

Rhipicephalus sanguineus sensu lato exposed to a dichloromethane *Acmella oleracea* extract: Acaricide activity and morphophysiological evaluations

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Abstract

The present study verified the efficacy of a dichloromethane *Acmella oleracea* (L.) R. K. Jansen extract against semi-engorged *R. sanguineus* s. l. female ticks and the effects of different concentrations on the germ cells and midgut epithelium of these ectoparasites through morphological and histological analyses. *In vitro* bioassays were performed (adult immersion test). For the cytotoxic evaluations, the semi-engorged females were allocated into five groups. The findings indicate that the dichloromethane *A. oleracea* extract was highly efficient against semi-engorged *R. sanguineus* s. l. females at concentrations higher than 10 mg/mL. The best results were obtained at 25 mg/mL, with an efficacy rate of 95%. The cytotoxic tests indicate that assessed the *A. oleracea* extract caused extensive damages to germ cells and the midgut epithelium of *R. sanguineus* s. l. ticks. The treatment affected the fertility of these ectoparasites, reducing their capacity to produce viable eggs and form the embryo and, consequently, to develop new individuals. Additionally, the treatment with the extract fraction affected the ectoparasites' nutrition, which could delay or impair their development, consequently decreasing their mobility to find a suitable host and preventing them from completing their life cycle and moving to the following instar. Thus, the dichloromethane *A. oleracea* extract is a promising and viable alternative to control *R. sanguineus* s. l. ticks

INTRODUCTION

Ticks are obligate hematophagous ectoparasites responsible for the transmission of several infectious agents (Dantas Torres, 2010) and the cause of injuries to host tissues, including humans. Some of the most important tick-borne diseases are Lyme borreliosis, Rocky Mountain spotted fever, viral encephalitis, ehrlichiosis, anaplasmosis, babesiosis and theileriosis (Dantas-Torres, 2010).

Rhipicephalus sanguineus sensu lato (Latreille, 1806) has been considered an efficient vector of a diversified group of pathogens, affecting both animals and humans (Dantas-Torres, 2008). *R. sanguineus* s. l. is a tick species widely distributed throughout the Americas, Africa and Australia and is probably the most prevalent among all ixodid tick species (Dantas-Torres, 2008, 2010).

Over the last decades, different commercial formulations aimed at controlling ectoparasites were launched in the veterinary market. These products are constantly replaced by new formulations, since ticks increasingly develop resistance to the active principles of many chemicals (Oliveira et al., 2008; 2009), mainly due to the indiscriminate use of such chemicals in an attempt to control infestations. Researchers and industries have made an effort to uncover products that are capable of acting directly on specific physiological mechanisms of the target parasites while, at the same time, offering no harm to non-target organisms, including humans (Oliveira et al., 2008, Oliveira et al., 2009).

The use of natural compounds, such as plant extracts, containing bioactive compounds, defined as active chemical substances from secondary metabolites, with the potential to control different pests, are a promising alternative (Denardi et al., 2010; Vendramini et al., 2012).

An example of a plant species containing bioactive compounds is *Acmella oleracea*, popularly known as Jambú and widely distributed throughout tropical and subtropical regions (Barbosa et al., 2016a). This species is a semi-erect herbaceous perennial with decumbent branches and yellow flowers arranged in globose chapters (Sharma et al., 2012).

Several biological activities of *A. oleracea* extracts have been reported in the literature. *Acmella oleracea* extracts were also shown to be useful as insecticides (Sharma et al., 2012).

The activities of aqueous (Chungsamarnyart and Jansawan, 1993), ethanolic (Chungsamarnyart and Jansawan, 1993) and hexane extracts have also been investigated (Castro et al., 2014; Oliveira et al., 2016). Castro et al. (2014), for example, evaluated the action of the hexane extract and attributed tickcide effects to the presence of spilanthol.

Therefore, the aim of the present study was to verify the efficacy of a dichloromethane *A. oleracea* extract against semi-engorged *R. sanguineus* s. l. female ticks and the effects of different concentrations on the germ cells and midgut epithelium of these parasites through morphological and histological analyses. The experiments were conducted to clarify the action of this extract on *R. sanguineus* ticks in order to provide data for the development of less toxic and harmful control methods and/or to improve existing methods for resistance avoidance and the preservation of both the environment and non-target organisms.

MATERIAL AND METHODS

2.1. Chemical Substance

2.1.1. Natural: *Acmella oleracea*

Acmella oleracea leaves, stems and inflorescences were collected in Belém, located in the state of Pará, Brazil (01°45'23"S and 48°50'37"W). A voucher specimen was identified and deposited (MG237142) at the Emílio Goeldi Museum herbarium, in Belém, Pará, Brazil.

2.1.2. Preparation of the *Acmella oleracea* extract

Experimental procedures were performed according to Barbosa et al. (2016b). The collected material (leaves, stems and inflorescences) was dried through air circulation in a climatized room (40°C), crushed using a mill and submitted to extraction with methanol alcohol using a soxlet equipment at 30 °C for 24 hours. The solvent was then evaporated using a rotary evaporator at 40°C.

2.1.3. Preparation of the dichloromethane *Acmella oleracea* extract

The methanol *A. oleracea* extract was solubilized in MeOH/H₂O (8:2), followed by successive extractions using a separatory funnel with hexane, followed by dichloromethane (CH₂Cl₂).

2.1.4. Chemical analyses

The dichloromethane *A. oleracea* extract was analyzed by isocratic high-performance liquid chromatography on a Hitachi Lachron Elite L-2000 HPLC (Merck) with a Lichrospher C18 5 µm (125×4 mm) column (Merck), using acetonitrile-water (30:70) as the mobile phase, at a flow rate of 1.0 mL/min, with a UV detector at 229 nm, running time of 10 min and a 20 µL injection volume (Internal Development).

2.2. *Rhipicephalus sanguineus* s. l. ticks (Latreille, 1806)

Semi-engorged *R. sanguineus* s. l. females, with about 5 days of feeding, were used throughout the experiment. The ticks were collected from dogs in the city of São Luís /MA, Brazil (02°31'51"S and 44°18'24"W) and maintained under controlled conditions (28°C, 85% relative humidity, and 12-h photoperiod) in a Biological Oxygen Demand (BOD) incubator at the Federal University of Maranhão (UFMA) São Luís Campus Animal Facility, in the state of Maranhão, Brazil. Unfed *R. sanguineus* s. l. couples (25 couple/infestation) were allowed to feed on naive New Zealand white rabbits, until reaching the semi-engorgement feeding stage. This stage was chosen to emulate field conditions, as the ticks remain attached to the host ingesting blood during this stage, causing host organism damage and transmitting pathogens.

2.3. Hosts

New Zealand White rabbits weighing between 3 and 3.5 kg were used as hosts. The animals had no prior contact with ticks or acaricides and were kept under controlled conditions (24°C and 12-h photoperiod) during the entire experiment, in cages and receiving *ad libitum* water and commercial food. This study was approved by the UNESP/SP/Brazil Ethics Committee for Animal Experimentation, under protocol n°6334/2014.

2.4. Extract doses

The initial extract concentration was established according to Castro et al. (2014) and Cruz et al. (2016). The 5 mg/mL, 6.25 mg/mL, 10 mg/mL, 12.5 mg/mL, 25 mg/mL and 50 mg/mL extract concentrations were obtained by diluting the extract in 50% ethanol and 1% DMSO. All concentrations were stored and labeled in Petri dishes until use. Each treatment was performed in duplicate.

For the cytotoxic assessments using semi-engorged *R. sanguineus* s. l. females, 6.25 mg/mL, 12.5 mg/mL and 25 mg/mL extract concentrations were used.

2.5. Bioassay (Adult Immersion Test - ATI)

The adult immersion test (AIT) was performed according to Drummond et al. (1973). Briefly, the semi-engorged *R. sanguineus* s. l. females were collected, weighed, washed under running tap water and dried using soft absorbent paper. The ticks were then allocated into eight groups containing 10 individuals each, as follows: Control Group I, exposed to distilled water, Control Group II, exposed to ethanol 50% and DMSO 1% and Treatment Groups 1 through 6, exposed to 5, 6.25, 10, 12.5, 25 and 50 mg/mL of the *A. oleracea* extract, respectively. All concentrations were obtained by diluting the *A. oleracea* extract in ethanol 50% and DMSO 1%.

The female ticks of each treatment group were immersed in each investigated *A. oleracea* extract concentration while the ticks belonging to the control groups were immersed in distilled water and ethanol 50% and DMSO 1%, respectively. After 5 minutes, the ticks were dried using absorbent paper, placed on Petri dishes and labelled for biological parameter analyses. All samples were kept in a BOD incubator under controlled conditions (28 ± 1°C, 80% humidity and 12 h photoperiod).

After oviposition, the egg mass produced by each group was weighed using an analytical scale, transferred to syringes and kept in a BOD incubator under controlled conditions ($28 \pm 1^\circ\text{C}$, 80% humidity, 12 h photoperiod). After 30 days, the females were weighed before oviposition, and the egg mass and hatching percentages were evaluated. The latter were obtained through visual evaluation of hatched larva performed by three people, according to Szabó et al. (1995). The efficacy of each treatment was verified through the analysis of the following parameters: **EPI** (egg production index) = (egg mass weight/ female weight before laying) $\times$ 100; **FR** (fecundity rate) = (EPI $\times$ HR)/100; **REI** (reproduction efficiency index) = (egg mass weight/ female weight before laying) $\times$ hatching percentage $\times$ 20,000; **%Rovip** (percentage of oviposition reduction) = [(EWcontrol – EWtreatment)/EWcontrol] $\times$ 100; **%Rhatch** (hatching reduction) = [(HRcontrol – HRtreatment)/HRcontrol] $\times$ 100; **C** (Efficacy of treatment) = (REI control – REI treated)/REI control $\times$ 100.

2.6. Statistical Analyses

The Biostat version 5.0 software was used for all statistical analyses. The average of each parameter for the different treatment groups were analyzed by the ANOVA and Tukey tests ($p < 0.05$).

2.7. Experimental cytotoxic evaluation model

Semi-engorged *R. sanguineus* s. l. females were allocated into the following groups: control group I (exposed to distilled water), control group II (exposed to a 50% ethanol + 1% DMSO solution), group III (exposed to the *A. oleracea* extract at 6.25 mg/mL), group IV (exposed to the *A. oleracea* extract at 12.5 mg/mL) and group V (exposed to the *A. oleracea* extract at 25 mg/mL). Subsequently, 150 semi-engorged *R. sanguineus* s. l. females were allocated into the following groups: 90 females were divided into three groups containing 30 females each (30 females for each concentration, in duplicate) and immersed for 5 minutes in the aforementioned *A. oleracea* extract concentrations. Concerning the control groups I and II, 60 females were immersed in distilled water and in a 50% ethanol and 1% DMSO solution for 5 minutes, respectively. Ticks were then dried using absorbent paper and placed in a BOD incubator ($28 \pm 1^\circ\text{C}$, 80% relative humidity and 12 h photoperiod) for 7 days. After 7 days of monitoring, all semi-engorged females were forwarded to histological analyses.

2.8. Histological analyses

All semi-engorged females were dissected. Tick ovaries and midgut were fixed for 24 hours in 37% formaldehyde, dehydrated in ethanol, embedded in Leica resin for 24 hours and transferred to plastic molds previously filled with polymerized Leica resin. All blocks were then sectioned (3 μm thickness) using a Leica RM 2255 microtome (Bio Rad), stained with hematoxylin and eosin on glass slides and examined under a photomicroscope. All histological equipment belonged to the Multiuser Laboratories at LAMP/UEMA, São Luis Campus, MA, Brazil.

RESULTS

3.1. Chemical analyses

Chemical monitoring was performed using high efficiency liquid chromatography. A chromatogram analyses of the dichloromethane *A. oleracea* extract revealed the presence of spilanthol at of 90%, with the chromatographic peak observed at a retention time of 16 minutes.

3.2. Bioassay (Adult Immersion Test - AIT)

The efficacy of dichloromethane *A. oleracea* extract on semi-engorged *R. sanguineus* s. l. females was evaluated using eight treatment groups, tested in duplicate. Concentrations higher than 10 mg/mL were highly efficient against the semi-engorged *R. sanguineus* s. l. females. At 12.5 mg/mL, a significant reduction in egg production (oviposition) and hatching percentage (68%) was observed. The best results were obtained for the 25 mg/mL dichloromethane *A. oleracea* extract, with an efficacy rate of 95%. AT 5, 6.25, 10, 12.5 and 50 mg/mL, control efficacy was of 27%, 41%, 59%, 68% and 85%, respectively. The bioassay results are described in Table 1.

Table 1

Average of weight of semi-engorged females before oviposition, egg mass weight, larval hatching percentage, egg production index, fecundity rate, reproduction efficiency index, percentage of reduction in oviposition, percentage of reduction of hatching and efficacy of treatment of *Rhipicephalus sanguineus* s.l. treated with different concentrations of *Acmella oleracea* extract fraction under laboratory conditions ($27 \pm 0.5^\circ\text{C}$ and $80 \pm 10\%$ RH) (Average $\pm$ standard deviation).

Treatments	Female weight before oviposition (FW)	Egg mass weight (EW)	Hatching percentage (HR)	Egg production index (EPI)	Fecundity rate (FR)	REI Reproduction efficiency index (REI)	Percentage of reduction in oviposition (Rovip)	Percentage of reduction of hatching (Rhatch)	Product Efficacy (C)
Control group I	2.55 ^a $\pm$ 0.03	1.34 ^a $\pm$ 0.06	89.24 ^a $\pm$ 2.16	52.36 $\pm$ 1.89	46.70 $\pm$ 0.71	933998.51 $\pm$ 14142.97			
Control group II	2.57 ^a $\pm$ 0.02	1.15 ^a $\pm$ 0.01	81.80 ^a $\pm$ 0.43	44.78 $\pm$ 0.37	36.63 $\pm$ 0.32	732667.93 $\pm$ 6491.04	13.682 $\pm$ 3.49	8.31 $\pm$ 1.76	21.55 ^a $\pm$ 1.07
5 mg/mL	2.56 ^a $\pm$ 0.02	1.08 ^a $\pm$ 0.12	80.48 ^a $\pm$ 0.60	42.24 $\pm$ 4.33	34.01 $\pm$ 3.76	680285.01 $\pm$ 75110.57	19.05 $\pm$ 5.36	9.78 $\pm$ 2.90	27.02 ^a $\pm$ 7.36
6.25 mg/mL	2.56 ^a $\pm$ 0.03	1.02 ^a $\pm$ 0.11	69.37 ^b $\pm$ 3.78	39.57 $\pm$ 3.94	27.55 $\pm$ 4.32	550947.72 $\pm$ 86312.88	24.15 $\pm$ 5.01	22.16 $\pm$ 6.21	41.06 ^b $\pm$ 8.67
10 mg/mL	2.58 ^a $\pm$ 0.02	0.81 ^b $\pm$ 0.04	61.23 ^{b,c} $\pm$ 7.77	31.42 $\pm$ 1.44	19.17 $\pm$ 1.50	383344.79 $\pm$ 30071.91	39.17 $\pm$ 5.35	31.22 $\pm$ 10.51	58.7 ^b $\pm$ 2.81
12.5 mg/mL	2.58 ^a $\pm$ 0.01	0.66 ^b $\pm$ 0.11	57.84 ^{b,c} $\pm$ 6.64	25.59 $\pm$ 0.66	14.77 $\pm$ 1.29	295457.84 $\pm$ 25789.87	50.46 $\pm$ 3.00	35.04 $\pm$ 9.11	68.38 ^c $\pm$ 2.46
25 mg/mL	2.57 ^a $\pm$ 0.02	0.27 ^c $\pm$ 0.03	20.39 ^{c,d} $\pm$ 1.28	10.65 $\pm$ 1.39	2.16 $\pm$ 0.16	43186.21 $\pm$ 3145.58	79.42 $\pm$ 3.37	77.12 $\pm$ 2.02	95.37 ^d $\pm$ 0.39
50 mg/mL	2.58 ^a $\pm$ 0.02	0.52 ^b $\pm$ 0.06	34.6 ^c $\pm$ 0.72	20.09 $\pm$ 2.41	6.94 $\pm$ 0.70	138800.88 $\pm$ 13979.01	61.13 $\pm$ 5.83	61.19 $\pm$ 1.77	85.132 ^d $\pm$ 1.65
FW = female weight (mg); EW = egg mass weight (mg); HR = egg hatchability rate (%); EPI = egg production index (); FR = fecundity rate (); REI = Reproduction efficiency index (); Rovip = percentage of reduction in oviposition (%); Rhatch = percentage of reduction of hatching (%); C = Product efficacy (%).									
Different letters in the same column indicate significant differences at the level of 5% ($p < 0.05$; ANOVA/Tukey).									

3.3. Histological analyses

The cytotoxic evaluation results are displayed in Figs. 1 and 2.

Discussion

The immersion tests performed on semi-engorged *R. sanguineus* s. l. females treated with the dichloromethane *A. oleracea* extract indicate high efficiency at concentrations higher than 10 mg/mL, considering decreased egg number and viability, reduced egg mass and reduced fertility and hatchability, among other biological parameters. The best results, of 95% efficacy, were obtained at 25 mg/mL. The 12.5 and 50 mg/mL extracts also provided excellent results, with control rates of 67% and 85%, respectively. Chungsamarnyart et al. (1991) and Chungsamarnyart and Jansawan (1993), when studying *R. microplus* larvae, obtained different results for aqueous and ethanolic *A. oleracea* extracts. The aqueous extract presented no acaricidal action at any concentration (Chungsamarnyart and Jansawan, 1993), while the ethanolic extract presented moderate action at 100 mg/mL (36% mortality after 48h) (Chungsamarnyart et al., 1991). Castro et al. (2014) reported positive results for engorged *R. microplus* females treated with a hexane *A. oleracea* extract, albeit at concentrations higher than 100 mg/mL (efficacy of 59%). Finally, Cruz et al. (2016) reported positive results for engorged *R. microplus* females treated with a methanolic *A. oleracea* extract at 50 mg/mL (efficacy of 85.2%).

Concerning the cytotoxic tests, the ovaries of individuals belonging to the control groups I and II presented typical morphohistological characteristics, i.e., ovaries containing oocytes in five developmental stages (I-V) (Denardi et al., 2004; Oliveira et al., 2005). Individuals

belonging to both groups showed no significant differences compared to each other. However, the immersion tests revealed control rates of 21.55% in control group II. The functional meaning of this data is not clear, considering that this characteristic was not confirmed by histological images, in which oocytes presented a completely normal aspect. No other oocyte alterations were observed, and this characteristic probably did not affect oocyte development in the studied ticks. Thus, it is concluded that the use of 50% ethanol and 1% DMSO did not interfere in the obtained results, only aiding in the extract dilution process.

Regarding the treatment groups, the assessed *A. oleracea* extract resulted in disorganized and vacuolated areas in oocytes in initial development stages (oocytes I, II and III). The damage was progressive, i.e., the higher the concentration, the heavier the damage, leading to more intense autophagic processes, including vacuolation, where cytoplasm portions become damaged and required degradation (Roma et al., 2011; Denardi et al., 2010). At higher concentrations, oocyte cytoplasm presented extensive vacuolated and disorganized areas occupying more than 50% of the cell, which can lead to death, probably through autophagy, a type of programmed cell death. Similar results were reported by Oliveira et al. (2008, 2009) and Roma et al. (2010, 2011) for *R. sanguineus* exposed to fipronil and permethrin, respectively.

The plasma membrane of oocytes in initial development stages presented numerous small to large pleats, resulting in slight to significant deformity, respectively. These alterations were also reported by Vendramini et al. (2012) for *R. sanguineus* treated with andiroba oil and Denardi et al. (2010) for ticks treated with neem aqueous extracts. This may have occurred due to the absence of the chorion, a resistant protective membrane (Denardi et al., 2004; Oliveira et al., 2005) which is fully deposited in oocytes in later development stages, as the plasma membrane alone was not capable to prevent the numerous damages caused by the *A. oleracea* extracts.

Another relevant alteration was observed in the oocyte nuclei in initial development stages, mainly at 12.5 mg/mL and 25 mg/mL, where pleated membranes and nuclear vacuoles were observed. As discussed previously, these structures indicate degenerative processes. Similar findings were reported by Oliveira et al. (2016) for *R. microplus* exposed to a hexane *A. oleracea* extract and Roma et al. (2010, 2011) for *R. sanguineus* exposed to permethrin.

Furthermore, the nucleoli were also fragmented. Other nucleoli alterations have been reported by other authors. For example, Denardi et al. (2010), Roma et al. (2010, 2011) and Vendramini et al. (2012) demonstrated nucleoli emergence as a ring-shaped compact mass after exposing *R. sanguineus* to neem, permethrin, andiroba oil and neem oil extracts, respectively. Such nuclear alterations indicate genetic germ cell material damage. This is a serious effect that can impair ectoparasite survival and which may be lethal or even transmitted to the next parasite generation, impairing the following cycle, as the hatched individuals (in case they are successful in hatching) will probably be less resistant or less adapted.

The present study also demonstrated that the investigated extract affected oocytes in initial development stages, reducing the number and size of yolk granules. The difference in staining regarding the yolk granules of treated individuals seem to indicate that their chemical composition itself was altered. These data are justified by extract action on the cell cytoplasm, resulting in damage and affecting the yolk granules, which are essential for embryo nutrition (Denardi et al., 2004; Oliveira et al., 2005). Consequently, the oocytes cannot store sufficient yolk to develop, i.e., would not be capable of reaching further development stages, not complete vitellogenesis or form the embryo, leading to impaired development of new individuals. Similar data have been reported by Oliveira et al. (2008, 2009) for *R. sanguineus* exposed to fipronil.

The assessed *A. oleracea* extract resulted in disorganized and vacuolated regions in mature oocytes (oocytes IV and V). Oocytes IV presented extensively disorganized areas, while oocytes V exhibited fewer damage. These findings allow us to infer that the chorion, a protective membrane in mature oocytes formed within stages IV and V (Denardi et al., 2004; Oliveira et al., 2005), was not capable of preventing *A. oleracea* extract compounds from penetrating oocytes IV, since degenerative processes were observed at this developmental stage. On the other hand, the chorion is fully deposited in stage V and is capable of preventing significant damage, since few and concentrated alterations were observed in the peripheral region of the investigated cells. These data are in contrast to those reported in the literature regarding ticks treated with other natural compounds, such as aqueous neem extracts (Denardi et al., 2010) and andiroba oil (Vendramini et al., 2012), as those compounds caused minimum damage to mature germ cells.

Considering the data obtained for oocytes in initial development stages and the fact that an immersion test was performed, it can be inferred that the extract compounds crossed the integument and the hemolymph until reaching the ovary, where they penetrated the oocytes via the plasma membrane, damaging intracellular components. On the other hand, oocytes IV displayed significant alterations, mainly in near the pedicel, which suggests that the extract compounds established another entrance path when the chorion began its deposition, i.e., via the pedicel. This is a very efficient entrance path, since a significant reduction in the number and size of the yolk granules in the cytoplasm, disorganized areas and extensive vacuolation throughout the cell were observed. According to Oliveira et al.

(2009) and Vendramini et al. (2012), extract compounds are able to cross the oocyte wall in ticks treated with fipronil and andiroba oil. However, Roma et al. (2010, 2011) and Denardi et al. (2010) exposed ticks to permethrin and an aqueous neem extract and reported that extract compounds are transferred to the oocytes via pedicel cells.

In the present study, the investigated *A. oleracea* extract caused damage to the midgut of *R. sanguineus* s. l. females. The first significant damage was regarding the shape of the generative cells (Stem cells), which became irregular and presented several plasma membrane deformities (ridges and scallops), which was sometimes ruptured, releasing cell content..

Another important alteration was generative cell (stem cells) disorganization and vacuolation. Round-shaped areas (probably lipid drops) were no longer observed due to weak or complete absence of staining. These results are justified by the action of the extract compounds, which damaged the plasma membrane, causing internal damage, including chemical cytoplasm composition alterations. Additionally, heavy vacuolation was observed in the cytoplasmic region in contact with the digestive cell. These structures confirm that these cell components were damaged and undergoing degradation processes, i.e., are evidence of a cell degeneration process (Scudeler et al., 2016). As generative cells are responsible for replacing all the dead cells throughout the digestive processes (Harrison and Foelix, 1999), the observed damage was severe and would certainly impair cell proliferation and differentiation, i.e., the damaged cells would produce faulty cells, or even be incapable of producing cells at all. Therefore, blood nutrient absorption would be prevented, leading to undernourished ectoparasites.

Another relevant alteration was associated with endosome number, size, location and color, digestive vacuoles and digestive residues found in the cytoplasm of digestive cells. Sessile digestive cells and residual sessile digestive cells presented significant decreases in endosome number and size, as well as digestive vacuoles and digestive residues, which turned a greenish color. This damage indicates that *A. oleracea* extract compounds affect digestive cells (destroying the plasma membrane, for example) resulting in impaired functions, including blood collection and intake (Agyei and Runham, 1995), blood cell lysis in digestive vacuoles (Sonenshine and Roe, 2014; Harrison and Foelix, 1999) and the formation of digestive residues (Sonenshine and Roe, 2014; Agyei and Runham, 1995; Harrison and Foelix, 1999). Other authors have reported alterations in digestive cells after treatment with natural and/or synthetic chemicals, such as Oliveira et al. (2014), who investigated *R. sanguineus* treated with fluazuron and Scudeler et al. (2016), who exposed insects to neem oil (*Azadirachta indica*).

Kathrina and Antonio (2004) reported that the secondary metabolites displaying pesticide effects are capable of inhibiting parasite feeding processes, as well as growth and development, reproduction or behavior. Maciel et al. (2010) and Menezes (2005) described three different modes of action for botanical substances on pests, namely toxic action (neurotoxic), action on organs or target molecules or action by ingestion. In the present study, the immersion and cytotoxic test results suggest that the *A. oleracea* extract compounds may act via the nervous system (neurotoxic action) or crossing the integument through the hemolymph, reaching internal *R. sanguineus* s. l. female organs, such as the ovary and midgut, affecting them indirectly and directly, respectively. The extract action via the nervous system was confirmed, as abnormal, repetitive and disordered movements were observed at some extract concentrations in the bioassays. The present study also suggests that *A. oleracea* extract compounds do not necessarily result in tick death, but instead, cause alterations which impair their biological success. As the extract used herein was obtained according to Barbosa et al. (2016b), through a liquid/liquid extraction method using a separatory funnel, organic solvents and fraction isolation, approximately 84–100% spilanthol contents are obtained, allowing for the inference that spilanthol caused the damages reported herein.

CONCLUSION

The present study demonstrated that different *A. oleracea* extract concentrations result in extensive damages to female *R. sanguineus* s. l. ticks. High efficacy in the control of semi-engorged females was observed, due to fertility reduction and consequent decreases in the production of viable eggs, embryo formation and development of new individuals. Additionally, the treatment with the extract fraction affected the ectoparasites' nutrition, which could delay or impair their development, consequently decreasing their mobility to find a suitable host and preventing them from completing their life cycle and moving to the following instar.

Declarations

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CONFLICT OF INTEREST

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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Figures

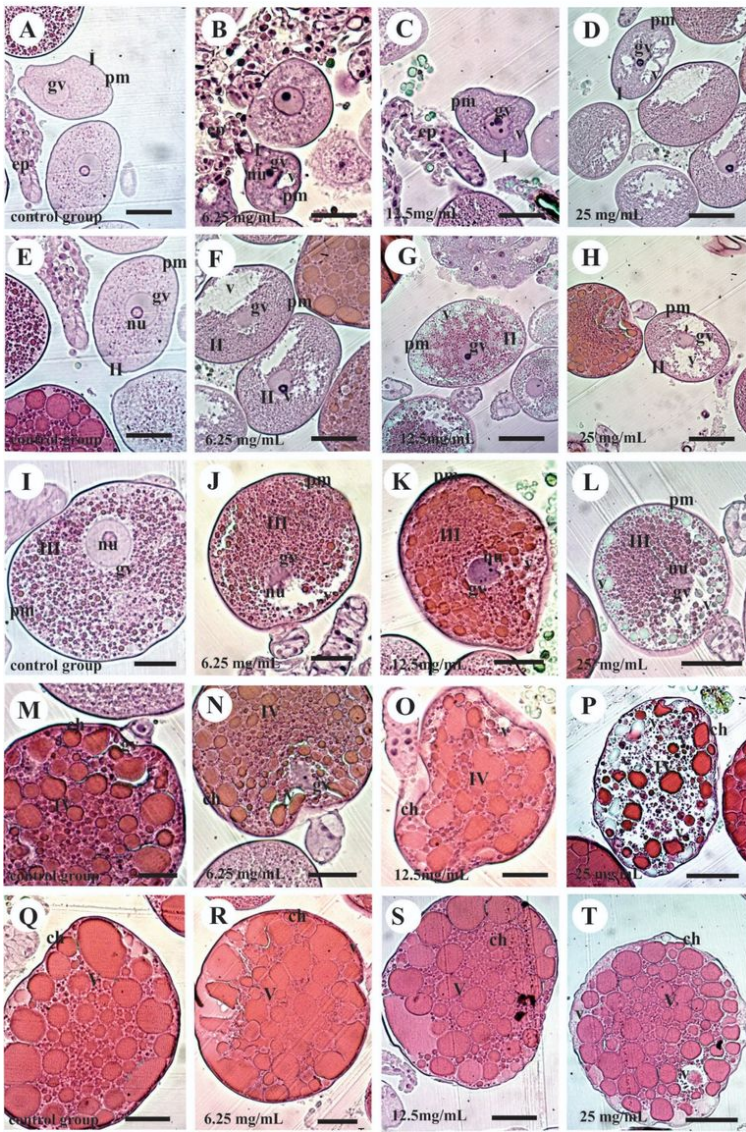


Figure 1

Histological sections of extract-treated semi-engorged *Rhipicephalus sanguineus* s. l. ovary. **A-T.** Hematoxylin and eosin (HE) staining. **A. E. I. M. Q.** Control I and II Groups. **B. F. J. N. R.** Group III (6.25 mg/mL). **C. G. K. O.S.** Group IV (12.25 mg/mL). **D. H. L. P. T.** Group V (25 mg/mL).

I= Oocyte **I**; **II=**Oocyte **II**; **III=**Oocyte **III**; **IV=** Oocyte **IV**; **V=** Oocyte **V**; **ch=** chorion; **ep=** ovary epithelium; **gv=** germ vesicle; **nu=** nucleolus; **p=** pedicel; **pm =** plasma membrane; **yg=** yolk granules, **v=** vacuoles. **Bars:** A-P= 50um, Q-T= 100um.

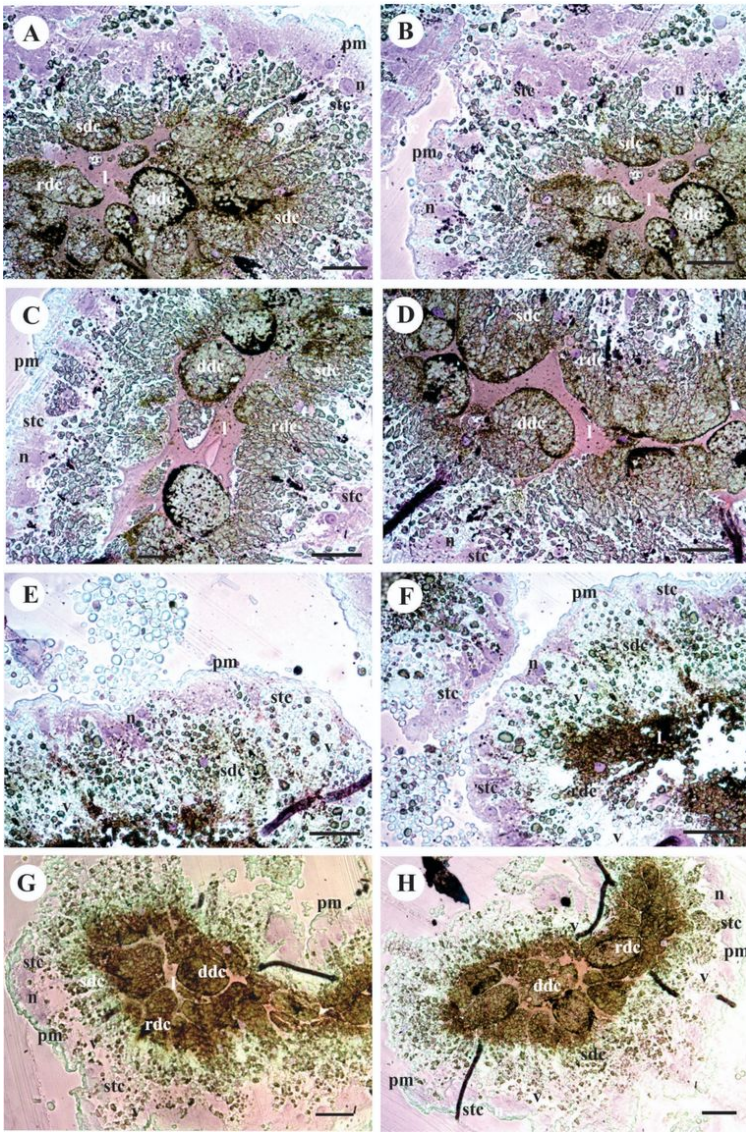


Figure 2

Histological sections of extract-treated semi-engorged *Rhipicephalus sanguineus* s. l. midgut. **A-H.** Hematoxylin and eosin (HE) staining. **A.** **B.** Control I and II Groups. **C. D.** Group III (6.25 mg/mL). **E. F.** Group IV (12.25 mg/mL). **G.H.** Group V (25 mg/mL)

sdc = Sessile digest cells; **rdc**=Residual Sessile digest cells; **ddc**=Detached digest cells; **dc**= digest cells; **l**= lumen; **stc**= Stem cells (generative cells); **n**= nuclei; **pm** = plasma membrane; **v**=vacuoles.

Bars: A, B, C, D, E, F, G, H, I, J, K, L= 50um