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Original Article

Chemical constituents of *Calotropis procera* latex and ultrastructural effects on *Haemonchus contortus*

Constituintes químicos do látex de *Calotropis procera* e efeitos ultraestruturais em *Haemonchus contortus*

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Abstract

This study aimed to evaluate the anthelmintic and ultrastructural effects of *Calotropis procera* latex on *Haemonchus contortus*. *C. procera* latex was twice centrifuged at 10,000×g and dialyzed to obtain a fraction rich in proteins, named LP (latex protein), and at 3,000 rpm to obtain a fraction rich in secondary metabolites, named LNP (latex non-protein). Specimens of *H. contortus* exposed to LNP, LP and PBS in the Adult Worm Motility Test (AWMT) were submitted to scanning (SEM) and transmission (TEM) electron microscopy to verify changes in their ultrastructure. Phytochemical tests in the LNP indicated the presence of phenols, steroids, alkaloids and cardenolides. High-Performance Liquid Chromatography (HPLC) characterized the presence of the compounds gallic acid and quercetin in the LNP. The protein content in the LP was 43.1 ± 1.1 mg/mL and 7.7 ± 0.3 mg/mL in LNP. In AWMT, LNP and LP inhibited the motility of 100% of the nematodes, with LNP being more effective than LP and ivermectin more effective than both ($p < 0.05$). Cuticle changes were observed by SEM and TEM in nematodes treated with LP and LNP. *Calotropis procera* latex has anthelmintic effects against *H. contortus*, causing damage to its cuticle and other alterations in its ultrastructure.

Keywords: Anthelmintic, nematode, cuticle, latex protein, secondary metabolites.

Resumo

Este estudo objetivou avaliar os efeitos anti-helmínticos e ultraestruturais do látex de *Calotropis procera* sobre *Haemonchus contortus*. Látex de *C. procera* foi centrifugado duas vezes a 10.000xg e dialisado para obter uma fração rica em proteínas, denominada proteínas do látex (LP). E centrifugado e centrifugado a 3.000 rpm, para obter uma fração rica em metabólitos secundários, denominada LNP (látex não proteico). Espécimes de *H. contortus* expostos à LNP, LP e PBS no Teste de Motilidade dos Nematoides Adultos (TMNA) foram submetidos a microscopia eletrônica de varredura (MEV) e de transmissão (MET), para verificar alterações em sua ultraestrutura. Testes fitoquímicos em LNP indicaram a presença de fenóis, esteroides, alcaloides e cardenolídeos. A presença dos compostos ácido gálico e quercetina em LNP foi caracterizada por Cromatografia Líquida de Alta Eficiência (CLAE). O conteúdo de proteínas em LP foi de 43,1 ± 1,1 mg/mL e de 7,7 ± 0,3 mg/mL em LNP. No TMNA, LNP e

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LP inibiram a motilidade de 100% dos nematoides, sendo LNP mais eficaz que LP, e a ivermectina mais eficaz que ambos (p <0,05). Alterações na cutícula de nematoides tratados com LP e LNP foram observadas por MEV e MET. O látex de *C. procera* apresenta efeito anti-helmíntico sobre *H. contortus*, causando danos à sua cutícula e outras alterações em sua ultraestrutura.

Palavras-chave: Anti-helmíntico, nematoide, cutícula, proteínas do látex, metabólitos secundários.

Introduction

Haemonchus contortus is one of the main gastrointestinal nematodes affecting the global production of sheep and goats and is considered the most pathogenic and prevalent in tropical and subtropical regions (Niciura et al., 2019; Santos et al., 2017). Infections by gastrointestinal nematodes can cause economic losses by reducing the productivity and fertility of herds, involving morbidity, weight loss and growth retardation, as well as mortality in cases of severe infection (Alowanou et al., 2019).

The resistance of gastrointestinal nematodes in small ruminants to commercially available anthelmintics is widely disseminated, making it necessary to adopt alternative measures to control these infections (Elandalousi et al., 2013; Ribeiro et al., 2015). Among the alternatives, the investigation of medicinal plants with anthelmintic potential has received much attention (Acharya et al., 2014; Hoste & Torres-Acosta, 2011).

The therapeutic properties of many plants traditionally used for medicinal purposes have been scientifically proven (Belemlilga et al., 2016; Macedo et al., 2012). The use of these plants has advantages, such as a low cost, biodegradability and a lack of ecological harm (Zhu et al., 2013). In addition, medicinal plants are also sources of bioactive compounds derived from primary and secondary metabolism, enabling the development of new drugs (Ribeiro et al., 2013).

In the validation of the anthelmintic properties of medicinal plants, *H. contortus* has been the target of several *in vitro* studies that used immature stages, such as eggs and larvae, or the adult parasite, as well as *in vivo* studies using sheep or goats that were naturally or experimentally infected (Cavalcante et al., 2016; Ribeiro et al., 2017). Associated with these tests, analyses have been proposed to understand the effect of phytotherapeutics or compounds derived from medicinal plants, such as the analysis of changes in the structure of eggs, larvae or adult nematodes by scanning or transmission electron microscopy (Araújo-Filho et al., 2018; Brunet et al., 2011; Cortes-Morales et al., 2019). Different microscopic techniques allow for verifying changes in the ultrastructure of the parasites and identifying their potential target structures (Sant'anna et al., 2013).

Calotropis procera (Aiton) W.T. Aiton, belonging to the family Apocynaceae, is a shrub that produces latex and is native to India and Africa, where it has traditionally been used for various medicinal purposes, including for the treatment of animal worms (Iqbal et al., 2005). Latex plays an important defence role in plants, acting against herbivorous insects, nematodes and phytopathogenic fungi (Ramos et al., 2019; Upadhyay et al., 2011).

The latex of *C. procera* has a wide pharmacological profile and has been investigated regarding its anthelmintic activity (Al-Qarawi et al., 2001; Cavalcante et al., 2016). In contrast, some components of this fluid cause toxicity in small ruminants (El Sheikh et al., 1991; Mahmoud et al., 1979). Some studies suggest that the insoluble fraction of *C. procera* latex is associated with the toxic effects of this fluid (Dewan et al., 2000; Kumar & Shivkar, 2004). In this respect, it is necessary to purify the compounds responsible for the therapeutic effect to separate them from those causing toxicity, as proposed also in this work.

Thus, this study aimed to evaluate the *in vitro* anthelmintic effect of two fractions (the soluble fraction and protein fraction) of *C. procera* latex against *H. contortus* and to verify the induction of changes in the morphology and ultrastructure of *H. contortus* exposed to these fractions.

Materials and Methods

Latex collection and purification

Specimens of *C. procera*, occurring naturally in two locations (S 3º47'25.82484" W 38º33'10.92672 and S 3º45'18.16668" W 38º33'21.86964) within the municipality of Fortaleza, Ceará state, Brazil, were identified and deposited in the Prisco Bezerra Herbarium of Ceará Federal University under registration number 58425. The latex of *C. procera* was obtained by breaking the vegetative apex and collecting latex in tubes containing the same proportion of distilled water (ratio of 1:1 v/v). *C. procera* latex was centrifuged at 3,000 rpm for 20 min, the

precipitate (insoluble fraction) was discharged and the supernatant was frozen for subsequent lyophilization, from which a product rich in secondary metabolites was obtained and named LNP (latex non-protein fraction)

A fraction rich in protein (LP) was obtained according to Ramos et al. (2013). First, *C. procera* latex was centrifuged at 10,000×g and 4°C, for 20 min. The supernatant was then dialyzed (molecular weight cut off of 8,000 Da) for 60 h in distilled water (4°C) for elimination of small molecules, such as secondary metabolites, followed by a second centrifugation step (10,000×g, 4°C, 20 min). The supernatant was then lyophilized to produce the LP.

Chemical and biochemical analysis

To identify the secondary metabolite classes, present in the LNP, phytochemical tests were performed with this fraction. Phenolic compounds present in the LNP were investigated by high-performance liquid chromatography. The soluble protein content was measured in both the LNP and LP. These assays are described below.

Phytochemical tests

Phytochemical analysis was performed according to the methodology of Matos (1988). Briefly, tests were carried out with specific reagents which form coloured compounds or precipitates as positive indication of determined class of secondary metabolite. The following classes were investigated:

Phenols, tannins and anthocyanins–an aliquot of the LNP (30 mg) was solubilized in 15 mL 70% ethanol and distributed in 3 test tubes. Two drops of ferric chloride solution were added to a tube, and colour changes between blue and green indicated the presence of phenols, and precipitate formation indicated the presence of tannins. The sample in tube 2 was acidified (pH 3) with hydrochloric acid solution (0.1 M), and in tube 3, it was alkalized (pH 8.5 and 11) with sodium hydroxide solution (0.1 M). Then, tubes 2 and 3 were heated. The different colour changes indicated the presence of various constituents.

Steroids and triterpenes–an aliquot of the LNP (10 mg) was solubilized in 5 mL chloroform and filtered with anhydrous sodium sulfate. Then, 1 mL acetic anhydride and 3 drops sulfuric acid were added. Blue-to-green coloration indicated the presence of steroids, and brown-to-red coloration indicated the presence of triterpenes;

Saponins–an aliquot of the LNP (20 mg) was solubilized in distilled water (5 mL) and shaken vigorously. A permanent foaming is indicative of saponin´s presence;

Alkaloids–an aliquot of the LNP (20 mg) was solubilized in 0.1 M hydrochloric acid (6 mL), filtered and distributed between 2 test tubes; then, three drops of Draggendorf or Mayer reagents were added. Precipitate formation indicated the presence of alkaloids.

The presence of cardiac glycosides was investigated by Keller Kelliani's test (Hassan et al., 2017). An aliquot of the LNP (20 mg) was solubilized in 5 mL distilled water, and 2 mL glacial acetic acid and a drop of ferric chloride solution were added. Then, 1 mL sulfuric acid was added. A brown ring at the interface indicated the presence of cardenolides.

High-performance liquid chromatography

An aliquot of the LNP (40 mg/mL) solubilized in HPLC-grade methanol was submitted to HPLC-DAD via a Shimadzu Prominence HPLC system equipped with an auto-sampler (SIL-20A) (Shimadzu, Kyoto, Japan), Shimadzu LC-20AD reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, an SPD-M20A diode array detector, and LC solution 1.22 SP1 software. A reversed-phase Shim-pack CLC-ODS (M) column (250 × 4.6 mm, 5 µm) was used.

Separation was performed with a linear gradient as previously described, with some modifications (Silva et al., 2011). Acetonitrile (C) and Milli-Q water (pH 2.8) (D) were used as the mobile phases with the following gradient elution programme: 0-15 min, isocratic elution with C:D (20:80 v/v); 17-25 min, linear change to C:D (40:60 v/v); and 25-40 min, isocratic elution with C:D (20:80 v/v). The flow rate was 1.0 mL/min, the injection volume was 20 µL, and the detection wavelength was 350 nm. The LNP sample was analysed in triplicate.

Chromatographic peaks were confirmed by comparing the retention time (t_R) and DAD spectra with those of reference standards. The reference standards were gallic acid and quercetin. Calibration curve for galic acid was $y= 3.10^{-8}$ x-0.0061 (r=0.9997); quercetin $y=2.10^{-8}$x-0.0001(r= 0.999). Gallic acid was obtained from Sigma Chemical

Co. (St. Louis, MO, USA), and quercetin was obtained by acid hydrolysis of rutin with 3% sulfuric acid, according Vila-Nova et al. (2012).

Protein dosage

The content of soluble proteins in LNP and LP was measured using the colorimetric method, as described by Bradford (1976). For this purpose, 2.5 mL of Bradford reagent was added to 100 µL of the LNP or LP in distilled water (1 mg/mL) and slightly shaken. The process was performed in triplicate. After 10 min, absorbance readings were performed at 595 nm. The protein content was estimated using a calibration curve of bovine serum albumin (BSA) as the standard reference.

Animal welfare

The procedures using sheep were approved by the Ethics Committee for Animal Use of Ceará State University (Protocol No. 7700820/2016).

Adult Worm Motility Test (AWMT)

This test was performed according to André et al. (2016). Adult parasites were obtained from an experimentally infected sheep harbouring a benzimidazole-resistant isolate of *H. contortus*. The level of infection was monitored using the McMaster technique with modifications (Ueno & Gonçalves, 1998), and the sheep were euthanized approximately 40 days after infection, exhibiting 20,000 eggs per gram (EPG). Immediately after slaughter, the abomasum was removed and briefly washed with phosphate buffered saline (PBS) at 37°C; then, the nematodes were collected from the mucosa.

The parasites, three females/well, were placed as soon as possible in 24-well plates containing PBS with 4% antibiotics (penicillin and streptomycin) and placed in a CO_2 incubator (5%) at 37°C for one hour. Then, different concentrations, determined in a preliminary test, of the LNP (1, 0.5, 0.25, 0.12 and 0.062 mg/mL) and LP (1.6, 0.8, 0.4, 0.2 and 0.1 mg/mL) diluted in PBS were added. Ivermectin (0.1 mg/mL) and PBS with antibiotics were used as positive and negative controls, respectively. Eight replicates were performed for each treatment and control. The motility of adult nematodes was verified by careful observation under a stereomicroscope after 3, 6, 12 and 24 h.

At the end of the test, twelve parasites were collected from each of the highest tested concentrations of the LNP (1 mg/mL) and LP (1.6 mg/mL) and from the negative control (PBS). The nematodes were fixed and subsequently subjected to scanning and transmission electron microscopy for analysis of ultrastructural changes. Nematodes treated with the LNP and LP were compared to nematodes not treated with *C. procera* latex fractions (negative control).

Scanning Electron Microscopy (SEM)

Haemonchus contortus obtained from the AWMT were fixed in 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer for a minimum period of 72 h. Subsequently, three washes were performed with PBS, followed by post-fixation with osmium tetroxide solution (1%) for 1 h for greater contrast of the material. Then, the parasites were washed three times with distilled water (10 min) and dehydrated in a gradual ethanol series (10, 20, 30, 60, 70, 80, 90 and 100%, 5 min each). The ethanol was then replaced with liquid carbon dioxide, and the samples were dried using an EMS 850 critical-point drying apparatus. The samples were mounted on stubs, coated with gold-palladium and observed with a Zeiss DSM-940A scanning electron microscope at an acceleration voltage of 15 kV. Alterations were observed in the morphology of the treated and control group parasites.

Transmission Electron Microscopy (TEM)

Haemonchus contortus obtained from the AWMT were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde and 0.1 M sodium cacodylate buffer, pH 7.1, for 48 h. The samples were then washed three times in sodium cacodylate buffer, post-fixed in 1% osmium tetroxide solution for 1 h and rinsed again with the same buffer. The samples were dehydrated in an increasing acetone series (30, 40, 50, 70, 90 and 100%, for 1 h each) and were encased in Spurr resin and polymerized at 60°C. Ultra-thin sections of 70 nm were obtained and collected in 400 mesh copper grids and counterstained with 1% uranyl acetate and 5% lead citrate. The samples were then analysed with

a JEOL 1400 Plus transmission electronic microscope at 60 kV. Changes were observed in the ultrastructure of the treated parasites compared to that of the parasites of the control group.

Statistical Analysis

In the AWMT, for each observed time (3, 6, 12 and 24 h), the percentage of motility inhibition of replicates of each treatment (the LNP, LP, Ivermectin and PBS groups) was calculated according to the following Equation 1:

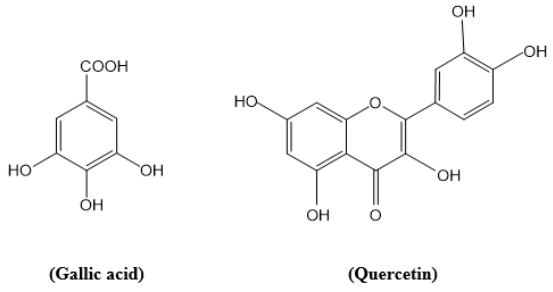
$$\text{Motility inhibition (\%)} = \frac{\text{number of motionless parasites}}{\text{total number of parasites per well}} \times 100 \quad (1)$$

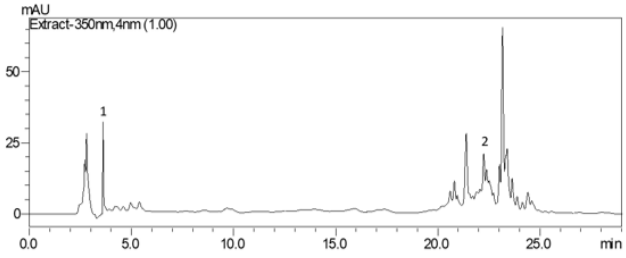
These data were submitted to the Shapiro-Wilk test for normality analysis ($p>0.05$), to be adequate to the criteria for using the ANOVA. Then, were submitted to two-way ANOVA and compared by the Bonferroni test, with a 5% significance level ($p<0.05$), using GraphPad Prism 5.01 software. The results are expressed as the mean ± standard deviation. The effective concentration to inhibit the motility of 50% of the adult nematodes (EC_{50}) was determined by probit regression using SPSS 22 for Windows, according to Araújo-Filho et al. (2019). The EC_{50} was measured at all times evaluated in the test (3, 6, 12 and 24 h).

Results

The latex of *C. procera* contained 87.3 ± 1.6% water and 12.7 ± 1.6% corresponded to dry mass. Based on the dry mass of the *C. procera* latex, the LNP and LP presented yields of 48.3 ± 1.3% and 5.8 ± 0.5%, respectively. The protein content, estimated by the Bradford method (1976), was 43.1 ± 1.1 mg/mL in the LP and 7.7 ± 0.3 mg/mL in the LNP. Secondary metabolites were separated from the LP by dialysis, while phytochemical tests indicated the presence of secondary metabolites, such as phenols, steroids, alkaloids and cardiac glycosides, in the LNP. The presence of gallic acid and quercetin was detected by HPLC in the LNP (Figure 1), where the retention times and UV-Vis spectrum of the compounds were similar to those of the analysed standards.

In relation to AWMT, the motility of *H. contortus* submitted to LNP and LP was shown in Figure 2, while the effect of LNP and LP on the inhibition of motility was shown in Table 1. The highest concentration of LNP (1 mg/mL)





Peak	Retention time	Compound	UV main peaks
1	3.546 min	Gallic acid	196 nm; 273 nm
2	22.244 min	Quercetin	202 nm; 280 nm; 334 nm

Figure 1. Representative high-performance liquid chromatograph of the non-protein fraction (LNP) of *Calotropis procera* latex. UV: Ultraviolet.

inhibited the motility of 100 ± 0.0% of parasites after 6 h of contact, while the highest concentration of LP (1.6 mg/mL) inhibited the motility of 95.8 ± 11.8% of parasites only after 12 h of exposure. Ivermectin (0.1 mg/mL) inhibited the motility of 100 ± 0.0% of parasites at the first observed time (3h), differing statistically from all LNP or LP concentrations tested at that time.

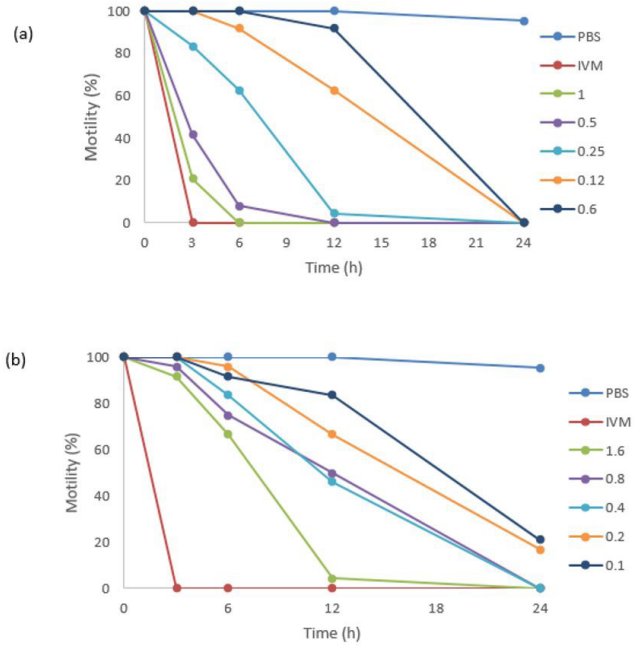


Figure 2. Motility of *Haemonchus contortus* (%) exposed to the *Calotropis procera* latex fractions: non-protein fraction-LNP (graph a - concentrations from 0.62, 0.12, 0.25, 0.5 and 1 mg/mL) and protein fraction-LP (graph b - concentrations from 0.1, 0.2, 0.4, 0.8 and 1.6 mg/mL). Phosphate buffered saline (PBS) and 0.1 mg/mL ivermectin (IVM) were used as negative and positive controls, respectively.

Table 1. Mean efficacy (percentage ± standard deviation) of non-protein (LNP) and protein (LP) fraction of *Calotropis procera* latex, ivermectin and phosphate buffered saline (PBS) on motility inhibition of *Haemonchus contortus*. The effective concentration to inhibit the motility of 50% (EC_{50}) of the adult worms submitted to LNP and LP was obtained for times 3, 6 and 12 h.

	Concentration (mg/mL)	3h	6h	12h	24h
LNP	1.0	79.2 ± 17.2 ^{B}	100.0 ± 0.0 ^{A}	100.0 ± 0.0 ^{A}	100.0 ± 0.0 ^{A}
	0.5	58.3 ± 29.5 ^{C}	91.7 ± 15.4 ^{A}	100.0 ± 0.0 ^{A}	100.0 ± 0.0 ^{A}
	0.25	16.7 ± 17.8 ^{D}	37.5 ± 11.8 ^{B}	95.8 ± 11.8 ^{A}	100.0 ± 0.0 ^{A}
	0.12	0.0 ± 0.0 ^{D}	8.3 ± 15.4 ^{C}	37.5 ± 11.8 ^{B}	100.0 ± 0.0 ^{A}
	0.06	0.0 ± 0.0 ^{D}	0.0 ± 0.0 ^{C}	8.3 ± 15.4 ^{C}	100.0 ± 0.0 ^{A}
	EC_{50} (mg/mL)	*0.50*	*0.27*	*0.13*	-
LP	1.6	8.3 ± 15.4 ^{D}	33.3 ± 25.2 ^{B}	95.8 ± 11.8 ^{A}	100.0 ± 0.0 ^{A}
	0.8	4.2 ± 11.8 ^{D}	25.0 ± 23.6 ^{BC}	50.0 ± 25.2 ^{B}	100.0 ± 0.0 ^{A}
	0.4	0.0 ± 0.0 ^{D}	16.7 ± 17.8 ^{BC}	54.2 ± 35.4 ^{B}	100.0 ± 0.0 ^{A}
	0.2	0.0 ± 0.0 ^{D}	4.2 ± 11.8 ^{C}	33.3 ± 25.2 ^{BC}	83.3 ± 25.2 ^{B}
	0.1	0.0 ± 0.0 ^{D}	8.3 ± 15.4 ^{C}	16.7 ± 17.8 ^{C}	79.2 ± 17.2 ^{B}
	EC_{50} (mg/mL)	*6.9*	*4.4*	*0.4*	-
PBS	-	0.0 ± 0.0 ^{D}	0.0 ± 0.0 ^{C}	0.0 ± 0.0 ^{C}	4.2 ± 11.8 ^{C}
Ivermectin	0.1	100.0 ± 0.0 ^{A}	100.0 ± 0.0 ^{A}	100.0 ± 0.0 ^{A}	100.0 ± 0.0 ^{A}

Capital letters compare means in the columns. Different letters indicate significantly different values ($p<0.05$).

The interactions between the factors (times and treatments) were statistically significant, with the time and treatment factors accounting for 32.6% and 43.5% of the total variation, respectively. The LNP and LP presented dose-dependent effects at 3 h, 6 h and 12 h, being possible to calculate their EC_{50} at each of these times (Table 1). For the 24 h period, it was not possible to obtain an EC_{50}, since all LNP concentrations and the three highest concentrations of LP inhibited the motility of 100 ± 0.0% of the nematodes. On the other hand, in the negative control group, only 4.17 ± 11.8% of the parasites lost their motility only at the last observed time (24 h).

In the SEM micrographs, changes were observed along the cuticle of *H. contortus* females exposed *in vitro* to the LNP and LP, including in the cephalic region and vulvar appendage (Figure 3). The alterations consisted of wrinkling of the cuticle, blister formation along the cuticle, and, in the case of the LP, deposition of material in the vulvar region. The vulvar appendage of *H. contortus* females was of the linguiform type (Figure 3), and in the LNP (B3)

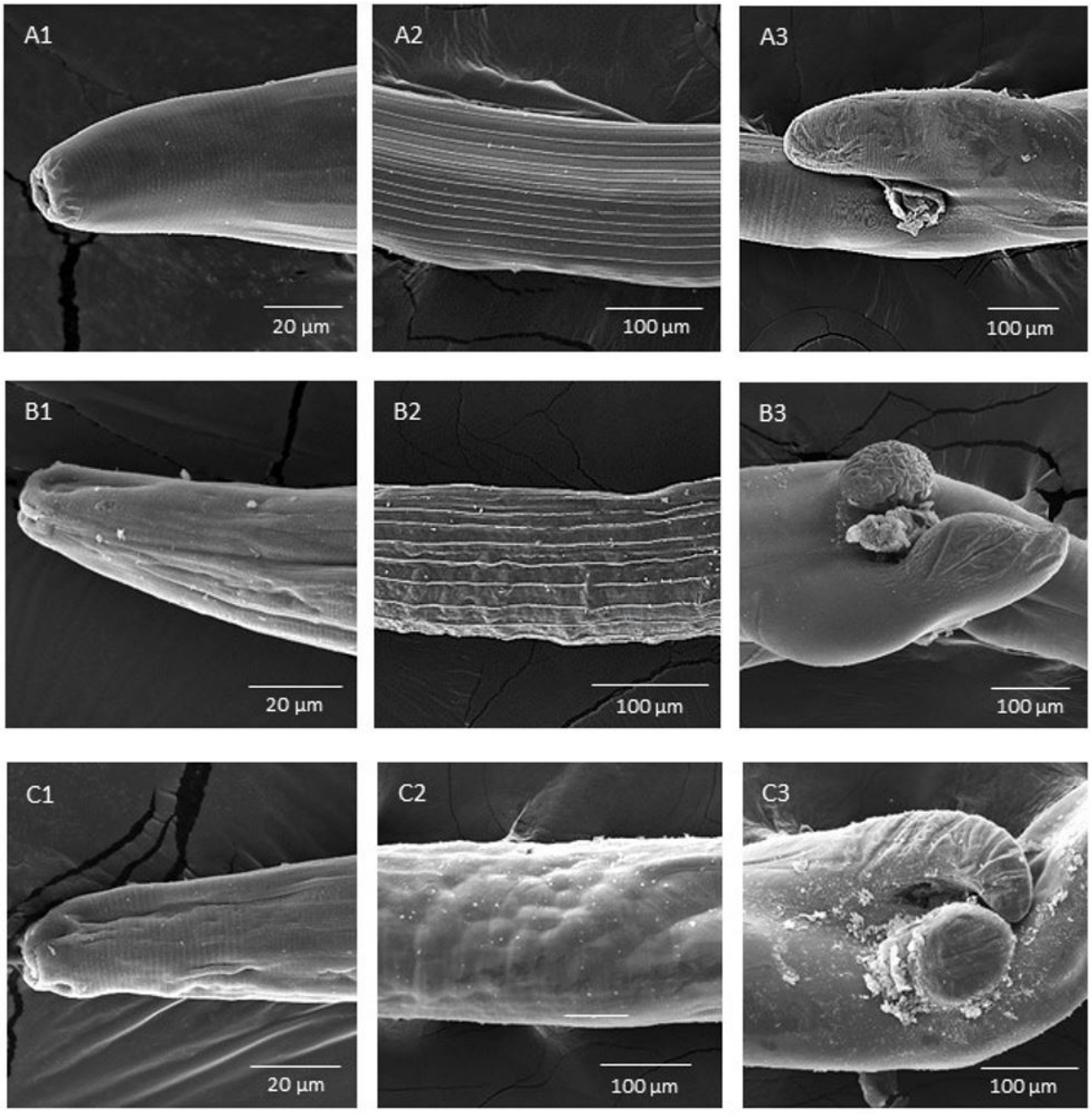


Figure 3. *Haemonchus contortus* females submitted *in vitro* to Phosphate buffered saline (PBS: A1, A2, and A3) and *Calotropis procera* latex fractions: 1 mg/mL of non-protein fraction (LNP: B1, B2, and B3) and 1.6 mg/mL of protein fraction (LP: C1, C2, and C3), and analysed by scanning electron microscopy (SEM). A1, B1 and C1: encephalic region; A2, B2 and C2: median portion; A3, B3 and C3: vulvar appendage. The wrinkling and formation of bubbles in the cuticle of nematodes exposed to the LNP and LP were observed. There was marked deposition of material in C3.

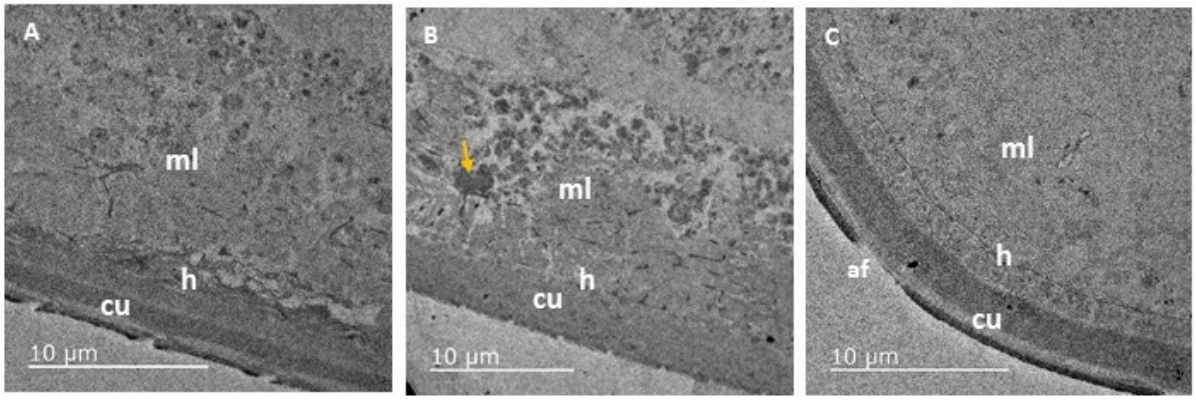


Figure 4. *Haemonchus contortus* females submitted *in vitro* to water (negative control, A) and *Calotropis procera* latex fractions: 1 mg/mL of non-protein fraction (LNP, B) and 1.6 mg/mL of protein fraction (LP, C), and analyzed by transmission electron microscopy (TEM). There was disorganization of cuticle layers and degradation of the muscular layer (A, B). af: annular furrow; cu: cuticle; ml: muscular layer; h: hypodermis. The arrow points to an electron-dense body.

and LP (C3) treated females, the presence of a lateral button was also observed. The set of longitudinal cuticular ridges (synlophe) of nematodes in the negative control group appeared well preserved.

The TEM results are shown in Figure 4. It was possible to observe disorganization of the cuticle of the nematodes treated *in vitro* with the LNP and LP. There was also degradation of the muscular layer of the parasites treated with the LNP, especially in the region with abundant mitochondria, also with the occurrence of electron-dense bodies. The muscular layers of the parasites treated with LP were entirely degraded, making it impossible to distinguish myofibrils and mitochondria in this tissue.

Discussion

In the present study, insoluble material of latex was removed via centrifugation to concentrate the bioactive molecules in the LNP. The concentration of total proteins in the LNP was 5.6 times lower than that in the LP, which corresponded to a purified fraction of the proteins of *C. procera* latex. The LP of *C. procera* was composed of basic proteins, with molecular masses ranging from 5 to 95 kDa, and presented proteolytic potential (Freitas et al., 2007). Proteins, such as cysteine, chitinases, peroxidases and osmotin have been identified in *C. procera* latex, including proceraine, proceraine B, calotropin DI and DII, calotropaine, calotropaine FI and FII, and CpOsm (Freitas et al., 2011; Ranjit et al., 2012; Singh et al., 2010).

Plant latex contains a wide range of specialized metabolites that are partially responsible for various activities, such as antibacterial, antifungal and anthelmintic activities; among these compounds are terpenoids, cardenolides, alkaloids and phenolics (Abarca et al., 2019). In *C. procera* latex, several metabolites have been identified, including cardenolides, alkaloids, steroids and phenols (Chundattu et al., 2016; Mohamed et al., 2015; Ranjit et al., 2012), which were also found in the present investigation. According to Murti et al. (2015), polyphenolic compounds show anthelmintic activity. Among these compounds, gallic acid and quercetin have already been reported in extracts of *C. procera* aerial parts (Khasawneh et al., 2011). Patel et al. (2015) and Kore et al. (2018) report significant activity of gallic acid and quercetin against *Pheretima postuma*, respectively. The *P. posthuma* (Annelida) is an earthworm used as a suitable model for screening of anthelmintic drug (Kore et al., 2018).

The latex of other plants, such *Carica papaya* and *Ficus* spp., has shown anthelmintic activity, and the proteins, especially cysteine peptidases, present in these fluids have been identified as responsible for the observed effect (Amorin et al., 1999; behnke et al., 2008). In this study, both the LNP and LP had promising anthelmintic effects on adult *H. contortus*, inhibiting the motility of parasites at low concentrations and in a short period of time. The results obtained were superior, for example, to the decoction of *Saba senegalensis* (Apocynaceae) leaves and an extract rich in hydrolysable tannins, which presented EC_{50} values of 6.79 and 3.54 mg/mL after 24 h of exposure, respectively (Acevedo-Ramírez et al., 2019; Belemlilga et al., 2016). However, the LNP and LP were not as effective as ivermectin (Table 1). The LNP was more effective than LP and had an EC_{50} lower than LP at all times tested (Table 1). Thus, it was possible that the effect of the LNP was caused by synergistic action among its compounds, including the proteases that were also present in the LP.

In vitro tests are used for preliminary investigation of the anthelmintic potential of natural products or isolated compounds, and tests using adult nematodes are considered more reliable than those using developing stages, such as eggs and larvae (André et al., 2016; Ribeiro et al., 2017). In addition, SEM and TEM analysis of the parasites submitted to these tests may present alterations that demonstrate the loss of homeostasis associated with the observed anthelmintic effect and may indicate the type of action of the bioactive compounds on the parasites (Behnke et al., 2008; Brunet et al., 2011; Martínez-Ortíz-De-Montellano et al., 2013).

In the present study, SEM allowed for the observation of damage in the *H. contortus* cuticle exposed to the LNP and LP, such as blistering and longitudinal and transverse wrinkling along the cuticle and on the vulvar appendage of the females. This damage was also verified in the *H. contortus* cuticle exposed to other plant-derived compounds, such as the monoterpenes carvacrol, acetylated carvacrol and thymol (André et al., 2016, 2017). Similarly, extracts rich in tannins, such as a decoction of *Spigelia anthelmia* (Ribeiro et al., 2017), an extract of hydrolysable tannins obtained from *Castanea sativa* (Acevedo-Ramírez et al., 2019) and extracts from leaves of *Onobrychis viciifolia* or *Lysiloma latisiliquum* (Martínez-Ortíz-De-Montellano et al., 2013), caused damage to the *H. contortus* cuticle similar to those observed in this study.

The presence of aggregated material in the vulvar region, as observed in the parasites treated *in vitro* with the LP, has already been reported for extracts rich in tannins, having been observed around the buccal capsule and in the anus of *H. contortus*, in addition to the vulvar region (Martínez-Ortíz-De-Montellano et al., 2013). These authors stated that the presence of aggregates in the vulvar region may affect the reproductive function of the nematodes due to mechanical obstruction of egg production or expulsion.

It is possible that changes observed in the cuticle of the LNP-treated nematodes were induced mainly by the cysteine peptidases present in the extract, since nematodes treated with the purified protein fraction (LP) showed more intense changes in the cuticle than those treated with the LNP. Cysteine peptidases from other plants induced cross-sectional wrinkling of the cuticle of *Heligmosomoides bakeri*, a rodent nematode, after 30 min of exposure (Behnke et al., 2008). It was also verified that cysteine peptidases were mainly responsible for the effect of papaya latex (*Carica papaya*) on the motility of *H. bakeri* in tests using cysteine inhibitors. In addition, *H. contortus* exposed to the ethyl acetate extract of *C. procera* latex exhibited inhibited motility but showed no changes in their cuticle (Cavalcante et al., 2016).

In TEM, the changes observed between the nematodes treated *in vitro* with the LNP and LP and those in the control group were disorganization of the cuticle, muscle layer degradation, especially in the region with abundant mitochondria, and the presence of electron-dense bodies. These changes have been observed in studies evaluating the effect of commercial anthelmintics or compounds with anthelmintic potential, with electron-dense bodies commonly being found in degraded tissues, as well as observed negative effects on mitochondria related to a decline in energy production (Brunet et al., 2011; Jiao et al., 2019; Rothwell & Sangster, 1996; Sant'anna et al., 2013).

The cuticle of nematodes consisted of three basic layers: the cortex, matrix layer and basal layer (fibre layer). These layers can be subdivided (Anya, 1966). In the nematodes treated with the LNP and LP, it was not possible to distinguish the basal layers, indicating the disorganization of these structures. The cuticle is a crucial structure for nematode development and survival (Page et al., 2014). In addition, the cuticle of nematodes is a potential site for drug uptake, where the pores present in the collagen matrix control the passage of molecules depending on their physicochemical properties, especially lipophilicity (Lanusse et al., 2018; Mottier et al., 2003, 2006). The entry and accumulation of anthelmintic drugs in parasites are determinants to achieve optimal efficacy, and the transcuticular route is the main entry path of anthelmintic drugs in parasites (Lanusse et al., 2018).

Conclusion

In conclusion, the latex of *C. procera* contained bioactive constituents with potential anthelmintic activity, such as proteins and compounds derived from secondary metabolism. The fractions of *C. procera* latex caused severe damage to the cuticle of *H. contortus*, as well as ultrastructural disorganization. This information corroborates the use of *C. procera* in traditional medicine, but further studies are needed to assess the *in vivo* anthelmintic efficacy and toxicological safety of latex of this species as well as its bioactive compounds.

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