Parasitology

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Chemical characterization and *in vitro* biological activity of *Cymbopogon citratus* extracts against *Haemonchus* spp. and *Trichostrongylus* spp. nematodes from sheep

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Research Article

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Abstract

Medicinal plants have been the focus of several studies due to their nematicide properties which can be used to control nematodes in sheep. No study has examined the morphological effects of *Cymbopogon citratus* on nematodes. Thus, this study evaluated the chemical composition, nematicidal activity and effects of *C. citratus* extracts on the morphology of eggs and infective larvae (L_3) of sheep. Aqueous and methanolic extracts and fractions of *C. citratus* were obtained and analysed *in vitro*. The *C. citratus* extracts were effective against *Haemonchus* spp. and *Trichostrongylus* spp. larvae and eggs. Ten fractions were obtained from *C. citratus*, six of which had high ovicidal activity at 1000 $\mu g\,mL^{-1}$, and two fractions had high activity at all tested concentrations. The phytochemical analysis identified the presence of compounds such as terpenoids, various ketones, esters, and fatty acids. The ultrastructural analysis showed deformations of the cuticle and wilting along the body of the nematodes at all concentrations. The muscular layer, intestinal cells and the mitochondria profile showed damage compared to the typical pattern. Ultra-thin sections of eggs treated with methanolic fractions of *C. citratus* presented modifications. This study showed the biological activity and effects of *C. citratus* on the gastrointestinal nematodes in sheep.

Introduction

Parasitic nematodes in small ruminants and other livestock can have major economic impacts on animal production worldwide. Host parasitism results in lower levels of weight gain, interference with food intake, delayed reproductive age and even death in severely affected animals (Hansen and Perry, 1994). Meanwhile, there is a significant economic burden related to the cost of anthelmintic treatments to control parasites (McLeod, 1995). In Brazil, sales in the animal health industry reached nearly US$1.5 billion in 2018, 53% of which was spent on ruminants and 29% on antiparasitic agents (Sindan, 2018).

Commercial anthelmintics are most commonly used to control nematodiasis. Anthelmintic resistance among ruminant nematodes, however, is undoubtedly one of the most serious challenges in the production of small ruminants, and in many countries, the presence of anthelmintic resistant nematodes in small ruminants has become the norm rather than the exception (Leathwick and Besier, 2014). A review of studies on anthelmintic resistance of gastrointestinal nematodes of small ruminants in Brazil was conducted by Salgado and Santos (2016) and showed research beginning in the 1960s in the South (sheep) and Northeast (goats), where livestock is economically significant, and in the Southeast (sheep), an important region for research and the economy.

Plants are an important resource that can provide alternatives to the conventional anthelmintic control methods of gastrointestinal nematodiasis (Hounzangbe-Adote *et al.*, 2005; Eguale and Giday, 2009; Kamaraj *et al.*, 2011; Ahmed *et al.*, 2013, 2014; Santos *et al.*, 2019). Plant-based therapies have numerous advantages over commercial anthelmintics, as they are biodegradable, cause no environmental damage, and have fewer residues (Chagas, 2004).

Cymbopogon citratus (Lemon grass), commonly called *Capim Limão*, *Capim Cidreira*, or *Capim Santo* in Brazil, is a herbaceous plant of the Poaceae family that is native to the tropical

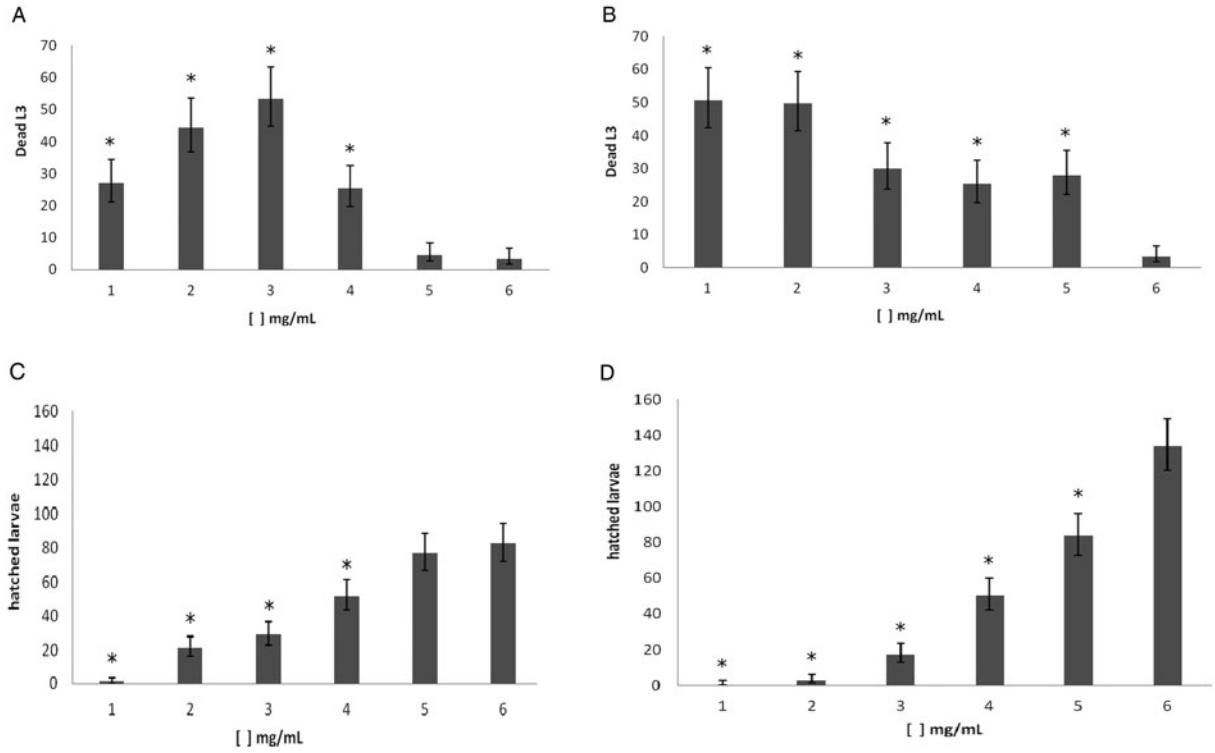

Fig. 1. Larvicidal and ovicidal assay with *Cymbopogon citratus* crude extract. Mean number of infective larvae (L_3) dead or hatched after a 48 h interaction with different concentrations of aqueous (A, C) and methanolic (B, D) extract. Concentrations in A and B: 25.0 mg mL^{-1} (1); 12.5 mg mL^{-1} (2); 6.2 mg mL^{-1} (3); 3.1 mg mL^{-1} (4); 1.6 mg mL^{-1} (5); and negative control with distilled water (6). Concentrations in C and D: 50.0 mg mL^{-1} (1); 25.0 mg mL^{-1} (2); 12.5 mg mL^{-1} (3); 6.2 mg mL^{-1} (4); 3.1 mg mL^{-1} (5); and negative control with distilled water (6). *Means are significantly different when compared to 3% DMSO and distilled water ($P < 0.001$).

regions of Asia, especially India (Gupta and Jam, 1978). *Cymbopogon citratus* has been identified as a medicinal plant species that can be used with domestic animals, with the highest use value reported at Colares Island, Pará State, eastern Amazon, Brazil. Tea/macerated leaves of *C. citratus* in water or food have been indicated for the medicinal treatment of helminths in dogs (Ritter *et al.*, 2012). The nematicidal activity of the plant has been analysed against the root-knot nematode (Oka *et al.*, 2000; Fabiyi *et al.*, 2018), pinwood nematode (Barbosa *et al.*, 2010) and gastrointestinal nematodes of small ruminants (Almeida *et al.*, 2003; Silva *et al.*, 2005; Macedo *et al.*, 2015, 2019). However, to date, no study has examined the morphological effects of *C. citratus* on nematodes. This study analysed the chemical composition of the plant and assessed its nematicidal activity and morphological and ultrastructural alterations of *C. citratus* extract and fractions on eggs and third-stage, infective larvae (L_3) of *Haemonchus* spp. and *Trichostrongylus* spp. nematodes in sheep.

Materials and methods

Collection and identification of botanical material

Cymbopogon citratus (Lemon grass) was collected at the Research Support Unit of the Centro de Ciências Tecnológicas e Agropecuárias, Universidade Estadual do Norte Fluminense – CCTA/UENF (21°S, 41°W). A voucher specimen (H8225) was deposited in the UENF herbarium, Campos dos Goytacazes City, Rio de Janeiro State, Brazil. Plant names were verified with http://www.theplantlist.org (accessed April 2018).

Collection of nematode eggs and third-stage, infective larvae (L_3)

Feces were collected directly from the rectum of 15 naturally infected sheep and refrigerated until analysis. The animals belonged to a small sheep breeding farm in the municipality of Campos dos Goytacazes. Eggs per gram (EPG) of feces were determined from 4 g of feces per animal as described by Gordon and Whitlock (1939). Feces were also cultivated to obtain L_3 as described by Bonadiman *et al.* (2006) and this larval stage was morphometrically identified at genera level as described by Van Wyk *et al.* (2004) and Van Wyk and Mayhew (2013).

Eggs were recovered from faecal samples with an EPG above 2000, as described by Bizimenyera *et al.* (2006). Briefly, the faecal samples were homogenized in distilled water and filtered through a series of sieves (24-, 48-, 80-,

Table 1. Substances postulated (Supplementary Fig. 1) by GC/MS analysis of the methanol extract of *C. citratus*, including retention time (R_t), percentage (%) of each component and molecular formulas

n	Compound	R_t	%	Molecular formula
1	*β*-linalool	13.692	1.11	$C_{10}H_{18}O$
2	*β*-citral	18.092	1.97	$C_{10}H_{16}O$
3	*trans* – geraniol	18.500	3.75	$C_{10}H_{18}O$
4	*α* – citral	18.983	1.38	$C_{10}H_{16}O$
5	Phytol	18.892	5.27	$C_{20}H_{40}O$
6	*α* – Tocopherol	56.150	0.72	$C_{29}H_{50}O_2$
7	Campesterol	58.775	0.93	$C_{28}H_{48}O$
8	Stigmasterol	59.500	1.43	$C_{29}H_{48}O$
9	*α* e *β* – Sitosterol	61.467	2.73	$C_{29}H_{50}O$
10	Linoleic acid	48.825	0.95	$C_{18}H_{32}O_2$
11	Cerin acetate (2*α*-acetoxyfriedelin)	66.150	11.8	$C_{32}H_{52}O_3$

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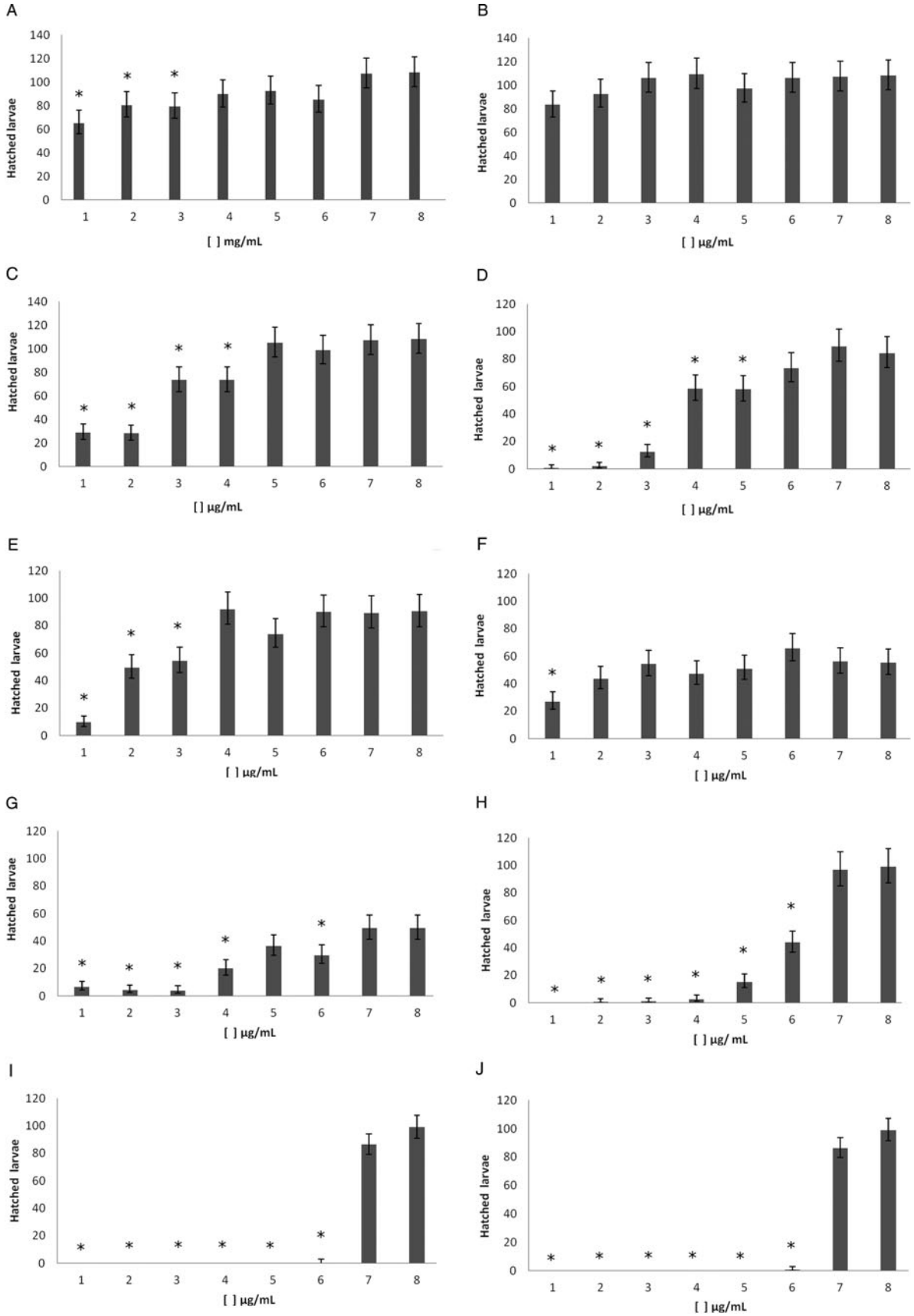

Fig. 2. Ovicidal assay with fractions obtained from *Cymbopogon citratus* methanolic extract. Mean number of hatched larvae after 48 h interaction with fractions: CCMF-01 (A); CCMF-02 (B); CCMF-03 (C); CCMF-04 (D); CCMF-05 (E); CCMF-06 (F); CCMF-07 (G); CCMF08 (H); CCMF09 (I); CCMF10 (J). Concentrations: 1000 μg mL^{-1} (1); 500 μg mL^{-1} (2); 250 μg mL^{-1} (3); 125 μg mL^{-1} (4); 62 μg mL^{-1} (5); 31 μg mL^{-1} (6); 3% DMSO (7); and distilled water (8). *Means are significantly different when compared to 3% DMSO and distilled water ($P < 0.001$).

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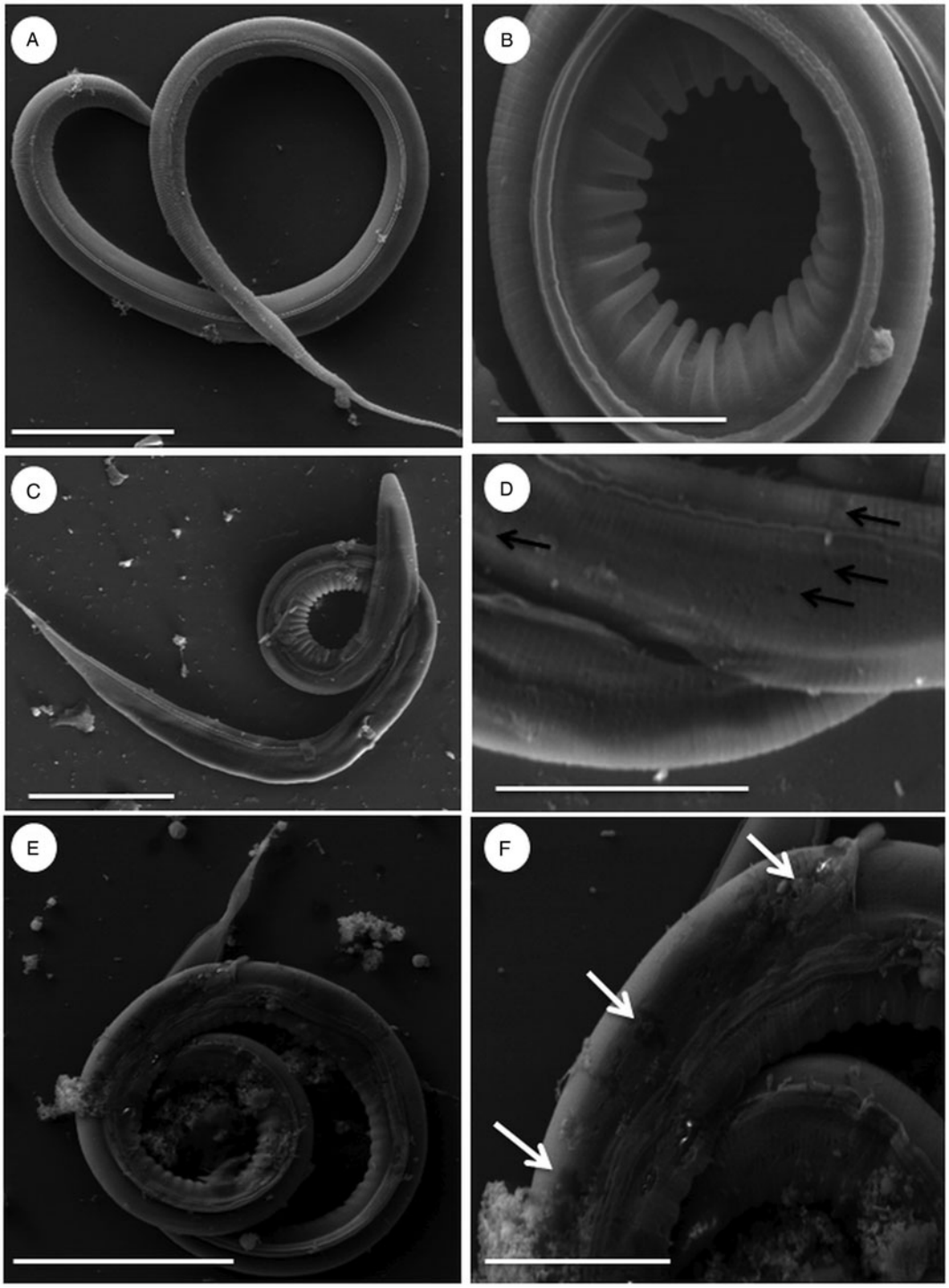

Fig. 3. S.E.M. micrographs of infective larvae (L_3) of *Haemonchus* spp. treated with *Cymbopogon citratus* crude extract. Control with 3% DMSO (A, B), aqueous (C, D) and methanolic (E, F) extract at a concentration of 25 mg mL^{-1}. C, D – L_3 cuticle with lesions (arrow); E, F – lesions (arrow) in the cuticle. Bars: 20 μm, 50 μm.

100-, 200-, 270-, and 400-mesh), the last of which trapped the eggs in the 38 μm pores (400-mesh). This sieve was washed, and the eggs and other contents were transferred to 15 mL Falcon tubes, which were then centrifuged at 1600***g*** for 5 min. This process was repeated with the addition of saline, and the supernatant was removed and filtered through a 400-mesh sieve to recover the eggs. The supernatant was discarded, and the eggs were washed liberally with distilled water to remove all traces of brine and then transferred to a conical sedimentation glass to settle for 2 h. Using a Pasteur pipette, the eggs were then retrieved and transferred to a Falcon tube.

Preparation of C. citratus *crude extracts*

Leaves of *C. citratus* were collected, chopped and dried in a greenhouse for 72 h at 30°C. Seventy grams of dried leaves were added to 2.5 L of methanol (MeOH) which was used for extraction. The solution was kept at room temperature for ten days, with the use of a rotary evaporator every 72 h, yielding 9.2 g of crude methanol extract. The crude MeOH extract was stored at 4°C until use. An aqueous extract was prepared from 63.3 g of dried leaves in 6 L of distilled water. The leaves were macerated for 24 h and then filtered, and the extract was frozen at −20°C and lyophilized. Two grams of lyophilized aqueous extract were obtained; the extracts

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were diluted in 3% Dimethyl sulfoxide (DMSO) to produce stock solutions.

In vitro *tests of anthelmintic activity with* C. citratus *crude extracts*

Stock solutions of 1.0 g mL^{-1} were used to test anthelmintic activity. Crude extracts at concentrations of 25.0, 12.5, 6.2, 3.1 and 1.6 mg mL^{-1} were used to assess larvicidal activity. Approximately 80 L_3 0.02 mL^{-1} were included in these tests. Crude extracts at concentrations of 50.0, 25.0, 12.2, 6.2 and 3.1 mg mL^{-1} and solutions of approximately 110 eggs 0.05 mL^{-1} were prepared for the hatching test. Negative controls were prepared using 3% DMSO. *In vitro* assays were performed in 24-well culture plates incubated at 27°C (L_3) and 30°C (eggs) for 48 h in a biochemical oxygen demand (BOD) incubator. To prevent further hatching, a drop of Lugol's solution was added to each well after 48 h (Coles *et al.*, 1992; Bizimenyera *et al.*, 2006). The number of hatched larvae and dead L_3 were counted using an inverted microscope. All assays were performed in triplicate.

Phytochemical analysis

The MeOH extract was submitted to liquid-liquid partition (MeOH/Hexane, 1:1, *v/v*). The crude MeOH extract was chromatographed over a silica gel column with a gradient of ethyl acetate/hexane, affording ten fractions. The crude MeOH extract and fractions were analysed by thin-layer chromatography and the first was also analysed by Gas Chromatography-Mass Spectrometry (GC-MS) with a GCMS-QP2010 Plus (Shimadzu, Kyoto, Japan), using a Factor Four/VF-5 ms column (30 m × 0.25 mm × 0.25 μm). The sample injection temperature was 250°C, with a split mode of injection. The sample was injected into the column at 1 mL min^{-1}. Chloroform was used as the eluent in the column dilution of the MeOH extract. This analysis identified the compounds and per cent mass present in each sample using the GCMS-QP2010 Series Software.

The fractions were tested on eggs and larvae at concentrations of 31, 62, 125, 250, 500 and 1000 μg mL^{-1}, as described above. Lethal concentration 50% (LC50) values were performed for the most active fractions of the MeOH extract.

Light and electron microscopy

For electron microscopy, samples were processed as described by Souza (2007). Briefly, the eggs and larvae were fixed for 24 h in 2.5% glutaraldehyde, 4% paraformaldehyde, 5 mM calcium chloride in 0.1 M cacodylate buffer, pH 7.2, post-fixed in 1% osmium tetroxide, 5 mM calcium chloride and 0.8 potassium ferrocyanide in 0.1 M cacodylate buffer. For S.E.M., the fixed eggs and L_3 were bonded on cover slips with the aid of poly-L-lysine to facilitate processing and visualization. The samples were dehydrated in an ethanol series, critical point dried with CO_2, mounted in stubs, sputter-coated with gold and examined in a Zeiss DMS962 scanning electron microscope operating at 15 KV. For TEM, the samples were dehydrated in an acetone series, infiltrated in resin Spurr, polymerised in an oven at 60°C, slices of 0.5 μm or 70 nm thickness were obtained and stained with 1% toluidine blue or contrasted in uranyl acetate and lead citrate, respectively. Samples were then examined using a light microscope or a Zeiss 900 transmission electron microscope operating at 80 kV. To produce the control micrographs (untreated eggs), fresh eggs were collected and processed for electron microscopy within 3 hours after collection.

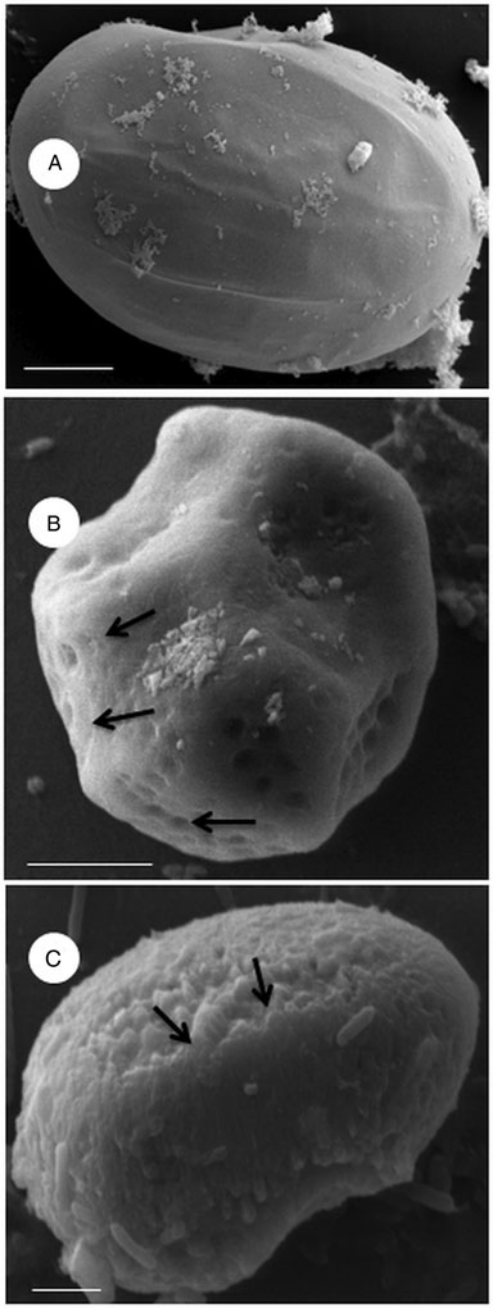

Fig. 4. S.E.M. micrographs of nematode egg treated with *Cymbopogon citratus* crude extract. Control with distilled water (A); methanolic (B) and aqueous (C) extract at a concentration of 50 mg mL^{-1}. Eggs treated with aqueous extract presented an irregular surface with numerous circular concave injuries (arrow) or with clusters of lumps (arrow) when treated with MeOH extract. Bars: 2 μm, 5 μm and 20 μm.

Statistical analysis

The analysis was performed based on generalized linear models, using the Poisson distribution and the GLIMMIX procedure in the Statistical Analysis System software (SAS System, Inc., Cary, NC, USA). In the case of significant difference, the Tukey test was applied.

Results

Faecal samples

Faecal samples revealed the presence of the *Haemonchus* and *Trichostrongylus* genera. *Haemonchus* spp. larvae were predominant (>85%).

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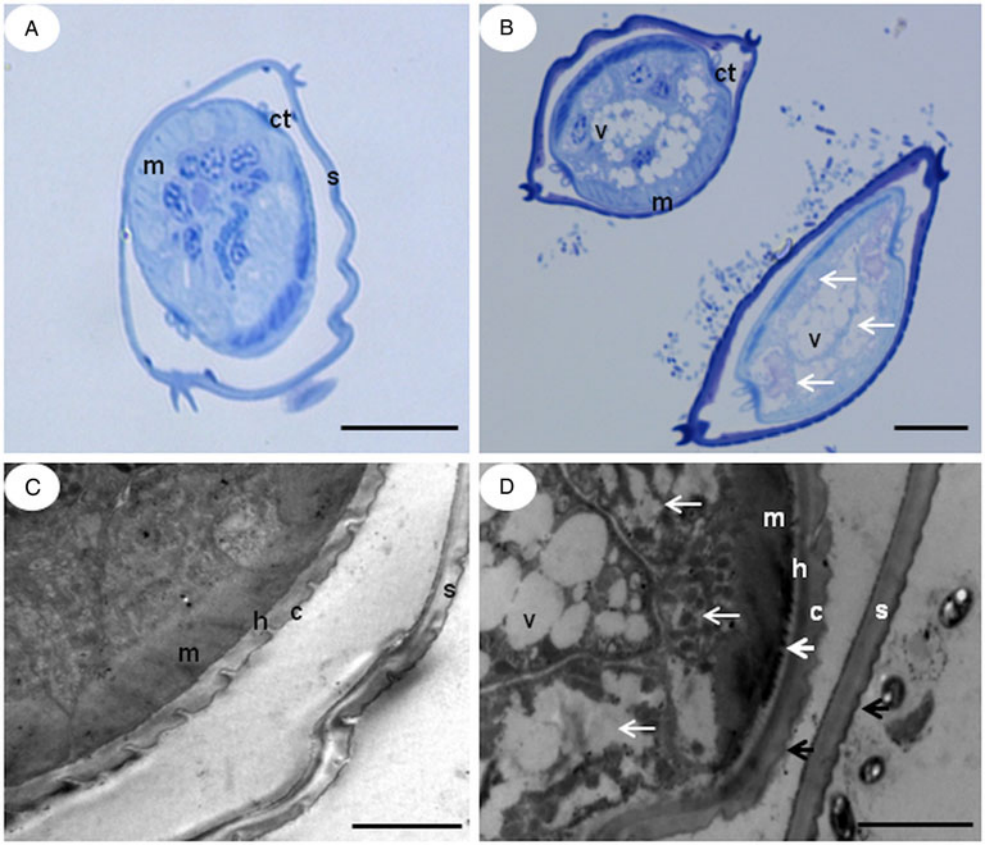

Fig. 5. TEM and semi-fine section micrographs of infective larvae (L_3) untreated (A, C) and treated (B, D) with *Cymbopogon citratus* aqueous extract 25.0 mg mL^{-1}. S – sheath, C – cuticle, H – hypodermis, M – Somatic muscle cells, V – Vacuoles, Ct – Longitudinal Cuticular thickening. Thin white arrows show the internal disorganization of the cells that make up the tissue. Large white arrow shows slight decoupling of the hypodermis-cuticle. Black arrows show the affected outer surface of the sheath and cuticle. Bars: a, b- 2 μ; c, d – 50 μ.

In vitro *anthelmintic action of* C. citratus *crude extract*

The aqueous and MeOH extracts of *C. citratus* showed significant larvicidal (Fig. 1A and B) and ovicidal (Fig. 1C and D) activity at all evaluated concentrations, except for the aqueous extract at a concentration of 1.6 mg mL^{-1}. Both extracts were active against *Haemonchus* spp. and *Trichostrongylus* spp. larvae.

Chromatography and C. citratus *fractions*

Ten fractions (*C. citratus* methanolic fraction CCMF-01 to CCMF-10) were used in the assays of biological activity to determine the active fraction. The phytochemical analysis identified the presence of compounds such as terpenoids (monoterpenes and steroids), various ketones, esters and fatty acids. From the *C. citratus* MeOH extract (Table 1), 11 major compounds were identified through GC-MS: four monoterpenes (linalool, *cis*-citral, *trans*-citral and *trans*-geraniol); one diterpene (phytol); four steroids (campesterol, stigmasterol, α-sitosterol and β-sitosterol); one triterpene (cerin acetate); linoleic acid and α-tocopherol (Supplementary Fig. 1). Among these, cerin acetate, *trans*-geraniol and phytol were particularly abundant.

Assay of biological action with C. citratus *MeOH fractions*

The fractions of *C. citratus* only showed ovicidal activity (Fig. 2), and mainly at concentrations of 1000 μg mL^{-1}: CCMF-03 (73.3%), CCMF-04 (99.3%), CCMF-05 (89.2%), CCMF-07 (86.6%) and CCMF-08 (100%) (Fig. 2C, D, E, G, and H). No larvae hatched after treatment with the fractions CCMF-09 and CCMF-10 at concentrations ranging from 62 to 1000 μg mL^{-1} indicating 100% ovicidal activity (Fig. 2I and J). Fractions CCMF-08, CCMF-09 and CCMF-10 were the only fractions that differed significantly from the control at all concentrations evaluated, while CCMF-02 was the only fraction that not differ significantly from the control. LC50 values of the fractions CCMF-09 and CCMF-10 were ~12 and 26 μg mL^{-1}, respectively (Supplementary Fig. 2).

Effects of C. citratus *extracts and fractions on the ultrastructure of* L_3 *and eggs*

Several changes were observed in the ultrastructure and morphology of eggs and L_3 after treatment with *C. citratus*. The cuticular surface of L_3 treated with 25.0 mg mL^{-1} of aqueous and MeOH extracts showed a modified appearance (Fig. 3C–E) that differed from the control (Fig. 2A and B). The cuticle showed lesions in the larvae treated with both aqueous (Fig. 3C and D) and MeOH extracts (Fig. 3E and F). Untreated eggs showed typical surface morphology (Fig. 4A), while eggs treated with MeOH extract presented an irregular surface with several injuries (Fig. 4B and C).

Ultra-thin or semi-fine sections of untreated L_3 revealed a typical internal morphology (Fig. 5A and C, 6A and B). For the treated L_3, we observed an irregular pattern of cuticle striation in many regions of the body (Fig. 5D). The sheath demonstrated strong interaction with the extract, with the external region being more intensely marked with dye (Fig. 5B) or electron-dense (Figs 5D and 6D). An alteration of the hypodermis was found with a slight decoupling from the cuticle (Figs 5D and 6C). Further, the muscular layer, intestinal cells and the mitochondria profile all showed damage compared to the typical pattern: muscle cells were degraded (Figs 5B, 5D, 6C and 6D); intestinal cells,

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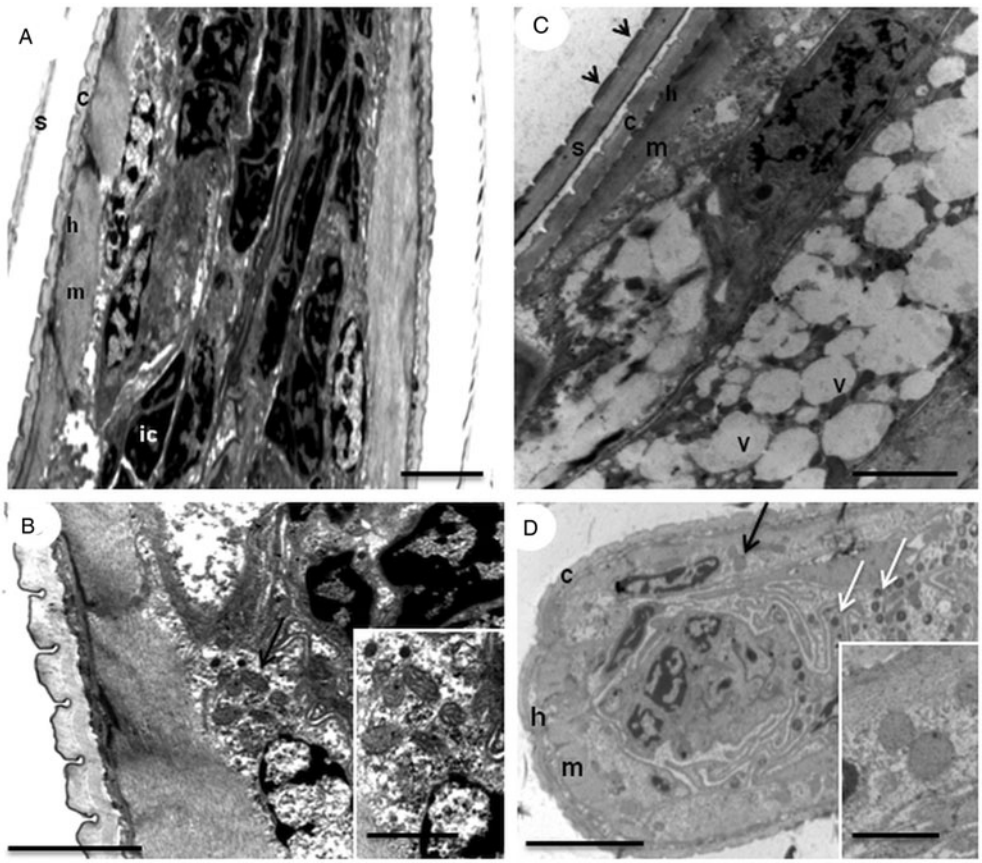

Fig. 6. TEM micrographs of infective larvae (L_3) untreated (A, B) and treated (C, D) with *Cymbopogon citratus* methanolic extract. Larvae were incubated with 25.0 mg mL^{-1} of the extract for 48 h at 27°C in BOD. Cuticle (C); hypodermis (h); somatic muscle cells (m); intestinal cells (ic); vacuoles (v); vesicles (white arrow); mitochondrial profiles (black arrow). Ultrastructural organization was not preserved in the treated larvae and showed vacuoles and vesicles. Mitochondria detail (inset). Bar: 1 μm, 2 μm and 5 μm.

when present, appeared distorted and numerous vacuoles filled the interior cavity (Figs 5B, 5D and 6C); and the mitochondria profile did not contain the matrix and cristae (Fig. 6D). Electron-dense vesicles were also observed filling the body cavity of the larvae (Fig. 6D).

Ultra-thin sections of untreated eggs revealed an internal morphology of the embryo in formation with organelles preserved (Fig. 7A), while eggs treated with methanolic fractions of *C. citratus* presented modifications (Fig. 7B–D). Eggs treated with fractions CCMF-08 and CCMF-10 at concentrations of 500 and 1000 μg mL^{-1}, respectively, showed a deformed embryo at an early stage of the embryogenesis process, while eggs treated with the CCMF-09 fraction at concentrations of 1000 μg mL^{-1} also showed deformed embryos but at a later stage of the embryogenesis process. Electron-dense vesicles were observed traversing the lipid and chitinous layers of the eggshell (inset Fig. 7D) and dispersed in the space between the eggshell and plasma membrane (arrow Fig. 7D).

Discussion

Research on medicinal plants has been increasing in recent years, and new discoveries are constantly being made. Due to interest in addressing the challenges that arise from the use of anthelmintics, particularly parasite resistance to these compounds, the biological activity of many plants has been tested. As such, interest from the pharmaceutical industry in developing new drugs from natural bioactive compounds has been growing, further promoting research on medicinal plants. In this study, extracts from the leaves of *C. citratus* demonstrated nematicidal activity against eggs and L_3 of gastrointestinal nematodes in sheep.

Both the aqueous and MeOH extracts of *C. citratus* showed nematicidal activity against eggs and L_3, but the MeOH extract showed greater effectiveness in terms of ovicidal and larvicidal activity. Previous studies on extracts and essential oil of *C. citratus* have shown effects on nematodes in animals. *C. citratus* aqueous extract reduced the number of *Haemonchus contortus* L_3 by 97% at a concentration of 224 mg mL^{-1} (Almeida *et al.*, 2003). Sheep treated with an oral dose of *C. citratus* alcoholic extract at a concentration of 20 mg kg^{-1} for 1, 3 or 4 consecutive days presented a significant reduction in EPG (Silva *et al.*, 2005). Meanwhile, *C. citratus* essential oil or decoction inhibited *H. contortus* egg hatching and larval development by 90–99% at concentrations of 0.62–10 mg mL^{-1} (Macedo *et al.*, 2015). *Cymbopogon citratus* essential oil nanoemulsion was also shown to inhibit 97.1% of *H. contortus* larvae hatching; however, it was not effective when used in live sheep infected with gastrointestinal nematodes (Macedo *et al.*, 2019). Moreover, *C. citratus* essential oil reduced the *H. contortus* burden by 38.5% in *Meriones unguiculatus* (gerbils) treated with 800 mg kg^{-1} (Macedo *et al.*, 2019). Thus, compounds in *C. citratus* have a lethal effect on nematodes.

Most of the studies on the chemical composition of *C. citratus* relate to its essential oil. Chemical compounds found in the essential oil vary according to the maturity of the plant (Tajidin *et al.*, 2012), environmental and geographical conditions (Abegaz *et al.*, 1983; Torres and Ragadio, 1996; Chisowa *et al.*, 1998; Bassolé *et al.*, 2011) and extraction method (Barbosa *et al.*, 2008; Mohamed Hanna *et al.*, 2012). Nevertheless, citral (a combination of geranial and neral) has been identified as the most abundant

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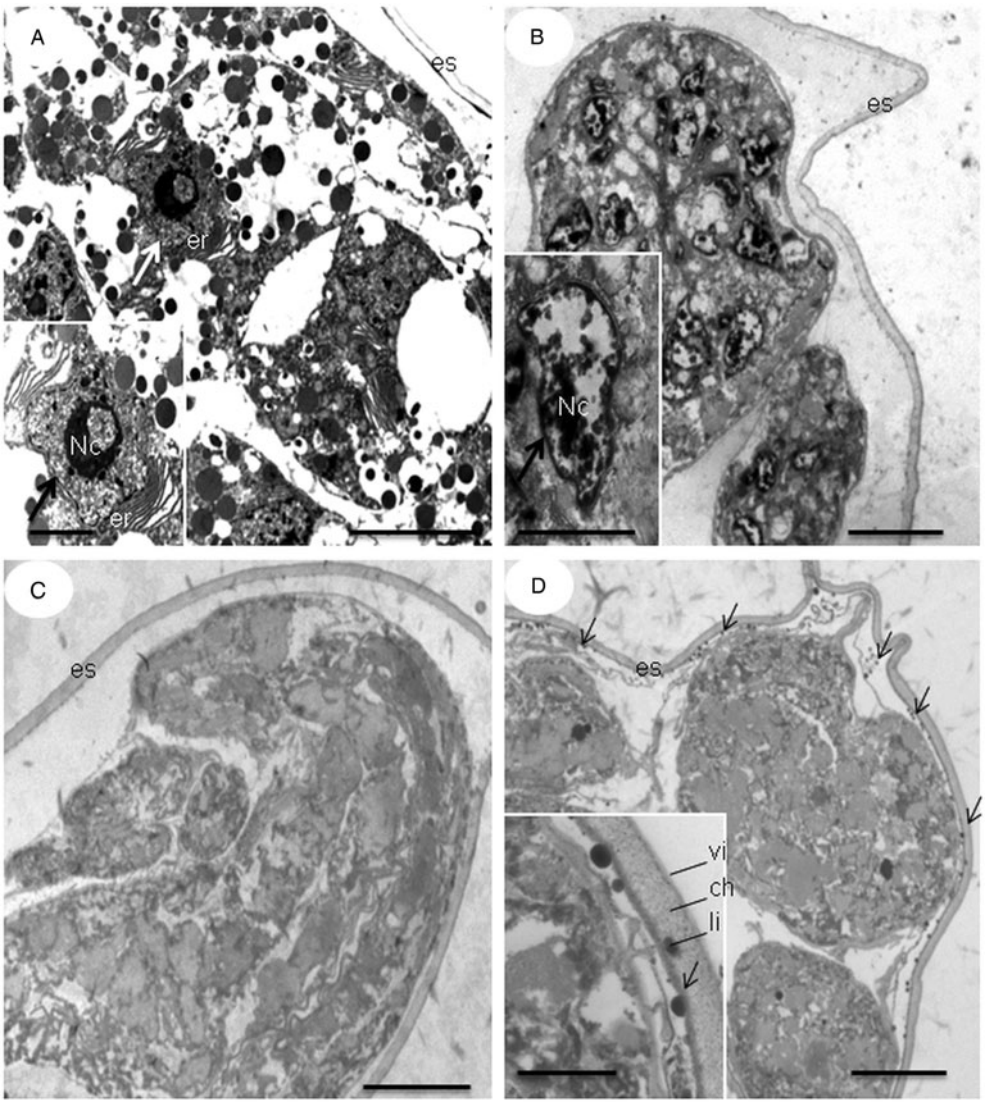

Fig. 7. TEM micrographs of nematode eggs treated with methanolic fractions of *Cymbopogon citratus*. Incubated egg with distilled water (A), and fractions CCMF-08 (B), CCMF-09 (C) and CCMF-10 (D) at concentrations of 500, 1000 and 1000 μg mL^{-1}, respectively. Nucleolus (Nc); rough endoplasmic reticulum (er); eggshell (es); vitelline layer (vi); chitinous layer (ch); lipid layer (li); nucleus (large arrow); vesicles (narrow arrow). Detail of the vesicles traversing the eggshell (inset). Bars: 2 μm and 5 μm.

compound (Saddiq and Khayyat, 2010). However, an exception was found for Ethiopian *C. citratus* essential oil that contained geraniol as its main component (Abegaz *et al.*, 1983). Similarly, analysis of the MeOH extract of *C. citratus* herein did not identify citral as the major compound, but rather a triterpenoid identified as cerin acetate (2α-acetoxyfriedelin).

Unlike the extracts, fractions of *C. citratus* only had an effect on nematode eggs. The lack of activity of *C. citratus* fractions against L_3 indicates that the various individual compounds of the fractions do not interfere with the survival of the larvae or a synergistic action of more fractions is necessary. In contrast, the extracts and fractions from *Combretum molle* and *Vernonia amygdalina* have shown similar effects on both nematode eggs and larvae (Ademola and Eloff, 2011).

Eggs and L_3 have different structural characteristics. The egg has a shell consisting of three layers, the inner lipid, medial chitin and outer vitelline layers (Bird and Bird, 1991), while L_3 has a double cuticle. The cuticle consists of four parts: a triple-layered epicuticle at the external surface, a cortical zone, a median zone and a basal zone. Biochemically, the cuticle consists of structural components, mostly collagen (insoluble in detergent), soluble proteins and low molecular weight components such as lipids (Decraemer *et al.*, 2003). The L_3 cuticle is covered with an additional sheath retained from the L_2. The nematicidal activity of the evaluated extracts was more intense in eggs than in L_3, which is likely due to the ability of the extracts to pass more easily through the eggshell than through the double cuticle of the L_3. As such, the extracts are able to reach the L_1 forming inside the egg, which is at a more fragile stage of life. This limited protective ability of the eggshell may vary with the stage of embryogenesis because the vitelline layer becomes more brittle and water-soluble toward the end of embryo formation (Bird and Bird, 1991). The L_2 cuticle retained by the L_3 functions as an extra protective sheath against environmental conditions (Hansen and Perry, 1994) and extracts have more difficulty crossing this barrier.

Few studies have evaluated the effects of plant extracts on nematode ultrastructure. In a previous study analysing the ultrastructural changes to L_3 of ruminant nematodes treated with *Onobrychis viciifolia* extract, Brunet *et al.* (2011) observed that in the ensheathed L_3, the lesions were mainly located in the hypodermis layer and the muscle cells. However, in the exsheathed L_3, the main damage seemed to occur in the intestinal cells, while changes in the hypodermis and muscular layers were less pronounced than in the ensheathed L_3. Furthermore,

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no alteration was observed in the sheath or the cuticle. Changes to the body surface of *H. contortus* were observed by S.E.M. after exposure to extracts of *Lysiloma latisiliquum* and *Onobrychis viciifolia* (Martínez-Ortíz-de-Montellano *et al.*, 2013) and *Acacia mearnsii* (Yoshihara *et al.*, 2015). All of these plants contain condensed tannins that are phenolic compounds, to which anthelmintic effects have been attributed. Here, *C. citratus* was shown to have an effect on the outermost walls of the body of L_3 of *Haemonchus* spp. and *Trichostrongylus* spp., i.e., sheath and cuticle. An irregular pattern striation, lesions and desquamations were observed in the cuticle and the sheath was electron-dense. Regions such as the hypodermis, muscular layer, intestinal cells and the mitochondria profile were also affected.

Previous studies using plants or synthetics compounds have demonstrated that their anthelmintic activity is related to the presence of terpenoids. Citral and lemonene (Macedo *et al.*, 2010), linalool and carvacrol (Zhu *et al.*, 2013), essential oil (Armstrong *et al.*, 2013), carvacrol (Andre *et al.*, 2016), thymol, carvacrol and eugenol (Hernando *et al.*, 2019), triterpenoids identified as urs-19(29)-en-3-yl acetate, (3β)-Urs-19(29)-en-3-ol and 1-(2',5'-dimethoxy phenyl)-glycerol (Cavalcante *et al.*, 2016), triterpenoid glycosides (saponins) identified as joazeiroside B and lotoside A (Gomes *et al.*, 2016) were some of the related compounds. Besides, other saponins revealed the presence of *in vitro* anthelmintic activity against nematodes eggs of goat (Botura *et al.*, 2013; Santos *et al.*, 2018), donkey (Maestrini *et al.*, 2019), sheep (Maestrini *et al.*, 2020) and L_3 of goat (Santos *et al.*, 2018). In a similar way, β-sitosterol steroid demonstrated anthelmintic activity against *Ascaris suum* (Villaseñor *et al.*, 2002) and sheep gastrointestinal nematodes (Giovanelli *et al.*, 2018). Interestingly, it was found that a hydroethanolic extract of the fungus *Pleurotus djamor* contained a fraction with ovicidal activity against *H. contortus*, constituted mainly of free fatty acids with less than 1% of β-sitosterol steroid (Pineda-Alegria *et al.*, 2017). Thus, terpenoids and β-sitosterol steroid found in *C. citratus* may be responsible for the anthelmintic effects described here.

Concluding remarks

This *in vitro* study of *C. citratus* plant extract is quite promising and validates popular knowledge of the medicinal properties of the plant. The extracts and fractions from *C. citratus* showed nematicidal activity in the assay as well as ultrastructural changes at various nematode life stages. In the continuing search for alternative means of controlling gastrointestinal nematodiasis, medicinal plants represent an important opportunity to pursue.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182020001432.

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