and, after 10 min, the number of constrictions was determined for 20 min. Samples of CP or RP (1, 5 or 25 mg/kg) dissolved in sterile saline (10 ml/kg) were given i.p. to mice 30 min before acetic acid administration. Control animals were given sterile saline or morphine (5 mg/kg, i.p.) as a positive control.

A formalin test was performed in male mice according to the method of Hunskaar et al. [26]. Formalin (1%) was administered (20 μl) to mice (n = 10) in the right hind paw, and the licking-time was measured over the interval of 0–5 min (neurogenic phase) and 20–25 min after administration (inflammatory phase). *C. procera* protein samples (CP and RP) at doses 1, 5 or 25 mg/kg (10 ml/kg) or morphine (5 mg/kg, i.p.) were injected 30 min before formalin administration. Control animals were injected with a similar volume of sterile saline (10 ml/kg). In two separate groups, animals were pretreated with naloxone 15 min before administration of CP or RP (50 mg/kg, i.p.) or morphine (5 mg/kg, i.p.), followed by formalin administration 30 min later.

2.11. Larvicidal activity of callus and root proteins

To investigate the larvicidal potential of CP and RP, the third instars of *Aedes aegypti* were produced as reported previously [27]. The larvicidal activity of callus and root proteins against *A. aegypti* was determined as described by Ramos et al. [8]. Correlation of the larvicidal and proteolytic activities was performed by determining proteolytic activity of CP and RP according to Freitas et al. [13]. Experiments were performed in triplicate.

2.12. Statistical analysis

Results are shown as the mean ± SEM and were considered statistically significant when $p < 0.05$ compared to the control sample as analyzed by ANOVA followed by Bonferroniïs post hoc test or ANOVA followed by Student–Neuman–Keuls post hoc test. Larvicidal effects were assessed by analysis of variance followed by Tukey's post hoc test. Statistics were assessed by using the GraphPad program (GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

In vitro plant tissue culture is a common strategy to develop biological sources of relevant medicinal molecules that are of therapeutic value. *C. procera*, a medicinal plant, is currently recognized as a source of such molecules. Even though the pharmacological characterization of root, bark or flower extracts has been performed, the

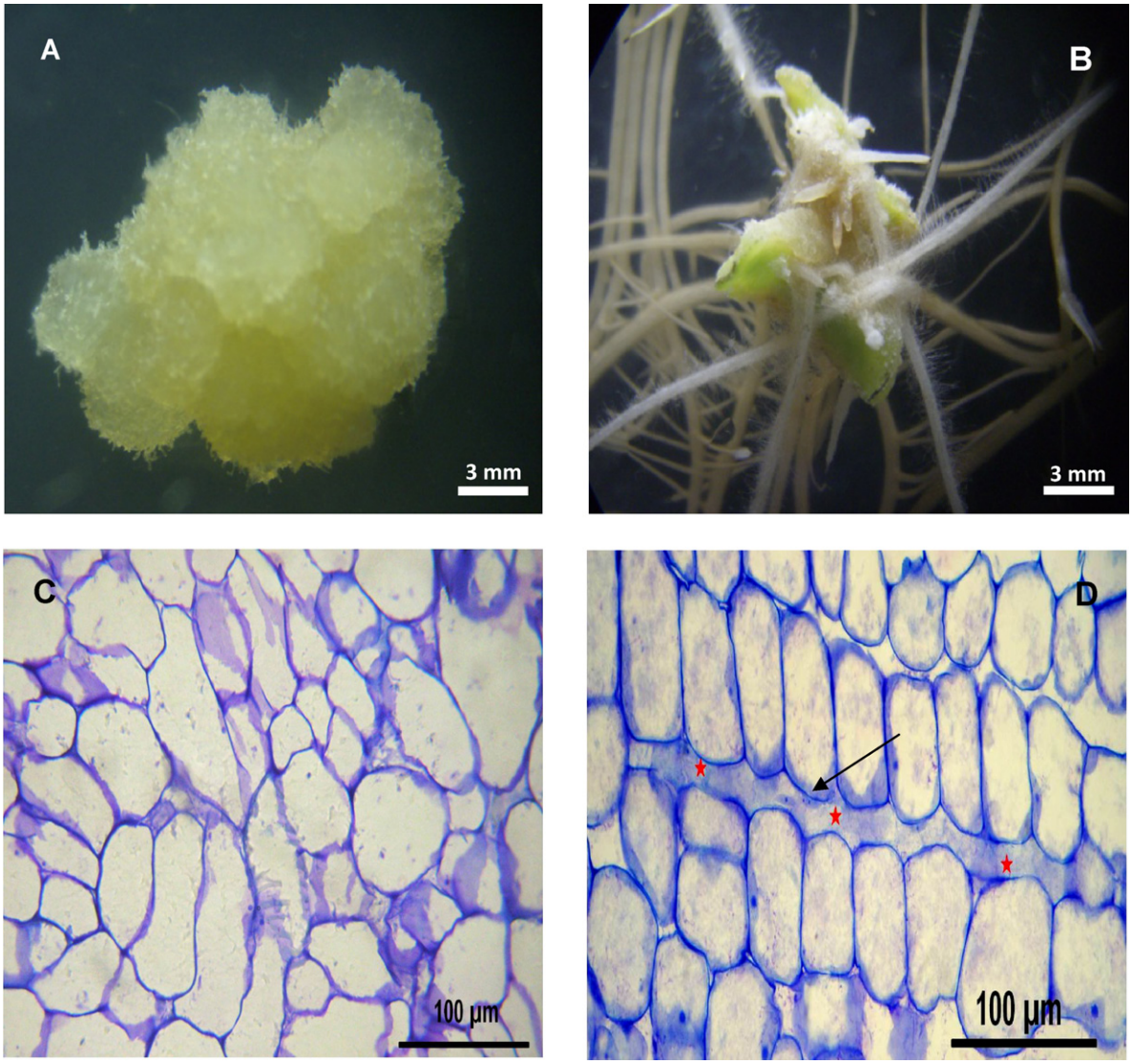

Fig. 1. General view of a callus obtained from hypocotyl (A) and adventitious roots obtained from cotyledonary explants (B) of *C. procera* cultivated in MS basal medium supplemented with 3 µM 1-naphthylacetic acid (NAA) and 4.6 µM kinetin (KIN). Histological views of longitudinal 5 µm sections of callus (C) and root (D) stained with Toluidine Blue (Images 640×). Callus was observed as a non-organized tissue without laticifer formation, whereas the roots exhibited organized and differentiated tissues, including laticifer formation. The laticifer tube is denoted by small stars. Arrow shows three nucleus, a typical characteristic of laticifer cells.

latex is described as the primary source of anti-arthritic, immune-stimulant and anti-tumor molecules. These activities have been reported extensively and characterized in the latex protein fraction [9,21].

To obtain a suitable source of these active molecules while avoiding predatory use of native plants, we established two rapid and inexpensive protocols to obtain *in vitro* cultivated callus and root tissues of *C. procera* for pharmacological studies. These protocols are based on experiments described by Suri and Ramawat [17]. Hypocotyls and cotyledonary explants, when cultivated in MS basal medium supplemented with 3.0 µM NAA and 4.6 µM KIN, produced calli and roots, respectively (Fig. 1A and B). We used the appropriate concentration of auxin and cytokinin as determined by Suri and Ramawat [17] as the optimal concentration to induce laticifer differentiation. These authors reported that they obtained calli, but not roots, from cotyledonary explants cultivated in the same media formulation, and they detected laticifer differentiation in this tissue. In this work, however, calli originating from hypocotyls did not differentiate laticifer (Fig. 1C). These structures were detected in the roots of differentiated cotyledonary explants (Fig. 1D).

Suri and Ramawat [17] showed that laticifer differentiation is a cytokinin-dependent process, and that kinetin was more effective for differentiation of this structure than other cytokinins. The authors reported the effects of the type, the concentration of auxin and the age of the cultures. Even NAA induced the differentiation of laticifer. The successive subcultures in media containing the natural auxin, Indol-3-ylacetic acid (IAA), were more effective for production of callus with differentiated structures. It was observed that the number of subculture cycles was proportional to the laticifer differentiation capacity. Suri and Ramawat [17] observed laticifer as early as 2 weeks after of culture, but higher differentiation was observed after 12 cycles of subculture (each cycle was 4 weeks). In this work, the callus was collected after 4–6 weeks of culture, at which time no laticifer differentiation was observed. We then investigated the pharmacological activities reported in latex in tissues with or without laticifers.

Soluble proteins were extracted from the freeze-dried cultured tissues and examined by two-dimensional electrophoresis (Fig. 2). Proteins were separated approximately in the same pH range. It is possible however, to observe distinct protein profiles. Differences in proteomes of CP and RP are expected since protein populations

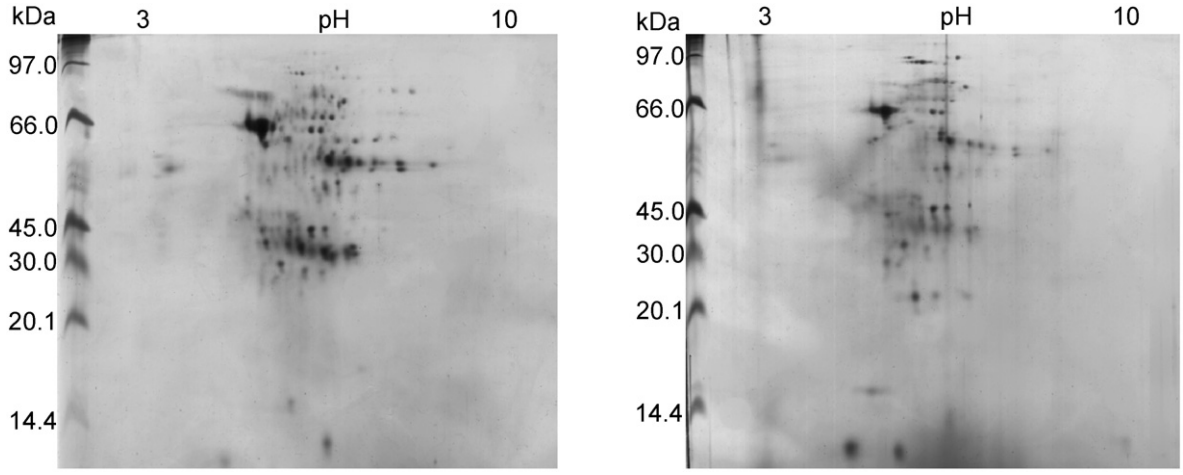

Fig. 2. Two-dimensional polyacrylamide gel electrophoresis (12.5%) of CP (left) and RP (right) separated in the pH ranges 3–10. Molecular weight markers (MW) were as follows: phosphorylase b (97.0 kDa); albumin (66.0 kDa); ovalbumin (45.0 kDa); carbonic anhydrase (30.0 kDa); trypsin inhibitor (20.1 kDa); and lactalbumin (14.4 kDa).

were obtained from distinct cells sources. It is therefore very limited to establish correspondences between both protein samples. Further analysis based on mass spectrometry will be necessary for the identification of CP and RP. Of note, CP and RP exhibited distinct protein profiles as compared to latex proteins (LP) previously reported [13]. LP exhibit highly basic proteins that were observed neither in CP or RP. It is likely identification of pharmacologically active proteins in these samples will require steps of purification.

Callus proteins (CP) and root proteins (RP) exhibited potent anti-inflammatory activity as indicated by the inhibition of neutrophil migration during carrageenan-induced peritonitis (Fig. 3). These data suggest that anti-inflammatory proteins expressed in the latex may be also found in calli and roots, despite the absence of laticifer tubes in the callus. Both samples were highly inhibitory at doses as low as 1 mg/kg. This corresponds to an effectiveness at least 5-fold greater than that observed for active proteins obtained from the latex under similar experimental conditions [14]. Anti-inflammation was confirmed when the activity of myeloperoxidase was measured in carrageenan-inflamed tissues (Fig. 4). CP and RP consistently inhibited neutrophil accumulation in paw edemas when animals were given 1 mg/kg of CP or RP. In previous studies of latex proteins, intravital microscopic analysis of mesenteric tissues revealed that inhibition of neutrophil influx by laticifer proteins reduced the adhesion and rolling of leukocytes. Furthermore, an increase in the serum nitric oxide (NO) level was observed [8]. As shown in Fig. 5, RP but not CP, induced serum NO production. Incremental NO production inhibited the expression of adhesion molecules, in turn, reducing cell migration [28,29]. These results are consistent with data using LP that suggests that proteins in the latex have distinct anti-inflammatory affects. Therefore, it is suggested that cultured calli and roots provide two distinct sources of anti-inflammatory proteins.

The anti-nociceptive activity of CP and RP was studied in two distinct models. Table 1 summarizes the anti-nociception measured in animals suffering from abdominal constrictions provoked by acetic acid treatment. CP consistently inhibited abdominal constriction at the minimum dose assayed, whereas RP was effective at higher doses. Our results demonstrate that CP was as active as the positive control (morphine). The doses that were active in this study were lower than those of latex proteins reported previously [15]. The effects of CP and RP were further tested in animals treated with formalin. The pain-relieving effect of CP and RP was studied during the neurogenic and inflammatory phases. Although statistically significant, the effect of CP during the neurogenic phase was less than that of the experimental control. CP only reduced the effects of formalin during the inflammatory phase at the higher doses (Fig. 6). In contrast, RP did not alter behavior during the neurogenic phase, but the licking time was effectively reduced during the inflammatory phase (Fig. 6). These results confirm that, as observed in anti-inflammatory trials, anti-nocicepitve proteins were produced in the cultured cells and roots of *C. procera*.

Of note, latex proteins from *C. procera* induced selective cytotoxicity in malignant cells as determined by *in vitro* and *in vivo* protocols [11,21]. Surprisingly, both CP and RP did not exhibit

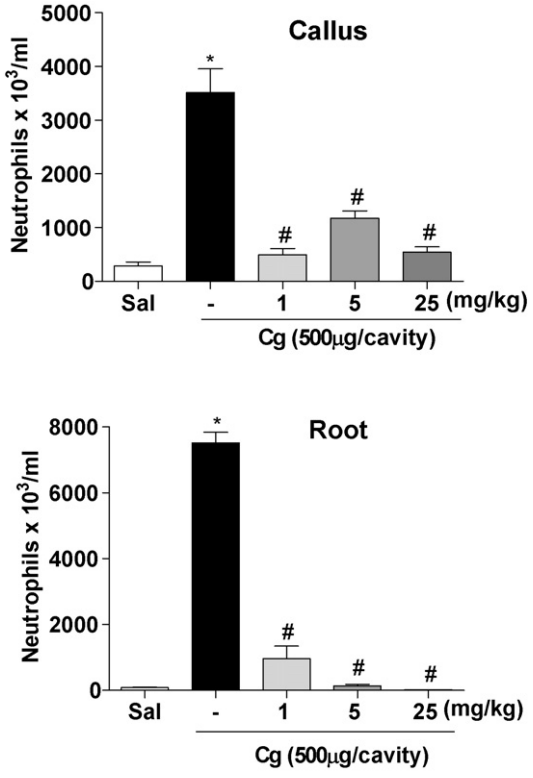

Fig. 3. Effects of callus and root tissue proteins on carrageenan-induced neutrophil migration in the peritonitis model. Rats were administered various doses of proteins extracted from *in vitro* culture of callus or roots from *C. procera* (1, 5 or 25 mg/kg; i.v.) 30 min before an i.p. injection of carrageenan. Neutrophils were counted in the peritoneal cavity 4 h later. Data represent the mean ± SEM ($n = 5$). $^{*}p < 0.05$ compared to saline, $^{\#}p < 0.05$ compared to carrageenan (ANOVA followed by Bonferroniïs post hoc test).

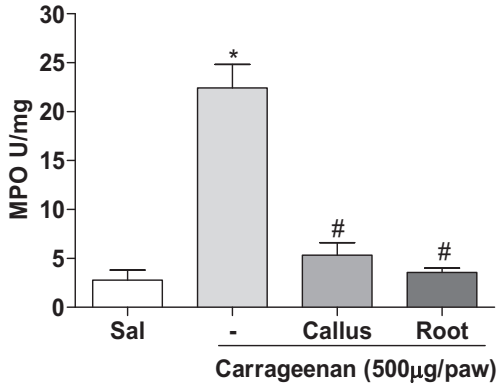

Fig. 4. The inhibitory effects of the CP and RP of *C. procera* on myeloperoxidase activity as measured in the paw edema tissue of rats given an intraplantar injection of carrageenan. The animals were treated 30 min before carrageenan injection with intravenous saline (Sal), CP or RP (1 mg/kg). Edema was determined as the amount of myeloperoxidase activity (MPO U/mg) 4 h after the inflammatory stimuli. Data represent the mean ± SEM ($n = 7$). $^{*}p < 0.05$ compared to Sal and $^{\#}p < 0.05$ compared to carrageenan (ANOVA followed by Bonferroni's post hoc test).

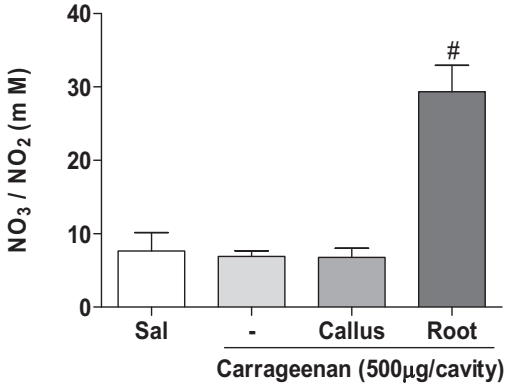

Fig. 5. Effects of laticifer proteins on the serum concentration of nitric oxide (NO_3/NO_2) in the peritonitis model. Carrageenan was injected into the peritoneal cavity of rats, and the serum concentration of NO was measured after 4 h. Callus or root (1 mg/kg) was administered 30 min before the inflammatory stimulus. Data represent the mean ± SEM ($n = 7$). $^{\#}p < 0.05$ compared to carrageenan (ANOVA followed by Bonferroni's post hoc test).

Table 1
Anti-nociceptive effects of callus and root tissue proteins of *C. procera* on acetic acid-induced writhing.

Experimental groups	Number of abdominal constrictions (20 min)	% Inhibition
Saline	10.9 ± 1.4	–
Morphine (5 mg/kg; s.c.)	2.2 ± 0.5*	79.8
Callus (mg/kg, i.v.)		
1	2.7 ± 0.9*	75.2
5	2.5 ± 0.9*	77.1
25	1.0 ± 0.4*	90.8
Root (mg/kg, i.v.)		
1	11.8 ± 1.8	15.7
5	5.2 ± 1.3*	62.8
25	4.6 ± 0.8*	67.1

Various doses were injected i.v. (1, 5 and 25 mg/kg) 30 min before injection of 0.6% acetic acid. Data represent the mean ± SEM ($n = 10$).
* $p < 0.05$ compared to saline (ANOVA followed by Student–Neuman–Keuls post hoc test).

an *in vitro* cytotoxic effect (Table 2) or *in vivo* suppression of tumor growth (Table 3). Despite the fact that the *in vivo* protective effects of laticifer proteins have been partially elucidated, very few studies have been done about the proteins involved in such activities. According to the cited studies [11,21] distinct proteins within the latex display *in vitro* cytotoxicity and *in vivo* anti-tumor activity. Since CP exhibit neither cytotoxicity nor anticancer activities, it is therefore suggested that laticifer proteins implicated in cytotoxicity and anti-cancer activities are not expressed in callus. However, this may not extend to the RP because root tissues were shown to form laticifer tubes (Fig. 1D). Lack of cytotoxicity and anticancer activity in RP may be due to a series of concerns including low concentration of cytotoxic and anti-cancer proteins; laticifers in cultured roots can be morphologically formed but still immature in biochemical and physiological terms and thus anti-inflammatory and ant-inocicceptive proteins were detected while cytotoxic and anti-cancer proteins not. Furthermore, CP and RP were not cytotoxic at 25 μg/ml, whereas laticifer proteins (LP) were active at doses below 2 μg/ml (Table 2). In addition, it should be noted that CP and RP produced anti-inflammatory and anti-nociceptive effects, suggesting that these molecules are distinct of those having anti-cancer activity. In this context, *in vitro*

Table 2
Cytotoxic activity of LP and proteins extracted from callus and root from *C. procera* on tumor cell lines.

Treatment	MDA-MB-435IC_{50} (μg/ml)	HL-60IC_{50} (μg/ml)	HCT-8IC_{50} (μg/ml)	SF-295IC_{50} (μg/ml)
LP	1.48 (0.98–1.90)	0.53 (0.34–0.66)	0.78 (0.72–0.90)	0.78 (0.72–0.89)
CP	>25*	>25*	>25*	>25*
RP	>25*	>25*	>25*	>25*

Data are presented as IC_{50} values and 95% confidence intervals from two independent experiments performed in duplicate using breast (MDA-MB-435), leukemia (HL-60), colon (HCT-8) and brain (SF295) cancer cells. Data represent the mean ± SEM.
* $p < 0.05$ compared to LP (ANOVA followed by Student–Neuman–Keuls post hoc test).

Table 3
The inhibitory activity of CP in mice transplanted with sarcoma 180 tumor cells.

Drug	Dose (mg/kg/day)	Liver (g/100 g body weight)	Spleen (g/100 g body weight)	Kidney (g/100 g body weight)	Tumor (g)	Inhibition (%)
Healthy mice						
0.9% Saline	–	4.59 ± 0.19	0.18 ± 0.03	1.46 ± 0.05	–	–
Mice transplanted with sarcoma 180 tumor						
Intraperitoneal route						
0.9% Saline	–	4.79 ± 0.15	0.72 ± 0.05	1.32 ± 0.08	4.26 ± 0.25	–
5-FU	25	4.77 ± 0.36	0.45 ± 0.02	1.20 ± 0.04	1.77 ± 0.30*	58.41
CP	50	4.05 ± 0.32	0.64 ± 0.05	1.24 ± 0.05	4.24 ± 0.38	0.61
CP	100	4.59 ± 0.28	0.67 ± 0.08	1.23 ± 0.04	3.80 ± 0.42	10.88

Data represent the mean ± SEM ($n = 10$).
* $p < 0.05$ compared to saline (ANOVA followed by Student–Neuman–Keuls post hoc test).

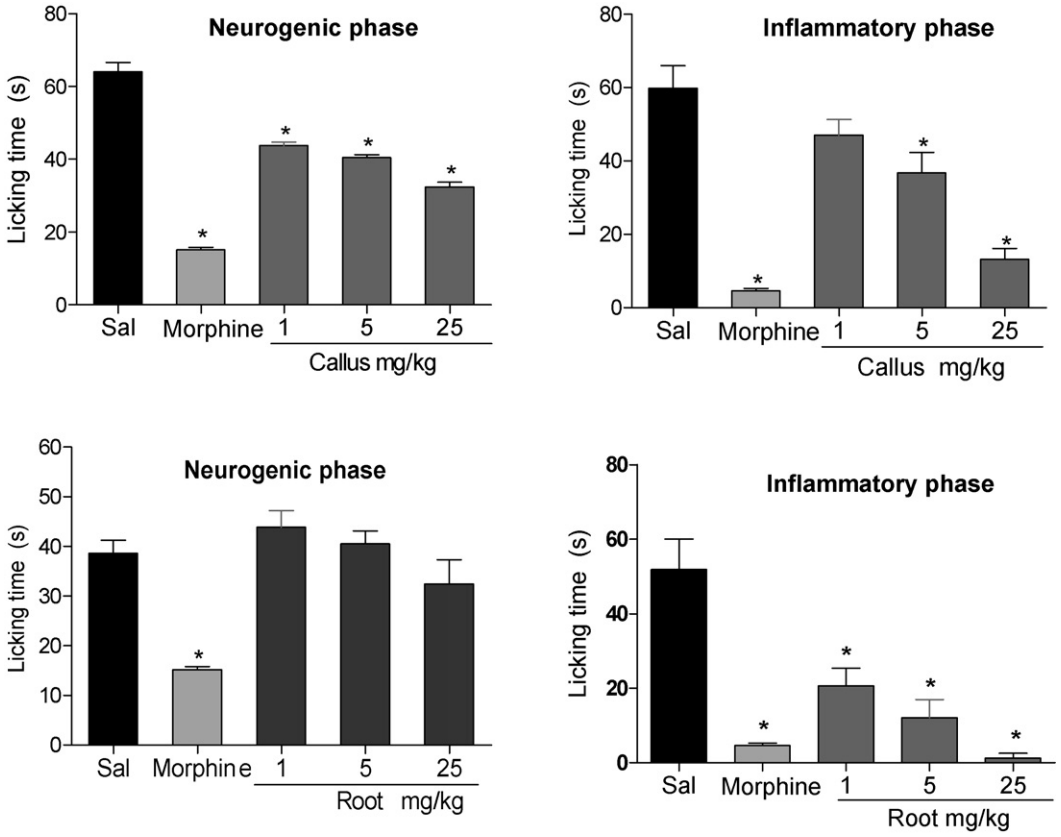

Fig. 6. Effects of root proteins on formalin-induced paw licking activity in mice (first phase, panel A; second phase, panel B). Morphine (5 mg/kg; s.c.) or root proteins (1, 5 and 25 mg/kg; i.v.) were injected 30 min before an intraplantar injection of 1.2% formalin. The licking time was recorded during the first 5 min (1st phase) and during the period of 20–25 min (2nd phase), A and B, respectively. Data represent the mean ± SEM ($n = 8$). *$p < 0.05$ compared to Sal (ANOVA followed by Student–Neuman–Keuls post hoc test).

cultures of *C. procera* represent a reliable protein source that will greatly contribute to the understanding of the pharmacological value of *C. procera* proteins.

The latex of *C. procera* exhibited cysteine-based proteolytic activity. This activity has been previously characterized and more recent studies suggest that this activity is, at least in part, the cause for larvicidal effects in laticifer proteins [13,27]. In our study, we examined the larvicidal effects of CP and RP following the same protocol. CP and RP were assayed after being dissolved in an experimental solution or treated with dithiothreitol (DTT), a known activator of cysteine proteinases. As seen in Table 4, CP and RP induced larvae death. However, the mortality rate diminished when the samples were pre-treated with DTT. This observation does not corroborate the results obtained with laticifer proteins, which, after DTT treatment, exhibited incremental proteolytic effects and mortality in the larvae [30]. The proteolytic activities of CP and RP were determined to be 0.36 μg/μl and 0.20 μg/μl, respectively. After DTT treatment, the activity were 0.58 μg/μl and 0.24 μg/μl.

Our study of *C. procera* extract activity revealed that calli and roots are suitable sources of pharmacologically active proteins. The anti-inflammatory and anti-nociceptive activity of CP and RP proteins were similar to those observed in latex protein fraction. However, purification and biochemical characterization of the active proteins in these cultures is necessary to confirm whether they are similar to those found in latex fluid. The lack of anti-cancer activity and differences in larvicidal activity of CP and RP compared to the latex protein samples confirm important differences between the protein profiles of calli, roots and latex.

Table 4
Larvicidal effects of CP and RP on the third instar of *A. aegypti*.

Samples	Mortality (%)	
	24 h	48 h
Ethanol (1%)	0	0
BSA (2 mg/ml)	0	0
DTT (0.25 mg/ml)	0	0
CP (2 mg/ml)	62.25 ± 6.2*	92.50 ± 7.5*
CP (2 mg/ml) + DTT (0.25 mg/ml)	12.50 ± 1.2	50.00 ± 9.1*
RP (2 mg/ml)	32.50 ± 3.2*	60.00 ± 4.0*
RP (2 mg/ml)+ DTT (0.25 mg/ml)	12.50 ± 1.2	37.50 ± 6.2*

Data represent the mean ± SEM ($n = 10$). Values within in the same column differ significantly (*$p < 0.05$) from the control (1% ethanol). Statistical significance was assessed by ANOVA followed by Tukey's post hoc test. Callus and root proteins were assayed with or without dithiothreitol (DTT). No larval death was documented after a 3-h observation. BSA: bovine serum albumin.

4. Conclusions

The pharmacological activity of the latex protein fraction of *C. procera* was investigated in two *in vitro* systems: calli and roots. The soluble protein fraction of these cells exhibited strong anti-inflammatory activity and also produced anti-nociceptive effects. However, the protein extracts failed to control the *in vitro* growth of malignant cells and to suppress tumors *in vivo*, which are two characteristics of latex proteins. The larvicidal activity of both protein extracts poorly correlated with the proteolytic activity. This is in contrast to the effects of latex. Our study provides insight into the potential biotechnological applications of latex molecules obtained from cultures from *C. procera*.

Cell culture protocols are still very useful tools for production and extraction of pharmacological relevant compounds [31]. This is the first report dealing with pharmacological activities of proteins obtained from cultured cells of *C. procera.* Similar to latex, callus and root proteins displaying pharmacological activities remain to be identified. Besides, results reported in this study support the culture of *C. procera* cells as a suitable source for purification of active proteins.

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