

# Cytotoxic Effects of Extract of *Acmella oleracea* in the Ovaries and Midgut of *Rhipicephalus sanguineus* Latreille, 1806 (Acari: Ixodidae) Female Ticks

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## Abstract

The present study investigated the effects of different concentrations of *Acmella oleracea* extract on the germinative cells and digestive processes of semi-engorged *Rhipicephalus sanguineus* females. For this experiment, 150 ticks were divided into five groups (30 individuals each). The animals were immersed for 5 min in different concentrations of the extract, distilled water, or ethanol 50%/DMSO 1%, dried, and kept in biological oxygen demand incubator for 7 days. The alterations were associated with the size of germinative cells and yolk granules; presence, size, and location of vacuoles in the cytoplasm of germinative cells; nuclear modifications in the germinative cells; damages to the nucleus and cytoplasm of the midgut generative cells; size of digestive cells; number of captured blood elements; accumulated digestive residues and digestive vacuoles in the digestive cells cytoplasm; and the number and distribution of proteins and polysaccharides in all the cells of both organs. The concentrations used in this study prevented an efficient and complete blood digestion by the midgut epithelial cells of the treated animals, resulting in the absence of the necessary nutrients to maintain the physiological events in the ectoparasites. In advanced stages, this can lead the ectoparasite to death. The germinative cells were highly impaired and probably not able to advance developmental stages (I–V) or complete vitellogenesis to be released during ovulation, which would prevent the females from originating a new individual. Thus, it can be concluded that the effects of *A. oleracea* are similar to those caused by chemical products widely recognized as effective to control ticks.

**Keywords:** Acaricide, control, extract, natural, *Rhipicephalus sanguineus*, tick

## INTRODUCTION

Ticks belong to one of the most important arthropod groups and have significant epidemiological importance, once they are hematophagous ectoparasites and transmit pathogenic protozoa, viruses, bacteria, and helminths,<sup>[1,2]</sup> mainly to mammals, including humans.

*Rhipicephalus sanguineus* are ticks of major medical and veterinary importance and have been described in the literature as the most widely distributed tick species worldwide.<sup>[2-5]</sup>

The use of synthetic acaricides is currently the main method to control ticks. However, the indiscriminate use of these chemicals can lead to the selection of resistant individuals and to the accumulation of chemical residues in

the environment, contaminating the soil and water streams, and consequently affecting other animals, including human beings.<sup>[6-8]</sup>

In this sense, natural compounds, substances obtained from plant extracts with acaricide action, would represent a promising alternative to control these pests.<sup>[9]</sup>

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*Acmella oleracea*, popularly known as Jambu, is a plant of the family Asteraceae, typical from the north region of Brazil with hot and humid climate. It is used as condiment, ingredient in popular medicine, and its pharmacological activities have been object to several studies.<sup>[10]</sup> Jambu also presents bactericidal, fungistatic and fungicidal,<sup>[11]</sup> larvicidal, and insecticidal properties.<sup>[12]</sup> Recent studies have demonstrated the highly efficient acaricidal activity of *A. oleracea* extracts against *Rhipicephalus Boophilus microplus* larvae and engorged females.<sup>[13]</sup>

*A. oleracea* is a small (30–60 cm tall), semi-erect herbaceous plant, with cylindrical fleshy stem and decumbent branches. The primary root has axial growth, with abundant lateral and adventitious roots in the stems and branches in contact with the soil. Its leaves are simple, with broadly ovate blade, and sparse hairs (trichomes) on both surfaces. The flowers are small and yellow, arranged in globose chapters with approximately 1 cm of diameter.<sup>[10]</sup>

The main biological effects of *A. oleracea* are attributed to the spilanthol or affinin [N-2-Methylpropyl]-2,6,8-decatrienamide or N-isobutyl-2*E*,6*Z*,8*E*-decatrienamide], an aliphatic alkamide with molecular formula C<sub>14</sub>H<sub>23</sub>NO, described as a yellowish viscous oil, obtained for the first time by Gerber in 1903.<sup>[14]</sup> Considering its diverse applications, spilanthol has aroused commercial interest and has been used in several formulations, such as food and drink additive, ingredient of cosmetic products, therapeutic agent, and insecticide to control insects and microorganisms in plants.<sup>[15]</sup>

Thus, the objective of the present study was to investigate the effects of different concentrations of *A. oleracea* extract on germinative and midgut cells of semi-engorged *R. sanguineus* ticks through morphological, histological, and histochemical techniques, comparing the results with those of the individuals from control Groups I and II. The results provide relevant information for the development of sustainable strategies and the improvement of current methods to control *R. sanguineus* ticks, minimizing environmental contamination, resistance development, and risks to nontarget organisms and as well.

## MATERIALS AND METHODS

### Chemical Substance

#### Natural: *Acmella oleracea*

The crude ethanolic extract from the aerial parts (flowers, leaves, and stem) of *A. oleracea* was provided by the Chemical Laboratory of Natural Products from the Pluridisciplinary Center of Chemical, Biological and Agricultural Research (CPQBA) University of Campinas (UNICAMP), Campinas, SP, Brazil, under the supervision of Dr. Rodney Alexandre Ferreira Rodrigues and support from postgraduate student Lais Thiemi Yamane.

The plants were cultivated in the experimental field of CPQBA/UNICAMP, located in Paulinia, SP, Brazil (geographic coordinates  $-22^{\circ} 47' 52''$  S,  $-47^{\circ} 6' 49''$ ). The seeds were

provided by the company Centroflora (Botucatu, SP, Brazil) and the aerial parts – flowers, leaves, and stem – were collected in April 2015. The plant was identified by Dr. John F. Pruski of Missouri Botanical Garden (USA), and a voucher specimen was deposited in CPQBA/UNICAMP Herbarium, Campinas, SP, Brazil, number 181,452. License for genetic testing (CGEN) number 010577/2014-9.

#### Drying and milling the plant material

The aerial parts (flowers, leaves, and stems) of *A. oleracea* were dried at 40°C under forced ventilation for 48 h until constant weight,<sup>[16]</sup> milled with a knife mill, and passed through a 48-mesh sieve –0.297 mm. The resulting material was placed in polypropylene-coated Kraft bags and kept in freezer at –20°C.

#### Preparing the crude ethanolic extract of *Acmella oleracea* (L.) R. L. Jansen

The extraction was performed through mechanical stirring in a stainless tank, at room temperature, using ethanol 96°GL (proportion 1:5 plant/solvent) for 1.5 h. The material was filtered three times to separate the residues.<sup>[16]</sup> The resulting crude extract was filtered, homogenized, and concentrated under vacuum in rotary evaporator at 40°C, lyophilized until a constant weight was attained, stored in amber flasks, and kept in freezer until use.

#### Analytical monitoring of spilanthol

The quantification of spilanthol in the extract was performed using a gas chromatograph coupled with a mass detector (GC–MS, Agilent® 5890 Series II mass selective detector Agilent® 5970 EI 70 eV) equipped with a fused silica column WCOT, HP5-MS, Agilent®, dimensions 30 mm × 0.25 mm × 0.25 mm. The analysis conditions were as follows: injector temperature: 220°C; detector temperature: 250°C, temperature program: 60°C–240°C (3°C/min), and sample injection using split mode at 1:40 ratio. Helium gas was used as carrier at 0.7 bar, 1 mL/min.

#### *Rhipicephalus sanguineus* Ticks (Latreille, 1806)

*R. sanguineus* semi-engorged females, weighing 27 mg on average (about 5 days of feeding), were used throughout the experiment. The ticks were provided by the Animal Facility of the Department of Biology, UNESP, Rio Claro campus/São Paulo, Brazil, where the colony is maintained under controlled conditions (28°C, 85% humidity and 12-h photoperiod) in a biological oxygen demand (BOD) incubator. Unfed *R. sanguineus* couples (25 couples/infestation) were allowed to feed on naive New Zealand white rabbits following Bechara *et al.*<sup>[17]</sup> until reaching the semi-engorged stage. The semi-engorged stage of the females was chosen due to the high parasitic efficiency in this phase.

#### Hosts

New Zealand white rabbits, weighing between 3 kg and 3.5 kg, were used as hosts. Rabbits were obtained from the Animal Facility of UNESP, Campus Botucatu/São Paulo, Brazil, and housed in the Animal Facility of UNESP, Rio Claro Campus/São Paulo, Brazil. The animals had not had

prior contact with ticks or acaricides and were kept under controlled conditions. During the entire experiment, the rabbits were maintained in cages, receiving water and commercial food *ad libitum*.

This study was approved by the Ethics Committee for Animal Experimentation of UNESP/SP/Brazil, protocol n°6334/2014.

### Extract dosage

Several doses were evaluated in preliminary tests (pilots) by diluting the extract in a solution of 50% ethanol +1% DMSO. After this bioassay, the efficacy of extract and the level of susceptibility of the semi-engorged females were evaluated, and the lethal concentration  $LC_{50}$  determined was 24.883 mg/mL. In this study, the concentrations corresponded to 25 mg/mL, 33 mg/mL, and 40 mg/mL of extract. All the concentrations of extract were kept in labeled volumetric flasks until the tests. Each treatment was conducted in duplicate.

### Experimental model

*R. sanguineus* semi-engorged females were divided into three treated groups: group III (25 mg/mL of extract), Group IV (33 mg/mL of extract), and Group V (40 mg/mL ppm of extract). The control Groups I and II were exposed to the placebo (distilled water) and solution of 50% ethanol +1% DMSO, respectively.

The 150 semi-engorged females of *R. sanguineus*, after being washed in a sieve with tap water, were dried on soft absorbent paper. Then, 90 females were divided into three groups of 30 females (30 females for each concentration –2 groups with 15 individuals – duplicates) and immersed for 5 min in Petri dishes containing the above different concentrations of extract. For control Groups 1 and 2, 60 females were immersed in distilled water and in a solution of 50% ethanol and 1% DMSO for 5 min, respectively.

Ticks were then dried in absorbent paper and placed in the BOD incubator ( $28 \pm 1^\circ\text{C}$ , 80% relative humidity and 12 h photoperiod) for 7 days. The observation period was established because frequently the effect of acaricides is not immediate but acts slowly on the physiology of the individual analyzed.<sup>[18]</sup>

After 7 days of monitoring, all the semi-engorged females were forwarded to histological and histochemical techniques.

## Methods

### Histology

All semi-engorged females maintained in the refrigerator for thermal shock anesthesia were dissected in a phosphate-buffered saline solution (NaCl: 7.5 g/L,  $\text{Na}_2\text{HPO}_4$ : 2.38 g/L, and  $\text{KH}_2\text{PO}_4$ : 2.72 g/L).

The ovaries and midguts were fixed for 24 h in 4% paraformaldehyde, dehydrated in ethanol, embedded in Leica resin for 24 h at  $4^\circ\text{C}$ , and transferred to plastic molds previously filled with polymerized Leica resin. After resin polymerization, all the blocks were sectioned at 3- $\mu\text{m}$  thickness slices using a Leica RM 2255 microtome (Bio-Rad) and stained

with hematoxylin and eosin, following routine histological procedures. The glass slides were examined in a Motic BA300 photomicroscope.

This device and other equipment were from the Histology Laboratory of the Biology Department at the Biosciences Institute, UNESP, Rio Claro Campus/São Paulo, Brazil.

### Histochemistry

To detect changes such as presence or absence, frequency, and distribution of proteins, polysaccharides, and lipids in the semi-engorged females of control and extract-treated groups, histological sections were prepared for the histochemical techniques listed below.

### Periodic acid–Schiff technique for polysaccharide detection (according to Junqueira and Junqueira)<sup>[19]</sup>

The engorged nymphs were fixed with aqueous Bouin. Slides with sections were immersed for 10 min in 0.4% periodic acid, washed with distilled water, and stained with Schiff's reagent for 1 h in the dark. The material was then washed thrice with sulfur water for 3 min each and rinsed with tap water for 30 min. Then, the material was stained with hematoxylin for 5 min. After drying, slides were clarified with xylol and mounted in Canada balsam.

### Bromophenol Blue Staining for protein detection (according to Pearse)<sup>[20]</sup>

Semi-engorged females were fixed with 4% paraformaldehyde. All slides were stained with bromophenol blue for 2 h at room temperature. Afterward, they were washed with 0.5% acetic acid for 5 min and tap water for 15 min; slides were quickly immersed in tertiary butyl alcohol, allowed to dry at room temperature, clarified, and mounted in Canada balsam.

## RESULTS

### Histology

#### Ovary

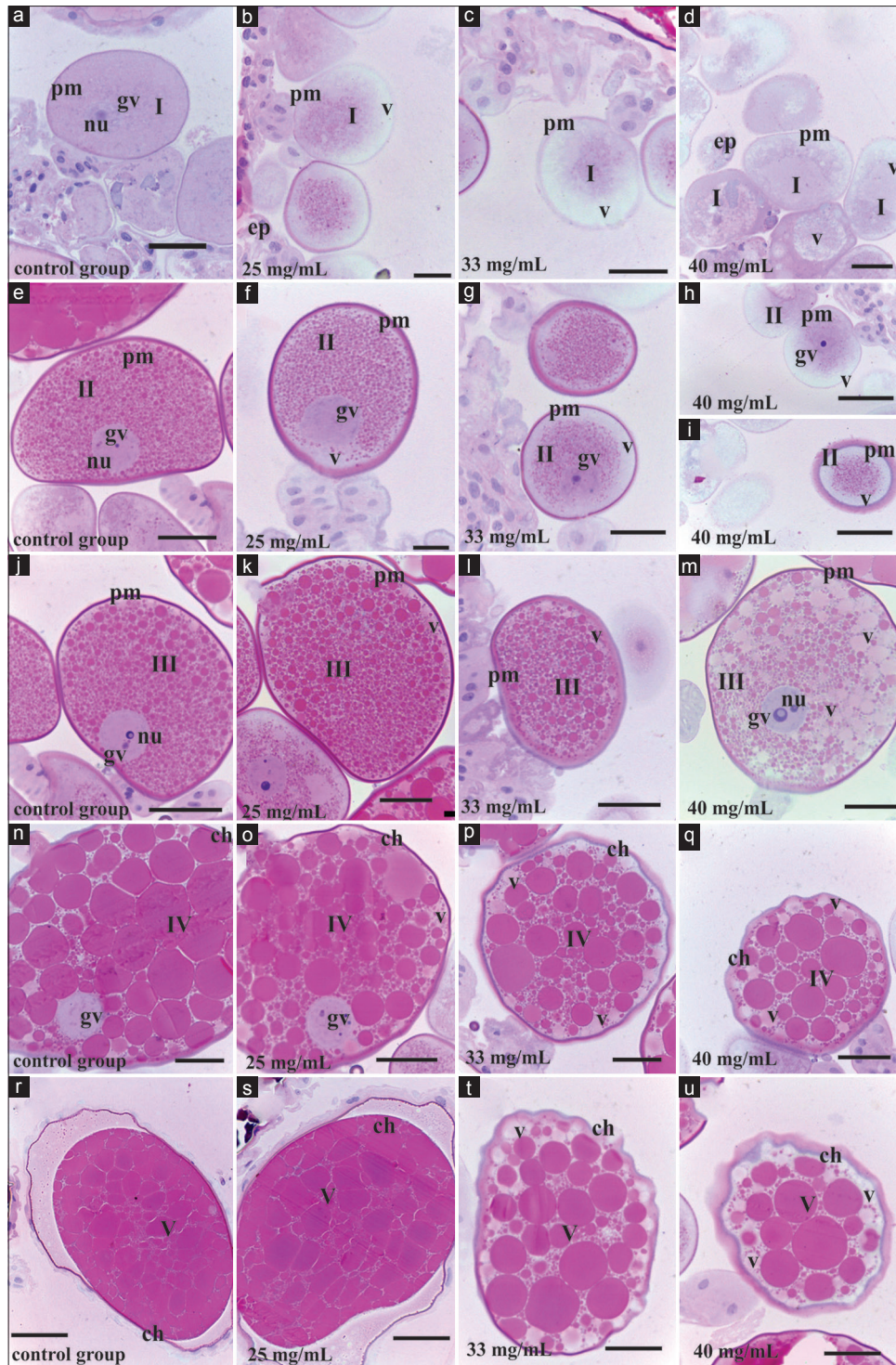
#### Control Groups I and II

The results obtained for the ovaries of semi-engorged females from the control groups are similar to those described by de Oliveira *et al.*<sup>[21]</sup> A summary of the main characteristics of the ovaries from female *R. sanguineus* ticks is presented as follows.

The *R. sanguineus* ovary is composed of a wall of epithelial cells and a great number of oocytes in five development stages, which are fixed to the wall through a mobile pedicel.<sup>[21]</sup>

Oocytes I are small elliptical cells with a germinal vesicle, which has a very evident nucleolus occupying a great part of the central cytoplasm. This central cytoplasm has a homogeneous aspect, without the presence of granulations. A thin plasma membrane [Figure 1a] surrounds these oocytes. Oocytes II are bigger than the first ones, are elliptical, and have a central germ vesicle. A thin and homogeneous cytoplasmic granulation is found in them. They are also surrounded by a thin plasma membrane [Figure 1e]. Oocytes III have medium size, and



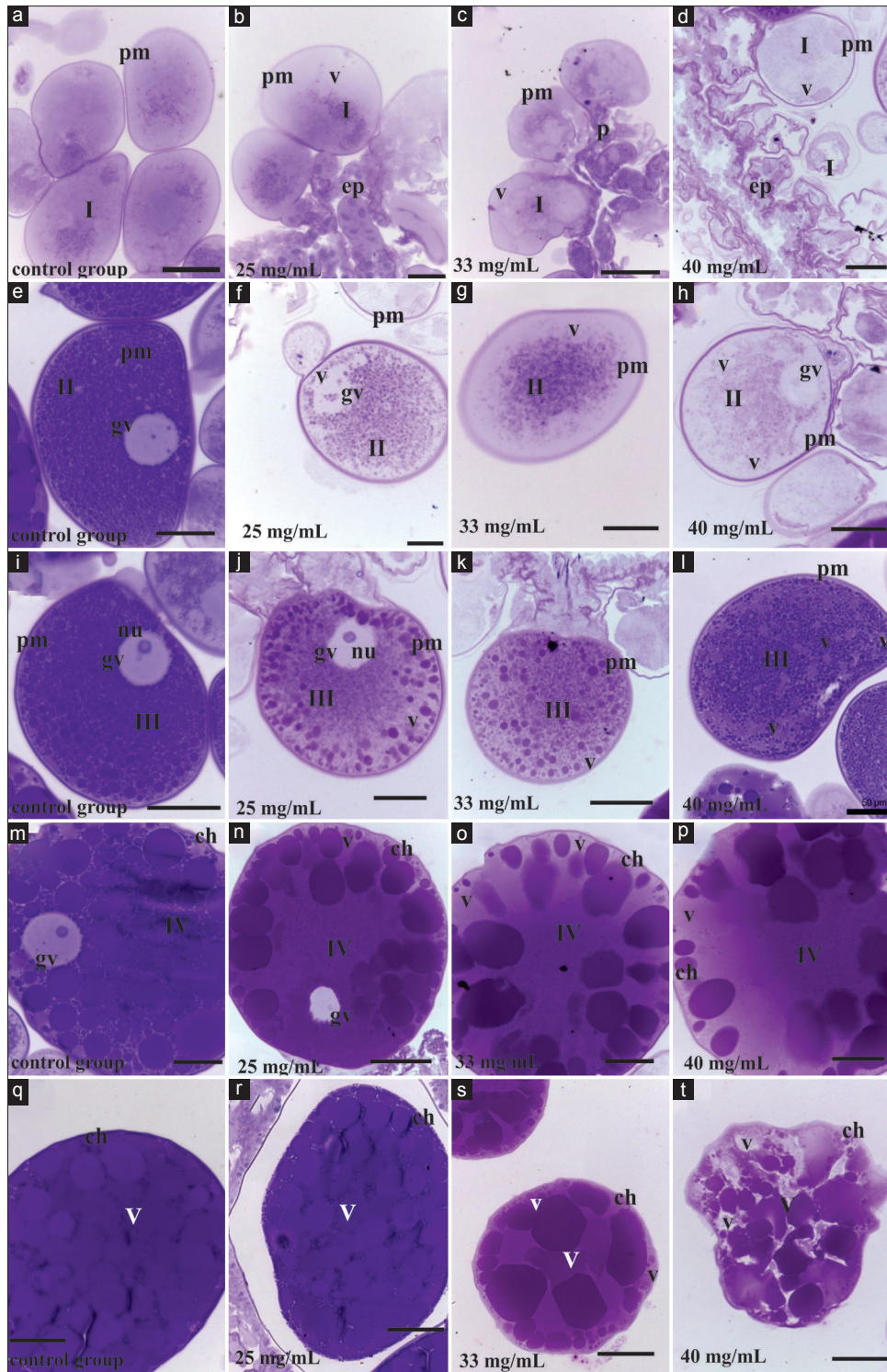


**Figure 1:** Histological sections of extract-treated *Rhipicephalus sanguineus* ovary. (a-u) H and E staining. (a, e, j, n, and r) Control I and II group. (b, f, k, o, and s) Group III (25 mg/ml). (c, g, l, p, and t) Group IV (33 mg/ml). (d, h, i, m, q, and u) Group V (40 mg/ml). I: Oocyte I; II: Oocyte II; III: Oocyte III; IV: Oocyte IV; V: Oocyte V; ch: Chorium; ep: Ovary epithelium; gv: Germ vesicle; nu: Nucleolus; p: Pedicel; pm: Plasmic membrane; yg: Yolk granules, v: Vacuoles. Bars (a-q) 50  $\mu$ m, (r-u) 100  $\mu$ m

their shapes vary from round to elliptical. The germinal vesicle occupies the oocyte pole projected toward the pedicel. The plasma membrane is thinner in this stage in comparison with the previous stages. The cytoplasm is filled with yolk granules of different sizes, the smaller ones occupy the central area,

and the bigger ones are located in the periphery [Figure 1j]. Oocytes IV are bigger than type III. They are round, and the germinal vesicle is not always seen, and when present, occupies the pole of the oocyte projected towards the pedicel. In this stage, chorion deposition begins and causes a thickening of





**Figure 2:** Histological sections of extract-treated *Rhipicephalus sanguineus* ovary. (a-t) PAS staining. (a, e, i, m, and q) Control I and II group. (b, f, j, n, and r) Group III (25 mg/ml). (c, g, k, o, and s) Group IV (33 mg/ml). (d, h, l, p, and t) Group V (40 mg/ml). I: Oocyte I; II: Oocyte II; III: Oocyte III; IV: Oocyte IV; V: Oocyte V; ch: Chorium; ep: Ovary epithelium; gv: Germ vesicle; nu: Nucleolus; p: Pedicel; pm: Plasmic membrane; yg: Yolk granules, v: Vacuoles. Bars (a-p) 50  $\mu$ m, (q-t) 100  $\mu$ m

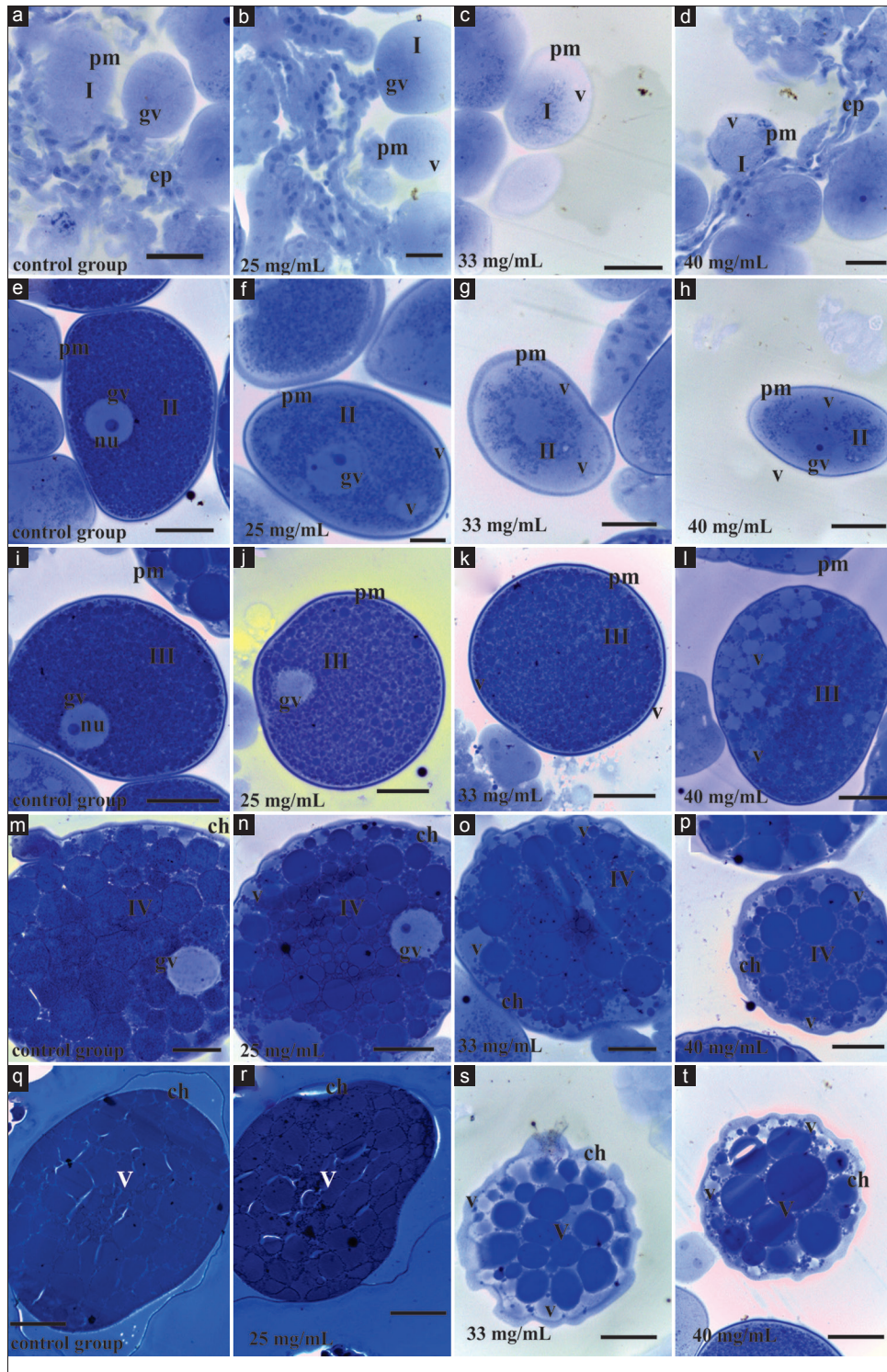
the oocyte wrap. The cytoplasm presents many yolk granules of several sizes that are randomly distributed [Figure 1n]. Oocytes V are the biggest germinal cells, with round shape, and the germinal vesicle can no longer be seen due to the big

and numerous cytoplasmic granules. The chorion is thick and is fully deposited [Figure 1r].

#### Group III (25 mg/mL of extract)

The oocytes of the individuals exposed to the extract showed





**Figure 3:** Histological sections of extract-treated *Rhipicephalus sanguineus* ovary. (a-t) Bromophenol blue staining. (a, e, i, m, and q) Control I and II group. (b, f, j, n, and r) Group III (25 mg/ml). (c, g, k, o, and s) Group IV (33 mg/ml). (d, h, l, p, and t) Group V (40 mg/ml). I: Oocyte I; II: Oocyte II; III: Oocyte III; IV: Oocyte IV; V: Oocyte V; ch: Chorium; ep: Ovary epithelium; gv: Germ vesicle; nu: Nucleolus; p: Pedicel; pm: Plasmic membrane; yg: Yolk granules; v: Vacuoles. Bars (a-p) 50  $\mu$ m, (q-t) 100  $\mu$ m

few morphological alterations in comparison with those belonging to control Groups I and II.

The cytoplasm of oocytes I contained large vacuoles located close to the peripheral region [Figure 1b].

Oocytes II showed vacuolation next to the pedicel region and around the germinative cell (next to the plasma membrane). In addition, the nuclear envelope lost its integrity and the plasma membrane was thickened [Figure 1f].

Oocytes III presented few modifications, which consisted in some folds in the plasma membrane and few small and round vacuoles occupying the region next to the cell periphery [Figure 1k].

Oocytes IV had small pleats in the membrane and small rounded vacuoles in the cytoplasm next to the cell periphery [Figure 1o].

Oocytes V showed shape alterations, presenting slightly pleated membranes [Figure 1s].

#### Group IV (33 mg/mL of extract)

The individuals belonging to treatment Group IV showed a greater number of altered oocytes in comparison with the ones from the previous group.

Oocytes I displayed large vacuolated regions in the periphery of almost 50% of the cells. In these oocytes, the germinal vesicle was of difficult visualization [Figure 1c].

Oocytes II showed large vacuolated areas surrounding the whole cell and located next to the pedicel, which reduced the number of yolk granules previously found in these sites. The germinal vesicle was of difficult visualization, and when detected, the nuclear envelope was irregular and ruptured. In some oocytes II, the plasma membrane was thickened [Figure 1g].

The oocytes III from this group showed similar modifications to oocytes III from the previous group (Group III), pleated plasma membrane, and few round and small vacuoles occupying the region next to the cell periphery [Figure 1l].

Oocytes IV lost their original shape; their morphology was irregular, and the envelope membranes thickened. Their cytoplasm contains small and round vacuoles between the yolk granules and next to the peripheral region of the cell and fewer and smaller yolk granules in comparison with the previous groups. The germinal vesicle is of difficult visualization. Some oocytes present ruptured yolk granules releasing their content into the cytoplasm [Figure 1p].

Oocytes V showed alterations in shape and in the cytoplasm. Their morphology is irregular, and the envelope shows various pleats. The cytoplasm contains smaller and fewer granules in comparison with the previous groups. Round vacuoles are observed next to the cell periphery [Figure 1t].

#### Group V (40 mg/mL of extract)

The ovaries of the individuals belonging to Group V showed numerous oocytes with significant histological alterations in comparison with those of the control Groups I and II and treatment Groups III and IV.

Oocytes I were rarely observed, some of them with extensive vacuolated regions in more than 50% of the cell, next to the cell periphery, and the germinal vesicle is seldom identified; others show large disorganized and vacuolated areas, with restricted and no longer homogeneous cytoplasm, in addition to deformed germinal vesicle [Figure 1d].

Oocytes II showed large vacuolated areas around the cell and yolk granules concentrated in the central region. When

detected, the germinal vesicle presented irregular morphology, with ruptured nuclear envelope. In some of these oocytes, the plasma membrane was intensely thickened [Figure 1h and i].

Oocytes III presented slightly pleated plasma membrane and small rounded vacuoles in the cytoplasm scattered among the yolk granules and next to the peripheral region [Figure 1m].

Oocytes IV showed alterations in shape and in the cytoplasm, with thickened and pleated envelope membranes. The cytoplasm contained smaller yolk granules in comparison with the previous groups. These vacuoles occupy the regions between the yolk granules and next to the cell periphery. The germinal vesicle is of difficult visualization. Some oocytes show ruptured yolk granules, whose content was released into the cytoplasm [Figure 1q].

Oocytes V showed significant alterations, with irregular shape, several folds, and thick envelopes. The cytoplasm contained large yolk granules (smaller than those from the previous groups), rounded vacuoles among the yolk granules and next to the cell periphery, and extensive vacuolated areas surrounding the whole cell. Some oocytes seem to have ruptured, releasing their content or shrinking [Figure 1u].

Exclusively for this treatment group, the ovary wall was damaged.

The epithelial cells lost their original shape, becoming either completely deformed, with pleated plasma membrane and reduced cytoplasm, or vacuolated, with flat morphology and pyknotic nuclei [Figure 1d, h and q].

### Midgut

#### Control Groups I and II

The midgut of the *R. sanguineus* from control Groups I and II showed an epithelium supported by a basal lamina and a tissue layer [Figure 4a-c]. The epithelium is pseudostratified and formed by digestive and generative cells [Figure 4a-c].

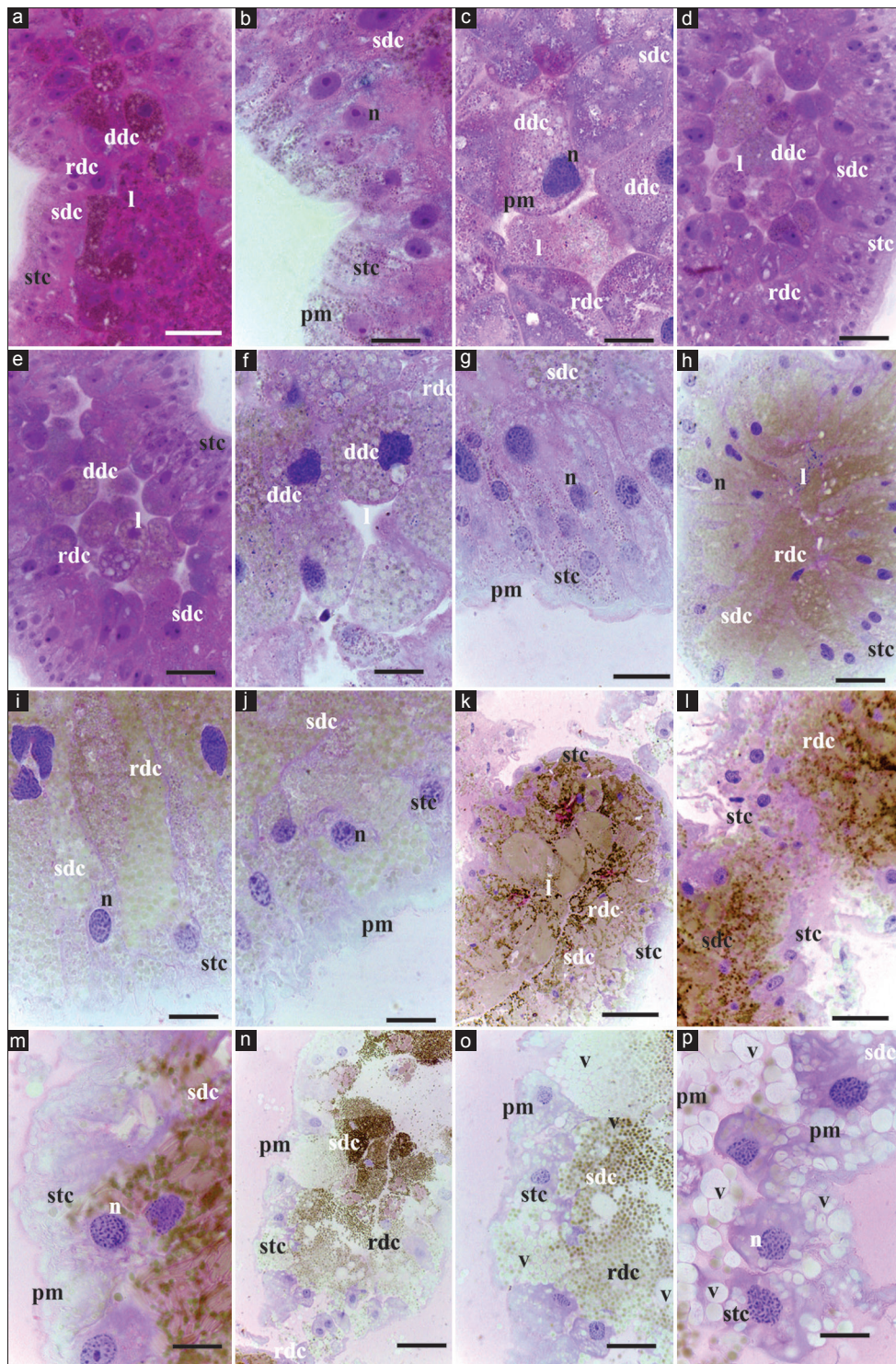
The generative cells (stem cells) are small, cubic or prismatic, and fully attached to the basal lamina. The nuclei of these cells are small, central, and rounded and the nucleoli are evident [Figure 4a-c]. The cytoplasm presents numerous and little stained rounded regions, vacuoles, and/or lipid drops [Figure 4a-c]. Externally, the plasma membrane limits the whole cell [Figure 4a-c].

The digestive cells are large and very frequent [Figure 4a-c]. Such cells undergo several developmental stages over the tick life cycle and engorgement process.

Three stages of digestive cells were found in the midgut of the *R. sanguineus* ticks belonging to control Groups I and II:

1. Sessile digest cells – Columnar cells, with large and rounded nuclei, numerous endosomes filled with blood (mainly red cells) and digestive vacuoles of various sizes, shapes and strongly stained by eosin [Figure 4a-c]
2. Residual Sessile digest cells – Cells with a particular shape, cytoplasm projected toward the lumen and with





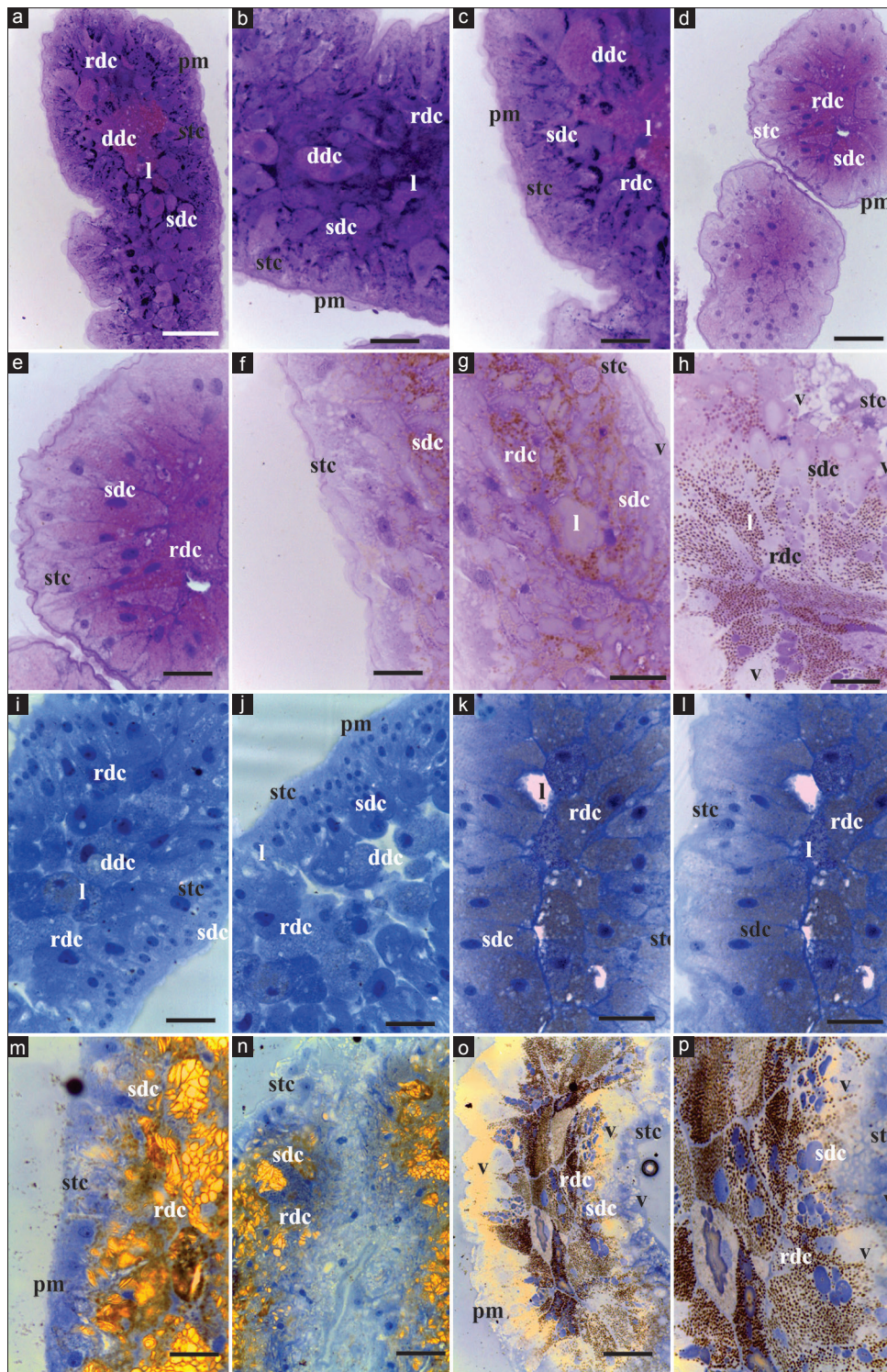
**Figure 4:** Histological sections of extract-treated *Rhipicephalus sanguineus* midgut. (a-p) H and E staining. (a-c) Control I and II group. (d-g) Group III (25 mg/ml). (h-j) Group IV (33 mg/ml). (k-p) Group V (40 mg/ml). sdc: Sessile digest cells; rdc: Residual sessile digest cells; ddc: Detached digest cells; l: Lumen; m: Muscular tissue; stc: Stem cells; n: Nuclei; pm: Plasmic membrane; v: Vacuoles. Bars (a, d, e, h, l, and o) 50  $\mu$ m; (b, c, f, g, i, j, m, and p) 20  $\mu$ m; (k and n) 100  $\mu$ m

little contact with the basal lamina, large and rounded nuclei, and the cytoplasm is filled with endosomes and digestive vacuoles [Figure 4a-c]

3. Detached digest cells – Spherical cells scattered in the midgut lumen, once they have detached from the basal

lamina. The cytoplasm contains several endosomes, digestive vacuoles, and residual hematinic bodies (final product of the intracellular digestion, accumulated inside the cell) [Figure 4a-c]. Their nuclei are large and round [Figure 4a-c].





**Figure 5:** Histological sections of extract-treated *Rhipicephalus sanguineus* midgut. (a-h) PAS staining. (a) Control I and II group. (b and c) Group III (25 mg/ml). (d and e) Group IV (33 mg/ml). (f-h) Group V (40 mg/ml). (i-p) Bromophenol blue staining. (i) Control I and II Group. (j) Group III (25 mg/ml). (k and l) Group IV (33 mg/ml). (m-p) Group V (40 mg/ml). sdc: Sessile digest cells; rdc: Residual Sessile digest cells; ddc: Detached digest cells; l: Lumen; m: Muscular tissue; stc: Stem cells; n: Nuclei; pm: Plasmic membrane; v: Vacuoles. Bars (a, d and o) 100  $\mu$ m; (b, c, e-n and p) 50  $\mu$ m

The midgut lumen of the individuals from control Groups I and II contains large amounts of blood, detached digest cells, projection of the apical cytoplasm of the residual sessile digest cells, and residues from the digestive processes.

These residues were released through exocytosis and/or by the lysis of digestive cells present in another phase of the digestion or life cycle and posteriorly incorporated to the fecal matter [Figure 4a].

**Group III (25 mg/mL of extract)**

The midgut of the semi-engorged females exposed to 25 mg/mL of *A. oleracea* extract showed similar morphological characteristics to those of the individuals from control Groups I and II.

The generative cells (stem cells), the digestive cells, the sessile digest cells, the residual sessile digest cells, the detached digest cells, and the muscle layer cells were not affected. The lumen presents a smaller amount of blood, revealed by the weak staining [Figure 4d-g].

**Group IV (33 mg/mL of extract)**

The midgut of the semi-engorged females exposed to 33 mg/mL of *A. oleracea* showed significant alterations in comparison with those of control Groups I and II. The extract caused modifications in the digestive cells in all stages and in the lumen as well.

The digestive cells are less frequent, the sessile digest cells are elongated, and the residual sessile digest cells present apical cytoplasm projected toward the lumen. Both accumulate fewer endosomes and digestive vacuoles. In addition to stained by eosin, these structures present dark green coloration [Figure 4h-j]. The cytoplasm also presents green coloration and the nuclei are elongated [Figure 4h-j].

Detached digest cells were not observed.

In this group, the midgut lumen is small, with a small amount of blood and filled with digestive cells, mainly by the apical projections of the residual sessile digest cells and residues from the digestive processes [Figure 4h-j].

**Group V (40 mg/mL of extract)**

The midguts of the semi-engorged females exposed to 40 mg/mL of *A. oleracea* extract presented significant alterations in comparison with the ones from the previous groups. The generative cells (Stem cells) underwent modifications in all developmental stages of the digestive cells and in the lumen as well. The midguts of the treated individuals showed two different morphological profiles:

a. Irregular generative cells (stem cells), with limited cytoplasm, without rounded and little-stained regions (probably vacuoles and/or lipid droplets); their nuclei are elongated or pyknotic, with blebs. Irregular plasma membrane in some cases twisted or detached from the basal lamina [Figure 4k-m]; few and very disorganized digestive cells. The sessile digest cells and the residual sessile digest cells are of difficult visualization, once their limits are covered by cytoplasmic granulations of various shapes and sizes. The cytoplasm presents regions of green coloration and contains endosomes and greenish digestive vacuoles, smaller in comparison with those from the other groups. The nucleus is elongated, and the plasma membrane is irregular [Figure 4k-m]. Detached digest cells are not observed, and the lumen presents varied content, with a small amount of blood, digestive cells, and residues [Figure 4k-m]

b. Irregular generative cells, cytoplasm with numerous round vacuoles of various sizes and nuclei with blebs. The digestive cells were less numerous and very disorganized. The sessile digest cells and residual sessile digest cells showed round vacuoles of various sizes, extensive vacuolated regions, and smaller and less frequent endosomes and digestive vacuoles in comparison with those of the control Groups I and II. The cytoplasm did not present green coloration, and the nuclei and plasma membrane were covered [Figure 4n-p]. Detached digest cells were not observed.

The midgut lumen did not contain blood; however, it was filled with digestive cells (sessile digest cells or Residual Sessile digest), with cells detached from the basal lamina and residues released through exocytosis and/or lysis of digestive cells [Figure 4k-p].

**Histochemistry*****Ovary and midgut***

The results of the histochemical tests for the detection of polysaccharides (PAS) and proteins (Bromophenol blue) in the ovaries and midguts of the *R. sanguineus* ticks submitted to *Acmella oleracea* extract were organized in tables for better understanding and comparison [Figures 2a-t; 3a-t; 5a-p]. [Tables 1-6]. The following is a brief summary of the results.

- Ovaries – In the control Groups I and II, polysaccharides and proteins were found in the oocytes. In the treatment groups (Groups III, IV, and V), the extract caused reduction in the number of protein elements and polysaccharides in Oocytes I, II, III, IV, and V
- Midguts – In the control Groups I and II, the histochemical techniques such as bromophenol blue and PAS showed moderate number of proteins and polysaccharides. In the treatment groups (Groups III, IV, and V), the extract caused many different damages.

**DISCUSSION**

The individuals from treatment Groups III, IV, and V submitted to the concentrations of 25 mg/ml, 33 mg/ml, and 50 mg/ml of *A. oleracea* extract, respectively, presented alterations in the ovaries and midgut cells when compared with the ones from control Groups I and II. The alterations were mainly associated with the size of the germinative cells and yolk granules; presence, number, size, and location of vacuoles in the cytoplasm of the germinative cells; nuclear alterations in the germinative cells; damages in the nuclei and cytoplasm of the midgut generative cells; size of the midgut digestive cells; number of captured blood elements; digestive residues accumulated and digestive vacuoles in the cytoplasm of the digestive cells; number and distribution of proteins; and polysaccharides in all the cells of both organs (ovary and midgut).

The histological analysis of the ovaries of the semi-engorged *R. sanguineus* females belonging to control groups I and II (groups I and II) presented the characteristics described by Oliveira et al.<sup>[21]</sup>. The ovary is constituted and oocytes in different



**Table 1: Histochemistry results of ovary of the tick *Rhipicephalus sanguineus* of control Groups I and II**

Oocytes stages	Vitelline elements	Germinal vesicle	Central region	Peripheral region	Chorium
I	Proteins [Figure 3a]	+	Cytoplasm ++, no granules	Cytoplasm ++, no granules	-
	Polysaccharides [Figure 2a]	-	Cytoplasm +, no granules	Cytoplasm +, no granules	-
II	Proteins [Figure 3e]	+	Thin granulation ++	Thin granulation ++	-
	Polysaccharides [Figure 2e]	-	Thin granulation ++	Thin granulation ++	-
III	Proteins [Figure 3i]	+	Small granules, ++	Medium granules, +++	-
	Polysaccharides [Figure 2i]	-	Small granules, ++	Medium granules, ++	-
IV	Proteins [Figure 3m]	+	Medium granules, +++	Larger granules, +++	+
	Polysaccharides [Figure 2m]	-	Medium granules, +++	Larger granules, +++	++
V	Proteins [Figure 3q]	+	Larger granules, +++	Larger granules, +++	+++
	Polysaccharides [Figure 2q]	-	Larger granules, +++	Larger granules, +++	+++

+: Weakly positive, ++: Moderately positive, +++: Strongly positive, -: Negative; Ø

**Table 2: Histochemistry results of ovary of the tick *Rhipicephalus sanguineus* of Group III**

Oocytes stages	Vitelline elements	Germinal vesicle	Central region	Peripheral region	Chorium
I	Proteins [Figure 3b]	+	Cytoplasm ++, no granules	Cytoplasm +, no granules	-
	Polysaccharides [Figure 2b]	-	Cytoplasm +, no granules	Cytoplasm +, no granules	-
II	Proteins [Figure 3f]	+	Thin granulation +	Thin granulation +	-
	Polysaccharides [Figure 2f]	-	Thin granulation +	Thin granulation +	-
III	Proteins [Figure 3j]	+	Small granules, ++	Medium granules, +++	-
	Polysaccharides [Figure 2j]	-	Small granules, +	Medium granules, ++	-
IV	Proteins [Figure 3n]	+	Medium granules, +++	Larger granules, ++	+
	Polysaccharides [Figure 2n]	-	Medium granules, +++	Larger granules, +++	++
V	Proteins [Figure 3r]	+	Larger granules, +++	Larger granules, +++	+++
	Polysaccharides [Figure 2r]	-	Larger granules, +++	Larger granules, +++	+++

+: Weakly positive, ++: Moderately positive, +++: Strongly positive, -: Negative

**Table 3: Histochemistry results of ovary of the tick *Rhipicephalus sanguineus* of Group IV**

Oocytes stages	Vitelline elements	Germinal vesicle	Central region	Peripheral region	Chorium
I	Proteins [Figure 3c]	+	Cytoplasm +, no granules	Cytoplasm +, no granules	-
	Polysaccharides [Figure 2c]	-	Cytoplasm +, no granules	Cytoplasm +, no granules	-
II	Proteins [Figure 3g]	+	Thin granulation +	Thin granulation +	-
	Polysaccharides [Figure 2g]	-	Thin granulation +	Thin granulation +	-
III	Proteins [Figure 3k]	+	Small granules, ++	Medium granules, +++	-
	Polysaccharides [Figure 2k]	-	Small granules, +	Medium granules, ++	-
IV	Proteins [Figure 3o]	+	Medium granules, +++	Larger granules, ++	+
	Polysaccharides [Figure 2o]	-	Medium granules, ++	Larger granules, ++	++
V	Proteins [Figure 3s]	+	Larger granules, ++	Larger granules, +	++
	Polysaccharides [Figure 2s]	-	Larger granules, ++	Larger granules, ++	++

+: Weakly positive, ++: Moderately positive, +++: Strongly positive, -: Negative

developmental stages fixed to an epithelial cell wall through a pedicel,<sup>[21-25]</sup> corroborating Till<sup>[26]</sup> for *R. appendiculatus*, Balashov<sup>[27]</sup> for *Hyalomma asiaticum*, Denardi et al.<sup>[28]</sup> for *Amblyomma cajennense*, Saito et al.<sup>[29]</sup> for *B. microplus*, Oliveira et al.<sup>[30]</sup> for *Amblyomma triste*, Sonenshine<sup>[31]</sup> for *Dermacentor variabilis* and *Dermacentor andersoni* and Sonenshine and Roe<sup>[32]</sup> for *Dermacentor variabilis*.

The ovaries of the females belonging to control groups I and II (groups I and II) did not show any differences between each other. Both presented ovaries in five stages of development (I-V) with typical morphological characteristics.<sup>[21,28-30,33]</sup>

The cytoplasm of the oocytes I and II from the individuals exposed to 25.0 mg/ml of *A. oleracea* extract presented large vacuoles located next to the cell periphery, indicating that the extract started causing damages at low concentrations. The oocytes of the individuals treated with 33.0 mg/ml of the extract showed large vacuolated regions in the cytoplasm and irregular or ruptured germinal vesicle envelope. In the individuals treated with 40.0 mg/ml of the extract, oocytes I and II were rarely observed and when present showed intense damages, such as disorganized and vacuolated regions, in addition to deformed germinal vesicle. These data suggest that the absence of the chorion (protective membrane, not deposited yet) would

**Table 4: Histochemistry results of ovary of the tick *Rhipicephalus sanguineus* of Group V**

Oocytes stages	Vitelline elements	Germinal vesicle	Central region	Peripheral region	Chorium
I	Proteins [Figure 3d]	+	Cytoplasm +, no granules	Cytoplasm +, no granules	-
	Polysaccharides [Figure 2d]	-	Cytoplasm -, no granules	Cytoplasm -, no granules.	-
II	Proteins [Figure 3h]	+	Thin granulation +	Thin granulation +	-
	Polysaccharides [Figure 2h]	-	Thin granulation -	Thin granulation -	-
III	Proteins [Figure 3i]	+	Small granules, ++	Medium granules, ++	-
	Polysaccharides [Figure 2i]	-	Small granules, +	Medium granules, ++	-
IV	Proteins [Figure 3p]	+	Medium granules, +++	Larger granules, ++	+
	Polysaccharides [Figure 2p]	-	Medium granules, ++	Larger granules, ++	++
V	Proteins [Figure 3t]	+	Larger granules, ++	Larger granules, +	++
	Polysaccharides [Figure 2t]	-	Larger granules, ++	Larger granules, ++	++

+: Weakly positive, ++: Moderately positive, +++: Strongly positive, -: Negative

**Table 5: Results of the Bromophenol blue histochemical test on the midgut of *Rhipicephalus sanguineus* of the control Groups I and II and Groups III, IV, and V**

	Cells	Control I and II	Group III (25 mg/mL)	Group IV (33 mg/mL)	Group V (40 mg/mL)
Midgut	Stem	+ [Figure 5i]	+ [Figure 5j]	+ [Figure 5k, l]	- [Figure 5m-p]
	Sessile digest cells	++ [Figure 5i]	++ [Figure 5j]	+ [Figure 5k, l]	+ [Figure 5m-p]
	Residual sessile digest cells	++ [Figure 5i]	++ [Figure 5j]	+ [Figure 5k, l]	+ [Figure 5m-p]
	Detached digest cells	- [Figure 5i]	- [Figure 5j]	Ø	Ø

+: Weakly positive, ++: Moderately positive, +++: Strongly positive, -: Negative; Ø: Not observed

**Table 6: Results of the polysaccharides (Schiff periodic acid) histochemical test on the midgut of *Rhipicephalus sanguineus* of the control Groups I and II and Groups III, IV, and V**

Cells	Control I and II	Group III (25 mg/mL)	Group IV (33 mg/mL)	Group V (40 mg/mL)
Stem	+ [Figure 5a]	+ [Figure 5b and c]	+ [Figure 5d and e]	- [Figure 5f-h]
Sessile digest cells	++ [Figure 5a]	++ [Figure 5b and c]	+ [Figure 5d and e]	+ [Figure 5f-h]
Residual sessile digest cells	++ [Figure 5a]	++ [Figure 5b and c]	+ [Figure 5d and e]	+ [Figure 5f-h]
Detached digest cells	- [Figure 5a]	- [Figure 5b and c]	Ø	Ø

+: Weakly positive, ++: Moderately positive, +++: Strongly positive, -: Negative; Ø: Not observed

be allowing the extract to penetrate through plasma membrane. The extract would be affecting the internal cell structures of the oocytes, and these structures would be stored and lysed into vacuoles. In this case, the lysis process would be highly efficient, considering the severity of the internal damages. Similar results were found by Oliveira *et al.*,<sup>[8]</sup> Roma *et al.*,<sup>[18]</sup> and Vendramini *et al.*<sup>[34]</sup> for *R. sanguineus* ticks exposed to fipronil, permethrin, and andiroba oil.

Autophagic vacuoles are mainly observed in cells undergoing processes of cytoplasm degeneration and/or organelle recycling,<sup>[35,36]</sup> which would justify the presence and increase in the number or organelles in oocytes I and II of the individuals treated with high concentrations of the extract, once these oocytes would be heavily damaged and would have to be eliminated in order to maintain the viability of the germinative cells and survive. de Oliveira *et al.*<sup>[7]</sup> reported intense autophagic processes in *R. sanguineus* ticks exposed to fipronil.

As the vacuolated areas increase, the area occupied by the yolk granules decreases, which indicates an impairment of the vitellogenesis, a process through which yolk is progressively

stored in the oocytes to originate mature oocytes.<sup>[18,37,38]</sup> Such impairment would prevent the oocytes from reaching more advanced developmental stages (III, IV, and V), and consequently affect fertilization and the formation of embryos. This process was also reported by Oliveira *et al.*<sup>[8]</sup> for *R. sanguineus* ticks exposed to fipronil and Denardi *et al.*<sup>[39]</sup> for *R. sanguineus* exposed to aqueous neem extract.

In addition, nuclear damages were detected in the oocytes I and II exposed to the concentrations of 33.0 mg/ml and 40.0 mg/ml of the extract. Such alterations indicate the occurrence of degenerative processes in the nuclear material.<sup>[18,40]</sup> Therefore, *A. oleracea* extract could be affecting the genetic material of oocytes I and II, causing irreversible damage to the cells, preventing them from advancing to advanced stages of development, and consequently, from completing vitellogenesis, ovulation, and fertilization. Even if fecundation is completed, the genetic material damages interfere in the viability of the eggs and embryos and cause the death of the germinative cells. Similar data were found by Denardi *et al.*<sup>[28]</sup> and Vendramini *et al.*<sup>[34]</sup> for *R. sanguineus* treated with neem oil and andiroba oil, respectively.



The oocytes III exposed to 25 mg/ml and 33.0 mg/ml of *A. oleracea* extract presented folds in the plasma membrane and few small and rounded vacuoles in the periphery of the cells. Those exposed to 40.0 mg/ml showed more vacuolated areas in the peripheral cytoplasm. The occurrence of more severe damages in the peripheral region next to the plasma membrane suggests that this would be the main entrance route for the extract, i.e., the extract would be uptaken from the hemolymph toward the interior of the oocyte through plasma membrane. Such a process has been reported by de Oliveira *et al.*<sup>[7,8]</sup> for *R. sanguineus* ticks exposed to fipronil. Roma *et al.*<sup>[18]</sup> and Vendramini *et al.*,<sup>[34]</sup> who studied *R. sanguineus* ticks treated with permethrin and andiroba oil, respectively, complement that chemical compounds can also penetrate through pedicel.

The plasma membrane of the oocytes II and III submitted to the concentrations of 25 mg/ml, 33.0 mg/ml, and 40.0 mg/ml of *A. oleracea* extract was thickened, which may have occurred to preserve the internal areas of the cells (cytoplasm and nucleus), once the membrane would lose permeability to prevent the extract from penetrating and consequently avoid possible damage. This process was also reported by Denardi *et al.*<sup>[37]</sup> for *R. sanguineus* ticks exposed to neem oil.

In the oocytes in more advanced developmental stages (IV and V), the extract caused less damage in comparison with oocytes I and II. The damages the extract caused to the oocytes in more advanced developmental stages (IV and V) can be justified by the presence of the chorion, protective membrane deposited by exocytic vesicles polymerized in the extracellular space between the basal lamina and the plasma membrane.<sup>[21,28-30,41]</sup> The basal lamina deposition starts in oocyte III<sup>[29]</sup> and is completed in oocyte V.<sup>[28]</sup> The fully deposited chorion is a thick membrane and represents an important barrier for the extract. Although the chorion is not fully capable to prevent the extract from penetrating oocytes IV and V, it is certainly efficient at minimizing such penetration<sup>[42,43]</sup>. Similar results were found by Oliveira *et al.*<sup>[7]</sup>, Oliveira *et al.*<sup>[42]</sup> and Remedio *et al.*<sup>[38]</sup> for *R. sanguineus* treated with fipronil, dinotefuran and neem oil.

The ovary wall of the semi-engorged *R. sanguineus* females from the control Groups I and II (Groups I and II) presented the same characteristics described by de Oliveira *et al.*,<sup>[21]</sup> similar to the ones described for other tick species.<sup>[28-30,33]</sup> The wall is consisted of a delicate layer of cubic epithelial cells with round nuclei limiting a narrow lumen. The ovary walls were damaged only in the individuals submitted to the treatment with 40 mg/ml of the extract. They lost their original shape, becoming deformed and with limited cytoplasm, presenting vacuolated cells with flat morphology and pyknotic nuclei. Such data indicate the occurrence of apoptosis<sup>[44]</sup> or autophagy<sup>[45]</sup> in the individuals treated. In the present study, probably autophagy is occurring, once the cells would be trying to eliminate the aggressing agent and the damaged cells, in an attempt to survive and ensure the development of the cells, the pedicel, and oocytes as well.

Histochemical techniques (PAS and bromophenol blue) were used to detect the presence of polysaccharides and proteins in the ovaries of the semi-engorged females from control Groups I and II and treatment groups.

Polysaccharides and proteins were found in the oocytes of the individuals belonging to control Groups I and II.<sup>[21]</sup> In the treatment groups (Groups III, IV, and V), the extract caused a gradual reduction in the number of protein elements and polysaccharides in oocytes I, II, III, IV, and V as the concentrations increased. These modifications occurred due to the vacuolation in the interior of the cells, the decrease in the number and size of yolk granules, and the decrease in affinity to the histochemical stain (in the cytoplasm, yolk granules, and membranes). Such data reveal that the treatment with *A. oleracea* extract affected the synthesis and storage of proteins and polysaccharides in the germinative cells of the *R. sanguineus* females treated. Consequently, several components of the germinative cells would become fragile deformed and the synthesis and storage of yolk would be compromised (quantitatively and qualitatively). As a result, the forming germinative cells would be so impaired that they would not be capable to originate a new individual. Similar data were found by Roma *et al.*<sup>[18]</sup> for *R. sanguineus* ticks treated with permethrin.

In addition to the ovaries, the midgut was used as a tool to investigate the action of *Acmella oleracea* extract. The midguts of the semi-engorged *R. sanguineus* females from control groups I and II presented the epithelial wall formed by different types of cells and supported by a basal membrane and a thin muscle tissue layer, corroborating Till, Balashov, Agbede and Kemp, Koh *et al.*, Sonenshine, Agyei and Runham, Harrison and Foelix and Sonenshine and Roe<sup>[1,26,27,31,46-51]</sup> for other tick species.

The midgut of the semi-engorged females exposed to 25 mg/mL of *A. oleracea* extract presented similar characteristics to those of control Groups I and II. The females exposed to *A. oleracea* extract at the concentration of 33 mg/mL showed alterations in the digestive cells (in all stages of development) and in the lumen. The sessile digest cells became elongated and the apical cytoplasm of the residual sessile digest cells was more projected toward the lumen. The sessile and residual sessile digest cells stored fewer endosomes and digestive vacuoles of greenish coloration. Detached digest cells were not observed. These data confirm the action of *A. oleracea* extract on the midgut of the ticks submitted to the treatment. The extract would be damaging the midgut epithelial cells, i.e., the plasma membrane of the digestive cells. Once the digestive cell membrane comprises receptors for blood cells,<sup>[31,49]</sup> the processes of blood uptake and the formation of endosomes and digestive vacuoles would be impaired.<sup>[1,31,49]</sup> The green coloration could be directly associated with the color of the extract applied to the ticks. Similar alterations were reported by Valotto *et al.*<sup>[52]</sup> and Scudeler and dos Santos,<sup>[53]</sup> who studied *Aedes aegypti* larvae and *Ceraeochrysa claveri* larvae exposed to natural compounds, respectively.

The semi-engorged females exposed to 40 mg/mL of *A. oleracea* extract showed several alterations in the generative cells (stem cells), in all developmental stages, and in the lumen as well. Some generative cells (stem cells) were irregular, with limited cytoplasm, elongated or pyknotic nuclei with blebs, and irregular plasma membrane, sometimes twisted or detached from the basal membrane; others were irregular and their cytoplasm presented numerous rounded vacuoles of different sizes and nuclei with blebs. These data suggest that the extract would be damaging the plasma membrane and penetrating into the epithelial generative cells, affecting the cytoplasm elements (captured by the vacuoles) and the nuclei of the cells. The presence of pyknotic nuclei and blebs confirms that the damages were intense and irreversible, i.e., the generative cells were heavily affected, no longer capable of playing the role of renovating the midgut epithelial cells<sup>[46,49]</sup> and replacing the midgut cells released from the basal lamina during the digestive process. Similar data were obtained by Ghribi *et al.*<sup>[54]</sup> and Scudeler *et al.*<sup>[55]</sup> for insects treated with biosurfactants and neem oil (*Azadirachta indica*), respectively.

Some sessile digest cells and residual sessile digest cells displayed extensive disorganized areas, absence of cytoplasmic limits, smaller endosomes, smaller digestive vacuoles, and greenish cytoplasm; others showed extensive disorganized regions, extensive vacuolated regions, absence of cytoplasmic limits, few endosomes, and digestive vacuoles. Detached digest cells were not observed in this group. Such results can be justified by the action of *A. oleracea* extract on the midgut epithelial digestive cells, probably by the direct contact of the extract with the midgut cells and posterior internalization. The digestive cells may have been so damaged that they would not be able to perform, completely or partially, the functions of uptaking the blood ingested during the feeding process, lysis of blood cells in the digestive vacuoles,<sup>[47,48]</sup> formation of residual hematinic bodies (final product of intracellular digestion, accumulated inside the cell)<sup>[46,50]</sup> and release of nutrients after the digestive processes,<sup>[31,49]</sup> consequently compromising the ectoparasite nutrition, the metabolism of the organ, and the viability of the individual. Scudeler and dos Santos<sup>[53]</sup> and Scudeler *et al.*<sup>[55,56]</sup> reported the occurrence of these processes in insects treated with neem oil.

The histochemical techniques such as bromophenol blue and PAS showed moderate number of proteins and polysaccharides in the midguts of the semi-engorged females from control Groups I and II. The females from the group exposed to 25 mg/mL of the extract did not show histochemical alterations after the treatment. In the groups exposed to 33 and 40mg/mL, the extract caused gradual decrease in the number of protein and polysaccharides, confirming that the extract would be affecting the midgut epithelial cells and consequently the digestive processes. These processes were also reported by Scudeler *et al.*<sup>[55]</sup> for *C. claveri* larvae treated with neem oil. In the present study, the damages observed in the oocytes and in the midgut cells were caused by the components of

*A. oleracea* (Jambu) extract. Spilanthol is the main active ingredient of Jambu and is responsible for most of its biological activities.<sup>[14,15,57]</sup> Further studies are needed to elucidate *A. oleracea* compounds and their action mechanisms.

## CONCLUSION

The results obtained in the present study suggest the occurrence of progressive damage caused by *A. oleracea* extract to the germinative and midgut cells of semi-engorged *R. sanguineus* ticks. The concentrations applied compromised the complete uptake and digestion of blood in the midgut epithelial cells of the animals treated, which results in the lack of the necessary nutrients to maintain the physiological processes of the ectoparasites. In advanced stages, such “malnutrition” can lead the ectoparasite to death. The germinative cells were so heavily impaired that they will probably not be able to advance the developmental stages (I–V) and complete vitellogenesis to be released during ovulation, which affects the female fertility and prevents the generation of a new individual.

Therefore, it can be concluded that the effects of *A. oleracea* extract are similar to those caused by acknowledged and efficient chemical products used to control ticks.

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## Conflicts of interest

There are no conflicts of interest.

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