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Research paper

Toxic action of *Acmella oleracea* extract on the male reproductive system of *Amblyomma cajennense* ticks

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ABSTRACT

The present study evaluated through morphohistological and histochemical techniques the effects of different concentrations of crude ethanolic extract of *A. oleracea* (EEAO) (Jambu) on the male reproductive system of *Amblyomma cajennense* sensu stricto (s.s.) ticks. The toxicity of this natural chemical was stablished, signalizing the promising potential of the compound as a strategy to control ectoparasites in the near future. For the experiment, 100 males fed on host rabbits with homogeneous weight ($p > 0.05$) were used. The ticks were divided into five groups (10 animals each): Control 1–exposed to distilled water; Control 2–exposed to ethanol 50% and DMSO 1%; Treatment 1–3–exposed to the concentrations of 6.2, 12.5 and 25 mg/mL of the EEAO, respectively, diluted in ethanol 50% and DMSO 1%, with exposure by immersion. After exposure, the males were dissected for the removal of the reproductive system and subjected to routine histological analysis with HE staining and histochemical techniques (PAS for the detection of neutral polysaccharides and Bromophenol blue to detect total proteins). The exposed individuals showed alterations in the glandular complex cells; however, the testes remained intact. The secretory cells of the multilobulated accessory glands presented intense cytoplasmic vacuolation. Additionally, the synthesis and secretion were reduced in the secretion granules, mainly concerning the polysaccharides, glyco- and lipoprotein elements, substances that will constitute the seminal fluid and enable the capacitation of spermatozoa in the female genital tract and also necessary for the formation of the spermatophore, which will encapsulate the mature spermatids. The alterations were dose-dependent, i.e., more intense and severe as the concentration of the product increased. .This experiment confirmed the cytotoxic potential of *A. oleracea* ethanolic extract in the concentrations of 6.2, 12.5 and 25 mg/mL on the reproductive system of *A. cajennense* s.s. male ticks.

1. Introduction

Ticks of the complex *Amblyomma cajennense* (Fabricius, 1787) have great medical and veterinary importance, once they parasitize different hosts, including the human beings. These ectoparasites are potential vectors of *Rickettsia rickettsii*, causative agent of Brazilian Spotted Fever or Rocky Mountain Fever (Beati et al., 2013; Guedes et al., 2005; Labruna, 2009; Soares et al., 2015; Tarragona et al., 2015), being found throughout the American continent, from the South of the U.S.A. and Central America to the North of Argentina (Beati et al., 2013; Estrada-Peña et al., 2014; Martins et al., 2016). With specific regard to Brazil, the species *A. cajennense* s. s. is spread in the western north of the Amazon Basin (Martins et al., 2016).

Currently, the most efficient and widely used method to control ticks is the use of chemical acaricides, mainly the synthetic ones; however, these substances present serious inconveniences, such as the high cost (Oliveira et al., 2011, 2015). Moreover, the indiscriminate use of such compounds induce the selection of resistant individuals, their toxicity can be lethal to non-target organisms and the residues accumulate in the environment, contaminating soils, water streams, fauna and flora, affecting animals in general and the human beings as well (Nolan, 1985; Oliveira et al., 2008; Pruett, 1999; Roma et al., 2009).

In this sense, the search for alternative control strategies is ongoing, and among these strategies is the use of vaccines and natural chemical

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acaricides, usually obtained from plant extracts (Castro et al., 2014; Oliveira et al., 2016).

Acmella oleracea is a plant of the family Asteraceae, found in tropical regions close to the Equator in Africa, South America and Asia. It is widely used in popular medicine as a potent local analgesic and anesthetic, and also presents fungistatic, fungicide and insecticide properties (Dubey et al., 2013; Favoreto and Gilbert, 2010).

Recent studies by Castro et al. (2014) and Oliveira et al. (2016) have demonstrated that crude hexane extracts obtained from the aerial parts of *A. oleracea* (flower, leaf and stem) have acaricide action, causing a high mortality rate in *Rhipicephalus (Boophilus) microplus* larvae and engorged females, in addition to interfering in the female reproductive system dynamics, significantly reducing oviposition and hatchability.

In ticks, the mating results in profound physiological changes that eventually results in egg production (Donohue et al., 2009). In the *Dermacentor variabilis*, for example, mating causes partially blood-fed female ticks to commence rapid engorgement to repletion and eventual detachment from the host and egg laying (Donohue et al., 2009). The peptidic male pheromone (engorgement factor α/β) transferred to the female during mating is known only from a single tick species, *Amblyomma hebraeum* (Donohue et al., 2009).

Thus, the present study had the objective to evaluate the effects of different concentrations of *A. oleracea* ethanolic extract on the morphophysiology of the reproductive system of fed *Amblyomma cajennense* s.s. males, in order to stablish whether this natural chemical has the potential to be used as a strategy to control this important ectoparasites.

2. Materials and methods

2.1. Obtaining the Acmella oleracea (L.) R. K. Jansen

The plants were cultivated in the experimental field (geographic coordinates 22° 47′ 52″ S, 47° 6′ 49″W) of the Chemical, Biological and Agricultural Research Center (CPQBA) of UNICAMP, Paulinia, SP, Brazil, and identified by PhD John F. Pruski from the Missouri Botanical Garden (USA). The voucher specimen was 181.452, deposited at the CPQBA/UNICAMP Herbarium (number 181,452). Authorization from Genetic Heritage Management Council (CGEN) was under number 010577/2014-9.

2.2. Preparing the crude ethanolic extract of Acmella oleracea

The crude ethanolic extract of the aerial parts of *A. oleracea* was obtained at Natural Products Chemistry Division from Chemical, Biological and Agricultural Research Center CPQBA/UNICAMP, Campinas, SP, Brazil.

The aerial parts (flowers, leaves and stem) of *A. oleracea* were dried and milled in a knife mill (Primotecnica®) using 0.5 mm sieve. The extraction was performed under mechanical agitation with 96° GL ethanol (1:5 plant/solvent, w/v ratio) in a stainless-steel vessel with 5 l' capacity for 1.5 h. The remaining residue was filtered and the extraction step was repeated twice with new solvent portion. The crude extract was filtered, pooled and concentrated in rotary vacuum evaporator at 40 °C. Then, the material was lyophilized until constant mass, stored in amber flasks and kept in freezer at −18 °C until use.

The chemical analysis of the extract was performed as Anholeto et al. (2017).

2.3. Amblyomma cajennense s.s. ticks

Ticks used in this study derived from a laboratory colony that started with adult ticks collected from the vegetation in Governador Jorge Teixeira Municipality, state of Rondônia, western Brazilian Amazon. Species identification followed Martins et al. (2016). For the bioassays, *A. cajennense* s.s. males adults weighing on average 10 mg were used. The ticks fed for 10 days on host rabbits provided by the Animal Facilities of UNESP, Botucatu Campus, SP, Brazil. The feeding stage was chosen to approximate the experiment to field conditions, once in this phase the ticks remain attached to the host ingesting blood, causing damages to the host organism and transmitting pathogens. The artificial infestations were performed according to the protocol described by Bechara et al. (1995). During the entire experiment, animals were maintained in cages in the Animal Facilities of UNESP, Rio Claro Campus, SP, Brazil, and received water and rabbit food *ad libitum*. This study was approved by the Ethics Committee for Animal Experimentation of UNESP, Rio Claro Campus, SP, Brazil, protocol number 11/2015.

2.4. Experimental design

The Adult Immersion Test (Drummond et al., 1973) was used for the tick exposure to the crude ethanolic extract of *A. oleracea*.

Adult ticks with homogeneous weights ($p > 0.05$), were divided into 5 groups (10 ticks each):

Control Group 1–exposed to distilled water;

Control Group 2–exposed to ethanol solvent at 50% and dimethyl sulfoxide (DMSO) at 1%;

Treatment 1–3–exposed to the concentrations of 6.2; 3.1 and 12.5 mg/mL of the crude ethanolic extract of *A. oleracea* aerial parts, respectively, obtained by dilution in ethanol 50% and DMSO 1%. The sublethal concentrations used in the treatment were based on studies by Anholeto et al. (2017) and all tests were performed in duplicate.

Prior to the experiment, ticks were washed in running tap water and dried with soft absorbent paper. The ticks were immersed in the concentrations for 5 min, dried in absorbent paper, mounted on labeled Petri dishes, and kept in BOD incubator (28 ± 1 °C, 85% humidity and 12 h photoperiod) for 7 days. This period was suggested by Oliveira et al. (2008) in studies with *R. sanguineus* sensu lato (s.l.) females subjected to fipronil, since the action of the acaricide is not immediate, acting slowly on the physiology of the individuals and the morphological alterations usually occur after seven days.

2.5. Morphophysiological evaluation

After exposed to sublethal concentrations of the crude ethanolic extract, the *A. cajennense* s.s. males were mounted on Petri dishes with phosphate buffered saline (NaCl 7.5 g/L, Na_2HPO_4 2.38 g/L and KH_2PO_4 2.72 g/L) and dissected using stereomicroscope.

2.5.1. Histology

a **Harris hematoxylin and aqueous eosin technique (Junqueira and Junqueira, 1983).**

The material was fixed in paraformaldehyde 4% for 48 h, and transferred to phosphate buffered saline (NaCl 7.5 g/L, Na_2HPO_4 2.38 g/L and KH_2PO_4 2.72 g/L), remaining for 24 h. Then, the samples were dehydrated in graded ethanol series 70%, 80%, 90% and 95% (30 min each bath), embedded in historesin containing an infiltration solution with 50 mL of (2-hydroxyethil) methacrylate and 0.5 g of dibenzoylperoxide, according to the manufacturer's orientation (*Leica Historesin Embedding Kit*®) for 24 h and included in plastic molds containing historesin and polymerizer (*Leica Historesin Kit*®). After, the blocks were sectioned in microtome Leica RM2265 (Leica®) at 3-μm thickness, mounted on glass slides, rehydrated in distilled water for 1 min and stained with Harris hematoxylin for 8 min. Then, the material was washed in running tap water for 3 min, stained with aqueous eosin for 5 min and washed in running tap water again. After drying at room temperature, the samples were rapidly immersed in xylol, covered with Canada balsam and a coverslip.

The permanent slides were analyzed and documented using bright-field microscope Leica DM750 (Leica®).

b **Histochemistry**

Histochemical tests were applied in order to detect the presence of polysaccharides (PAS technique – periodic acid Schiff) according to Junqueira and Junqueira (1983) and proteins (Bromophenol blue technique) according to Pearse (1985).

3. Results

3.1. Histology

3.1.1. Control groups I and II

The reproductive system of *A. cajennense* s.s. males fed for 10 days on host rabbits (Control Groups I and II) showed typical characteristics, previously described by Anholeto et al. (2015) and Sampieri et al. (2014), subdivided into two regions: anterior and posterior. The anterior region consists of a multilobulated accessory gland complex (Fig. 1**E–H**) associated with a pair of seminal vesicles and connected to the ejaculatory duct (Fig. 1**A**). The glandular complex is subdivided into four distinct regions: a) anterodorsal (Fig. 1**E**), b) laterodorsal (Fig. 1**F**), c) posterodorsal (Fig. 1**G**) and d) posterolateral (Fig. 1**H**) lobes differing according to their location, morphology and type of synthetized molecules (proteins, polysaccharides and lipids) that will constitute the spermatic fluid and the spermatophore. The posterior region contains a pair of elongated and tubular testes, connected to the multilobulated accessory gland complex through deferent ducts.

The testes are surrounded by a simple squamous epithelium, which penetrates the organ dividing it into spermatocytes, and are subdivided into three distinct regions, classified according to the germ cells developmental stages, as follows: a) proximal region (Fig. 1**B**), in contact with the deferent ducts, which are connected with the accessory gland complex. It contains the primary spermatids in early stages of development (immature); b) medial or intermediate region (Fig. 1**C**), where the germ cells are in the intermediate stage of development (secondary spermatids) and c) distal or posterior region (Fig. 1**D**) where the mature spermatids are found.

3.1.2. Treatment I – 6.2 mg/mL

The males of this group, exposed to the crude ethanolic extract of *A. oleracea* at the concentration of 6.2 mg/mL showed morphological alterations in the reproductive system in comparison with the individuals from the control groups I and II (Fig. 1**I–M**). The testes and the laterodorsal and posterodorsal lobes of the accessory glands did not present significant alterations, while the cells of the anterodorsal lobe had the cytoplasm full of large vacuoles (Fig. 1**I**). The nuclei of these cells were irregular and strongly stained with hematoxylin, suggesting the onset of pyknosis (Fig. 1**I**). The secretion granules in the lobe lumen were less frequent than the ones observed in the individuals belonging to the Control Group (Fig. 1**I**). The secretory cells of the posterolateral lobes showed cytoplasmic granules strongly stained with hematoxylin and the nuclei of these cells were irregular and strongly stained (Fig. 1**M**).

3.1.3. Treatment II – 12.5 mg/mL

In this group, the males showed more intense morphological alterations than those from Treatment I (Fig. 1**J** and **N**). The testes were intact; however, alterations were observed in the accessory glands. The cells of the anterodorsal lobe had the cytoplasm intensely vacuolated and with few secretion granules (Fig. 1**J**). The nuclei of the cells were irregular and strongly strained by hematoxylin (Fig. 1**J**).

The cytoplasm of the cells in the posterolateral lobes was reduced due to the presence of large vacuoles (Fig. 1**N**). The nuclei of these cells were irregular and intensely stained by hematoxylin (Fig. 1**N**). In comparison with the Control Group, fewer secretion granules were observed (Fig. 1**N**).

The laterodorsal, posterodorsal and posterolateral lobes showed similar results to the ones found in Treatment I.

3.1.4. Treatment III – 25 mg/mL

The results obtained for the males exposed to the crude ethanolic

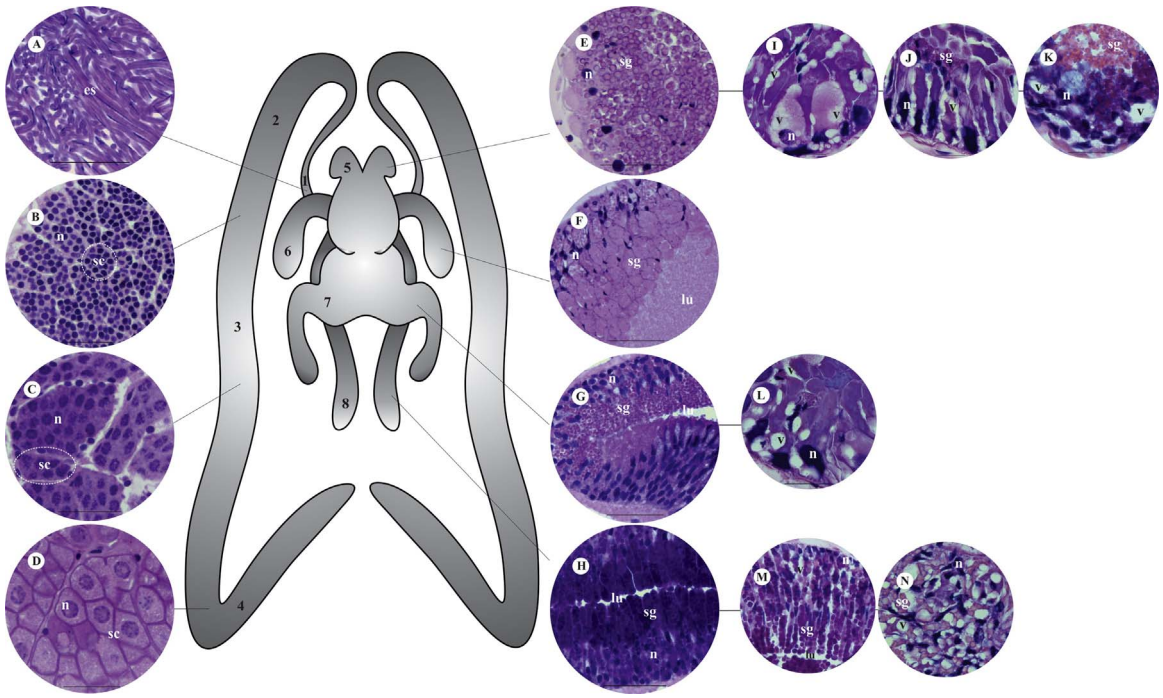

Fig. 1. Schematic representation and histological sections of *Amblyomma cajennense* s.s. male reproductive system stained by hematoxylin and eosin **A** = Histological section of the seminal vesicle – Control I and II. **B–D** = Histological sections of the testes – Control I and II. **E–H** = Histological sections of the multilobulated accessory gland complex (agc) – Control I and II. **I;M** = Histological sections of the agc of the males exposed to 6.2 mg/mL of the EEAO. **J;N** = Histological sections of the agc of the males exposed to 12.5 mg/mL of the EEAO. **K;L** = Histological sections of the agc of the males exposed to 25 mg/mL of the EEAO. **agc** = accessory gland complex. **es** = elongated spermatid. **sc** = spermatocytes. **sg** = secretion granules. **lu** = lumen. **n** = nucleus. **v** = vacuole. **1** = seminal vesicle. **2** = proximal region of the testes. **3** = medial region of the testes. **4** = distal region of the testes. **5** = anterodorsal lobe. **6** = laterodorsal lobe. **7** = posterodorsal lobe. **8** = posterolateral lobe. Bars **A–D** = 50 μm; **E-N** = 20 μm.

extract of *A. oleracea* at the concentration of 25 mg/mL, reveled the occurrence of the most severe morphological alterations in the accessory glands (Fig. 1**K** and **L**); however, the morphology of the testes was not affected.

The cells of the laterodorsal lobe had the cytoplasm vacuolated and with few secretion granules, damages observed in great part of the tissue (Fig. 1**K**).

In the posterodorsal lobes, the secretory cells had the cytoplasm moderately stained by eosin, in addition to secretion granules and large round-shaped vacuoles occupying most part of it. The nuclei of these cells were irregular and strongly stained by hematoxylin (Fig. 1**L**).

The laterodorsal lobes showed similar morphology to the Control Group, i.e., without alterations.

3.2. Histochemistry

In addition to histological techniques, histochemical tests were performed on the male reproductive system of *A. cajennense* males for the detection of polysaccharides (PAS)(Fig. 2) and total proteins (Bromophenol blue)(Fig. 3). For a better understanding, the results are shown in tables (Tables 1–2).

a **PAS reaction for the detection of neutral polysaccharides**

b **Bromophenol blue reaction for the detection of total proteins**

4. Discussion

The most efficient and widely used control method is based on synthetic chemicals; however, it has to be periodically replaced, once the active ingredients induce the selection of resistant ticks (Abbas et al., 2014; Crampton et al., 1999; Nolan, 1985).

Under this perspective, the search for acaricides based on plant extracts has been intensified, considering the indiscriminate use of synthetic acaricides and their toxic effects on the environment, non-target organisms and on the human health as well (Borges et al., 2011).

In this regard, plants as the ones from the family Meliaceae, including the genera *Melia* (Oelrichs et al., 1983)*, Trichilia, Toona, Aglaia, Azadirachta* (Martinez, 2002)*, Carapa* (Vendramini et al., 2012), and the family Asteraceae, as *Calea serrata, Artemisia annua* (Chagas et al., 2011; Ribeiro et al., 2008) and *Acmella oleracea* have been acknowledged as promising alternatives to control populations of arthropods (Castro et al., 2014; Oliveira et al., 2016).

Thus, the present study had the objective to evaluate the effects of *A. oleracea* ethanolic extracts at the sublethal concentrations of 6.2, 12.5 and 25 mg/mL on the morphophysiology of the reproductive

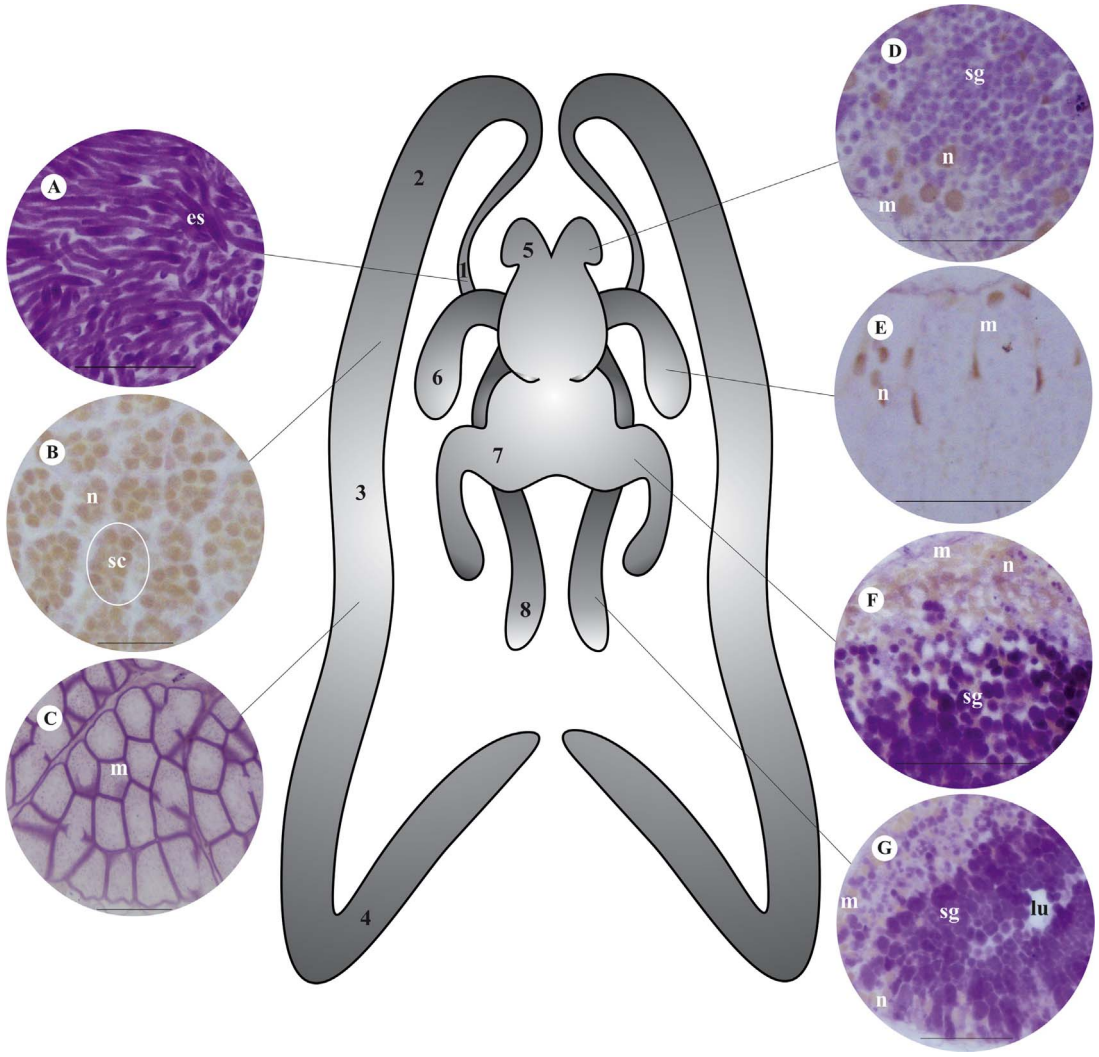

Fig. 2. Schematic representation and histological sections of the male reproductive system of *Amblyomma cajennense* s.s., subjected to PAS reaction (periodic acid Schiff) **A** = Histological section of the seminal vesicle – Control I and II. **B-C** = Histological sections of the testes – Control I and II. **D–G** = Histological sections of the multilobulated accessory gland complex (agc) – Control I and II **agc** = accessory gland complex **es** = elongated spermatid **sc** = spermatocytes. **sg** = secretion granules. **m** = membrane **lu** = lumen **n** = nucleus **v** = vacuole **1** = seminal vesicle **2** = proximal region of the testes **3** = medial region of the testes **4** = distal region of the testes **5** = anterodorsal lobe **6** = laterodorsal lobe **7** = posterodorsal lobe **8** = posterolateral lobe. Bars **A–C** = 50 µm; **D–G** = 20 µm.

Table 1
Results of the PAS (periodic acid Schiff) histochemical test on the male reproductive system of *A. cajennense* s.s. for the detection of neutral polysaccharides.

	Organ region	Spermatids	Control I and II	Figure	Treatment Group I (6.2 mg/mL)	Treatment Group II (12.5 mg/mL)	Treatment Group III (25 mg/mL)
Testes	Proximal	*membrane*	–	**2B**	–	–	–
		cytoplasm	–		–	–	–
	Medial	*membrane*	+	**2C**	Ø	Ø	Ø
		cytoplasm	+		Ø	Ø	Ø
	Distal	*membrane*	Ø		Ø	Ø	Ø
		citoplasm	Ø		Ø	Ø	Ø
Multilobulated accessory gland	Lobes	Secretory cells					
	Anterodorsal	*membrane*	–	**2D**	–	Ø	–
		cytoplasm	+ +		+ +	Ø	+ +
		secretion granules	+ +		+ +	Ø	+ +
	Laterodorsal	*membrane*	–	**2E**	–	–	–
		cytoplasm	–		–	–	–
		secretion granules	–		–	–	–
	Posterodorsal	*membrane*	–	**2F**	–	–	–
		cytoplasm	+ + +		+ + +	+ + +	+ + +
		secretion granules	+ + +		+ + +	+ + +	+ + +
	Posterolateral	*membrane*	–	**2G**	–	Ø	Ø
		cytoplasm	+ + +		+ + +	Ø	Ø
		secretion granules	+ + +		+ + +	Ø	Ø
	Region	Eloganted spermatids					
	Seminal vesicle	*membrane*	–	**2A**	–	–	–
		cytoplasm	+ + +		+ + +	+ + +	+ + +

+, weakly positive; + +, moderately positive; + + +, strongly positive; −, negative; Ø, region not observed.

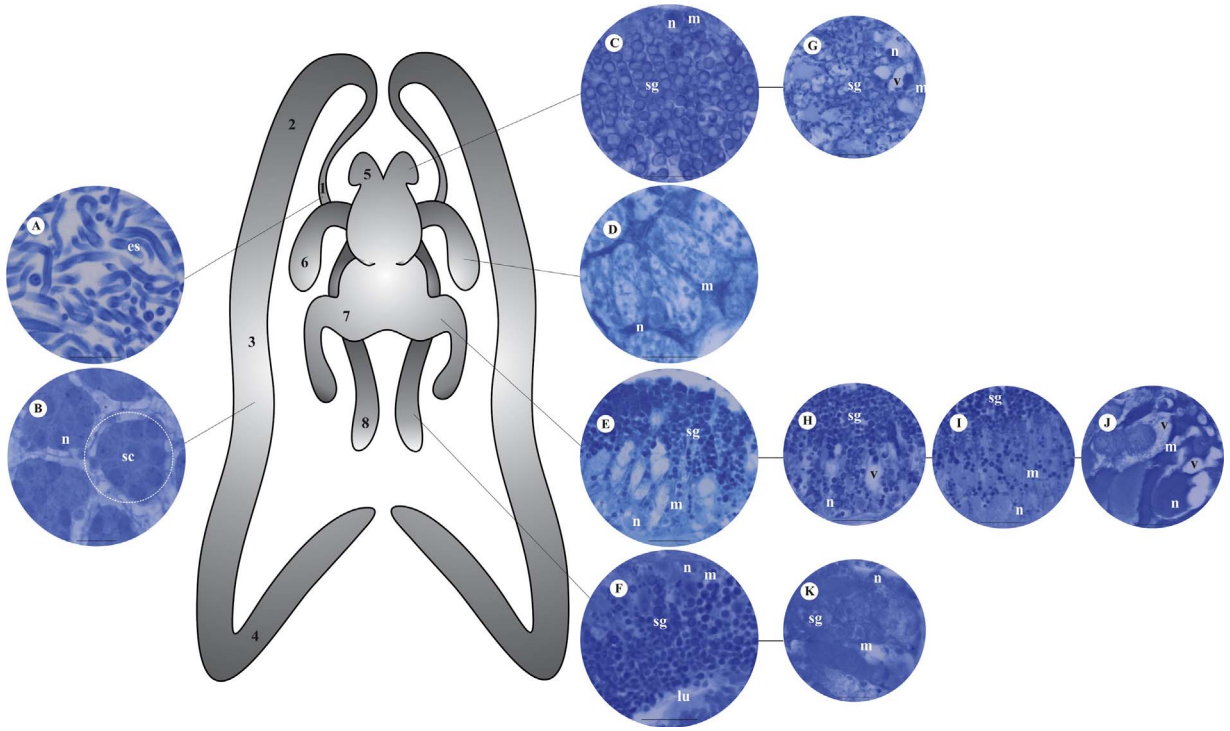

Fig. 3. Schematic representation and histological sections of the male reproductive system of *Amblyomma cajennense* s.s. subjected to Bromophenol blue reaction **A** = Histological section of the seminal vesicle – Control I and II. **B** = Histological section of the testes – Control I and II. **C-F** = Histological sections of the multilobulated accessory gland complex (agc) – Control I and II **G–H** = Histological sections of the agc of the males exposed to 6.2 mg/mL of the EEAO **I;K** = Histological sections of the agc of the males exposed to 12.5 mg/mL of the EEAO **J** = Histological sections of the agc of the males exposed to 25 mg/mL of the EEAO **agc** = accessory gland complex **es** = elongated spermatid **sc** = spermatocytes **sg** = secretion granules **m** = membrane **lu** = lumen **n** = nucleus **v** = vacuole **1** = seminal vesicle **2** = proximal region of the testes **3** = medial region of the testes **4** = distal region of the testes **5** = anterodorsal lobe **6** = laterodorsal lobe **7** = posterodorsal lobe **8** = posterolateral lobe. Bars **A** and **B** = 50 μm; **C–K** = 20 μm.

Table 2
Results of the histochemical test with Bromophenol blue on the male reproductive system of *A. cajennense* s.s. for the detection of total protein.

	Organ region	Spermatids	Control I and II	Figure	Treament Group I (6.2 mg/mL)	Figure	Treatment Group II (12.5 mg/mL)	Figure	Treatment Group III (25 mg/mL)	Figure
Testes	Proximal	*membrane*	Ø		Ø		Ø		Ø	
		citoplasm	Ø		Ø		Ø		Ø	
		nucleus	Ø		Ø		Ø		Ø	
	Medial	*membrane*	+ +	**3B**	+ +		+ +		+ +	
		cytoplasm	+ + +		+ + +		+ + +		+ + +	
		nucleus	+ + +		+ + +		+ + +		+ + +	
	Distal	*membrane*	Ø		Ø		Ø		Ø	
		cytoplasm	Ø		Ø		Ø		Ø	
		nucleus	Ø		Ø		Ø		Ø	
Multilobulated accessory gland	Lobes	Secretory cells								
	Anterodorsal	*membrane*	+ +	**3C**	+ +	**3G**	Ø		+ +	
		cytoplasm	+ +		+ +		Ø		+ +	
		secretion granules	+ +		+ +		Ø		+ +	
		nucleus	+ + +		+ + +		Ø		+ + +	
	Laterodorsal	*membrane*	+ + +	**3D**	+ + +		+ + +		+ + +	
		cytoplasm	+		+		+		+	
		secretion granules	+ +		+ +		+ +		+ +	
		nucleus	+ + +		+ + +		+ + +		+ + +	
	Posterodorsal	*membrane*	+ +	**3E**	+ +	**3H**	+ +	**3I**	+ +	**3J**
		cytoplasm	+ +		+ + +		+ +		+ +	
		secretion granules	+ + +		+ + +		+ + +		Ø	
		nucleus	+ +		+ +		+ +		+ +	
	Posterolateral	*membrane*	+ +	**3F**	+ +		+ +	**3K**	+ +	
		cytoplasm	+ +		+ +		+ +		+ +	
		secretion granules	+ + +		+ + +		+ + +		+ + +	
		nucleus	+ +		+ +		+ +		+ +	
	Region	Eloganted spermatids								
	Seminal vesicle	*membrane*	+ + +	**3A**	+ + +		+ + +		+ + +	
		cytoplasm	+ + +		+ + +		+ + +		+ + +	
		nucleus	+ + +		+ + +		+ + +		+ + +	

+, weakly positive; + +, moderately positive; + + +, strongly positive; −, negative; Ø, region not observed; * presence of negative cytoplasmic vacuoles.

system of *Amblyomma cajennense* s.s. male ticks.

According to Sampieri et al. (2016), the male reproductive system of *A. cajennense* comprises a pair of tubular testes, responsible for the production and maturation of germ cells until they reach spermatid stage and a complex of multilobulated accessory glands that produce and secrete the spermatic fluid and synthetize the spermatophore. The morphohistological and histochemical analyses performed on the individuals from control groups I and II in this study corroborate Sampieri et al. (2016).

In the testes of the *A. cajennense* ticks analyzed in this study, the germ cells were enclosed by a simple squamous epithelium, where the spermatocytes are formed. According to Sonenshine and Roe (2014), the development of the spermatids occurs in two phases: a) the spermatogenesis, characterized by the mitotic and meiotic divisions of the spermatogonia and spermatocytes, originating the spermatids and b) the spermiogenesis, final phase of the spermatid development (differentiation), originating the spermatozoa. The spermatogenesis in most species from the family Ixodidae begins in the final nymph stage and is concluded soon after ecdysis, as demonstrated by Oliveira et al. (2012) in *Rhipicephalus sanguineus* s.l. However, such dynamics is not a general rule, once in some species the spermatogenesis is concluded only when the male reaches the adult stage and the feeding process starts, as is the case of *Dermacentor variabilis* (Sonenshine and Roe, 2014). Furthermore, some spermiogenic phases are dependent of other physiological processes, such as the development of the adult feeding process and the production of spermatophore and ejaculatory secretions (Feldman-Muhsam and Borut, 1983, 1978; Reger, 1974, 1961; Sonenshine and Roe, 2014). The most advanced spermiogenic stage observed in this study was the mature spermatid, classified by Sampieri et al. (2016) as spermatid V and described as elongated filiform cells (Reger, 1974, 1961; Sampieri et al., 2016).

Some studies developed on the male reproductive system of ticks have reported that the developing germ cells (during spermatogenesis and spermiogenesis) would be sensitive to any physiological alterations occurring in the animal organism, including exposure to substances with proven toxic potential (Sampieri et al., 2016). However, the present study showed that the ethanolic extract of *A. oleracea* at the concentrations of 6.2, 12.5 and 25 mg/mL did not cause significant damage in the spermatids in initial or final phases of development. This can be explained by the fact that, when the spermatids reach the final stages of spermatogenesis, i.e., when they are fully differentiated, the spermatic cell is encapsulated, having then a protective barrier against the direct action of toxic agents, such as the extract analyzed here (Montasser et al., 2005; Resler et al., 2009; Roshdy, 1966; Shepherd et al., 1982). These data oppose Montasser and Amin (2010), who analyzed the testes ultrastructure in *Argas persicus* ticks exposed to ivermectin a macrocyclic lactone, at the concentration of 400 µg/kg host. The authors observed the rupture of the spermatids plasma membranes and organelles, in addition to the presence of multivesicular bodies and numerous vacuoles in the cytoplasm of these germ cells, alterations that prevent the normal development of the spermatids, and, consequently, of the spermatozoa.

The individuals exposed to the crude ethanolic extract of the aerial parts (flower, leaf and stem) of *A. oleracea* at the concentrations of 6.2, 12.5 and 25 mg/mL differently from the ones belonging to the control groups, showed significant morphological alterations in the secretory cells of the accessory gland complex. Such alterations were more intense and severe as the concentration of the chemical increased (dose-dependent), corroborating the results obtained by Sampieri et al. (2015), who exposed male *A. cajennense* ticks to the esters of ricinoleic acid obtained from castor oil.

The ethanolic extract of *A. oleracea*, caused intense and severe cytoplasmic vacuolation in the secretory cells of the accessory gland complex, which in turn reduced the synthesis and storage of the

secretion granules that would constitute the spermatic fluid and the spermatophore (Sampieri et al., 2015; Sonenshine and Roe, 2014). These data corroborate Sampieri et al. (2015), who studied the action of castor oil esters on *A. cajennense* s.l. ticks and observed the significant cytoplasmic vacuolation in the secretory cells of the accessory glands in the individuals treated with higher concentrations of the acaricide, offered to the host rabbits via commercial food. The cytoplasmic vacuoles observed in these cells would probably be autophagic, and would be removing from the system the organelles damaged by the toxic components of the extract. Moreover, this vacuolation could represent the recycling of damaged portions of the cytoplasm, in an attempt to preserve the viability of the cell (Carvalho and Recco-Pimentel, 2012; Junqueira and Carneiro, 2013).

In addition to the intense cytoplasmic vacuolation in the cells of the accessory gland complex, the secretion granules produced by them were reduced, signalizing a drop in the synthesis and secretion of polysaccharides, glycoprotein, mucoprotein, lipoprotein and glycosaminoglycan, elements that constitute the seminal fluid. The lack of these substances can impair the process of spermatozoa capacitation in the female genital tract, and inhibit the formation of the spermatophore (mature spermatids capsule) (Garcia-Fernandez et al., 1998; Sonenshine and Roe, 2014). These are important alterations, once they directly affect the fertilization of the females, as the male sperm and spermatids are transferred to the female genital tract via spermatophore (Anholeto et al., 2015; Sampieri et al., 2015; Sonenshine and Roe, 2014). Oliver and Brinton (1972) studied *Dermacentor variabilis* and *Ornithodoros moubata* ticks and reported that the spermatids capacitation in the female reproductive tract would be activated by a polypeptide produced by the male accessory glands. Lomas and Kaufman (1992) reported the presence of a chemical factor produced by the accessory glands of *A. hebraeum* ticks. After released into the seminal fluid, this substance would act on the degeneration of the salivary glands of the female. The same authors suggested that other chemical factors produced in the male genital tract would influence female engorgement; however, the chemical nature of such factors has not been clarified yet. Thus, alterations in the morphophysiology of the male glandular complex affects the synthesis of the secreted products and, consequently, alters the fertilization and reproduction processes (Sampieri et al., 2015).

The results presented here clearly show that the *A. oleracea* ethanolic extract at the concentrations of 6.2; 12.5 and 25 mg/mL caused morphohistological and physiological damages to the accessory gland complex of *A. cajennense* males; however, such damages were not sufficient to affect the germ cells. Nevertheless, this extract can be considered a promising alternative to control *A. cajennense* ticks once it was able to inhibit the production and secretion of seminal fluids, which would hinder the ectoparasites reproductive process. These findings open the possibility to use the ethanolic extract obtained from the flowers, leaves and stems of *A. oleracea*, as a control strategy.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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