Cytotoxic Effects of Extract of *Acmella Oleraceae* (Jambú) in *Rhipicephalus Microplus* Female Ticks

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ABSTRACT The present study analyzed the effects of different concentrations of the hexane extract of *A. oleraceae* (HEAO) (Jambú) on the germ cells of semi-engorged *Rhipicephalus microplus* female ticks, through a morpho-histological study, evaluating the effectiveness of the extract in the genesis of the individuals. To perform this analysis, 100 semi-engorged females were divided into five groups with 20 individuals each: groups I and II, respectively constituted by distilled water control and 50% ethanol + 1% DMSO, and groups III, IV, and V constituted by treatment with HEAO in the concentrations of 12.5, 25.0 and 50.0 mg/mL, respectively. All the ticks were immersed in the different concentrations of the extract or in distilled water for 5 minutes, dried and conditioned in BOD incubator for 7 days. The individuals of the treatment groups revealed the action of this extract showing alterations in the germ cells of the females from the different groups when compared with those from the groups I and II (control groups). These alterations were mainly related to the size and shape of the oocytes; number of yolk granules; presence, number, size and location of vacuoles in the cytoplasm of all the germ cells; and the presence of nuclear alterations in these cells as well. Thus, it was demonstrated that the concentrations of HEAO affected the germ cells of *R. microplus* ticks. The effects of the extract are similar to those caused by renowned and efficient chemical products used to control these ticks. *Microsc. Res. Tech. 79:744–753, 2016.*

INTRODUCTION

Rhipicephalus microplus is the main hematophagous ectoparasite of bovines and is responsible for serious economic losses, caused by blood spoliation, retardation of calf development and milk production, and transmission of pathogens that cause diseases such as babesiosis and anaplasmosis (Ghosh et al., 2006). The main method to control the *R. microplus* is based on the use of synthetic acaricides, and the frequent use of these compounds has led to a selection of resistant ticks (Mendes et al., 2011), with consequent inefficiency of treatments, increase in the dosages and number of applications (Furlong et al., 2007). The use of these acaricides can also lead to environmental contamination (soil and water), and poisoning of human beings and animals (contaminating milk and meat with residues), affecting directly and indirectly the health of the communities involved in food production (Graf et al., 2004; Roel, 2001).

In the search for new methods to control these ectoparasites, medicinal plants emerge as a viable and promising alternative, due to the great variability of species, low cost and accessibility in particular regions (Agnolin et al., 2010). Over the last years, many essential oils and extracts of natural plants have showed promising effects to control *R. microplus* (Castro et al., 2014; Garcia et al., 2012; Singh and Chaturvedi, 2012a,b).

The cattle tick has a high biotic potential (Oliver, 1989); therefore, products and substances to impair their reproductive process are of great interest in the control of these populations. *A. oleracea* is a medicinal plant from the Asteraceae family and has several therapeutic functions, presenting an increasing demand worldwide (Prachayasittikul et al., 2013). The hexane extract of *A. oleracea* demonstrated high efficiency on *R. microplus*, proven by the significant reduction in the oviposition and hatchability of eggs, interfering in the reproductive system of this ectoparasite (Castro et al., 2014).

The present study had the objective to verify the effect of different concentrations of the hexane extract of *A. oleraceae* (HEAO) on the germ cells of semi-engorged *Rhipicephalus microplus* ticks through a morpho-histological study, evaluating the impact of the extract in the genesis of new individuals (interfering or not), providing fundamental data for the development and improvement of methods to control this

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Abbreviation: HEAO, hexane extract of *A. oleraceae*

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ectoparasite, causing less harm to the environment and non target organisms.

MATERIAL AND METHODS

Natural Substance: *Acmella Oleracea*

Aerial parts of *A. oleracea* were collected at Anidro do Brasil Extrações S.A. pharmaceutical chemical company, located in the city of Parnaíba, Piauí, Brazil. Reference plant specimens were deposited in the Herbarium CEN of the *Embrapa Recursos Genéticos e Biotecnologia*, Brasília, Distrito Federal, Brazil, under the number 81102.

Preparation of Extracts

The plant material was dried at 40°C, ground in a hammer mill (1 mm sieve), and used for three consecutive extractions one per hour with hexane solvent, being kept under constant stirring and heating at 40°C for 2 h. The hexane was evaporated under reduced pressure using a rotary evaporator until complete removal of the solvent and formation of the hexane extract.

Chemical Analysis

The hexane extract of *A. oleracea* was analyzed by isocratic high-performance liquid chromatography using a Hitachi Lachron Elite L-2000 (Merck) with a Lichrospher C18 5 μm (125 × 4 mm) column (Merck), acetonitrile-water (30:70), a flow rate of 1.0 mL/min., and a UV detector at 229 nm, with a running time of 10min and a 20-μL injection volume (Internal Development) (Castro et al., 2014). The external standard used was 95.9 ppm of spilanthol.

Rhipicephalus Microplus Ticks

R. microplus semi-engorged females, weighing 70 mg on average, were used throughout the experiment. They were collected from naturally infested calves. The semi-engorged stage of the females was chosen due to the high parasitory efficiency in this phase.

Dosage

The initial concentration of extract was defined based in Castro et al. (2014). Several concentrations were evaluated in preliminary tests (pilots) by diluting of extract in 50% ethanol and 1% DMSO. In this study, the concentrations corresponded to 12.5 mg/mL, 25 mg/mL and 50 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. microplus semi-engorged females were divided into three treated groups: group III (12.5 mg/mL), group IV (25 mg/mL) and group V (50 mg/mL). The control group I was exposed to the placebo (distilled water) and the control group II was exposed to 50% ethanol and 1% DMSO.

The 100 semi-engorged females of *R. microplus*, after being washed in a sieve with tap water, were dried on soft absorbent paper. After that, 60 females were divided into three groups of 20 females (20 females for each concentration—two groups with 10 individuals—duplicates) and immersed for 5 minutes in Petri dishes containing the above different concentrations of extract. The control group I and II were also composed of 20 females that had been immersed in distilled water and in 50% ethanol and 1% DMSO, respectively. Ticks were then dried in absorbent paper and placed in the BOD incubator (28 ± 1°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 (Roma et al., 2010). After the monitored for 7 days, all the semi-engorged females forwarded to histological and histochemical techniques.

Methods

Histology. All the semi-engorged females maintained in the refrigerator for thermal shock anesthesia were dissected in a phosphate buffered saline-PBS solution (NaCl 7.5 g/L, Na_2HPO_4 2.38 g/L e KH_2PO_4 2.72 g/L).

The ovaries were fixed for 24 h in 4% paraformaldehyde, dehydrated in ethanol, embedded in Leica resin for 24 h at 4°C and transferred to plastic moulds previously filled with polymerized Leica resin. After resin polymerization, all the blocks were sectioned at 3 μm 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 equipments were from the Histology Laboratory of the Biology Department at the Biosciences Institute, UNESP, campus of Rio Claro-SP, Brazil.

RESULTS

Histology

Control Group (Group I and II). The ovary of the semi-engorged *Rhipicephalus microplus* females of the control group showed typical *R. microplus* characteristics as described by Saito et al. (2005). Below, a summary of the main characteristics.

The ovary of *R. microplus* consists of an epithelial cells wall and the oocytes (germ cells) at different developmental stages (I to V) attached to the epithelial wall through a pedicel (Oliveira et al., 2005; Saito et al., 2005).

The oocytes I are small and elliptical cells, and the germ cell has a prominent nucleus, with homogeneous cytoplasm and no yolk granules, covered by a thin plasmic membrane (Fig. 1A); the oocytes II are larger compared with oocytes I, elliptical, with central germinal vesicle and thin and homogeneous cytoplasmic granulation (Fig. 1B); the oocytes III show intermediate size, round shape, the germinal vesicle occupy the pole turned to the pedicel and the cytoplasm is full of granules of different sizes; the smaller granules are located in the central region and the larger ones are seen in the periphery (Fig. 1C); the oocytes IV are larger than oocytes III, rounded, the germinal vesicle is rarely seen, occupying the oocyte pole, the cytoplasm shows many yolk granules of different sizes distributed beyond the beginning of the chorium deposition (Fig. 1D); the oocytes V are the largest germ cells,

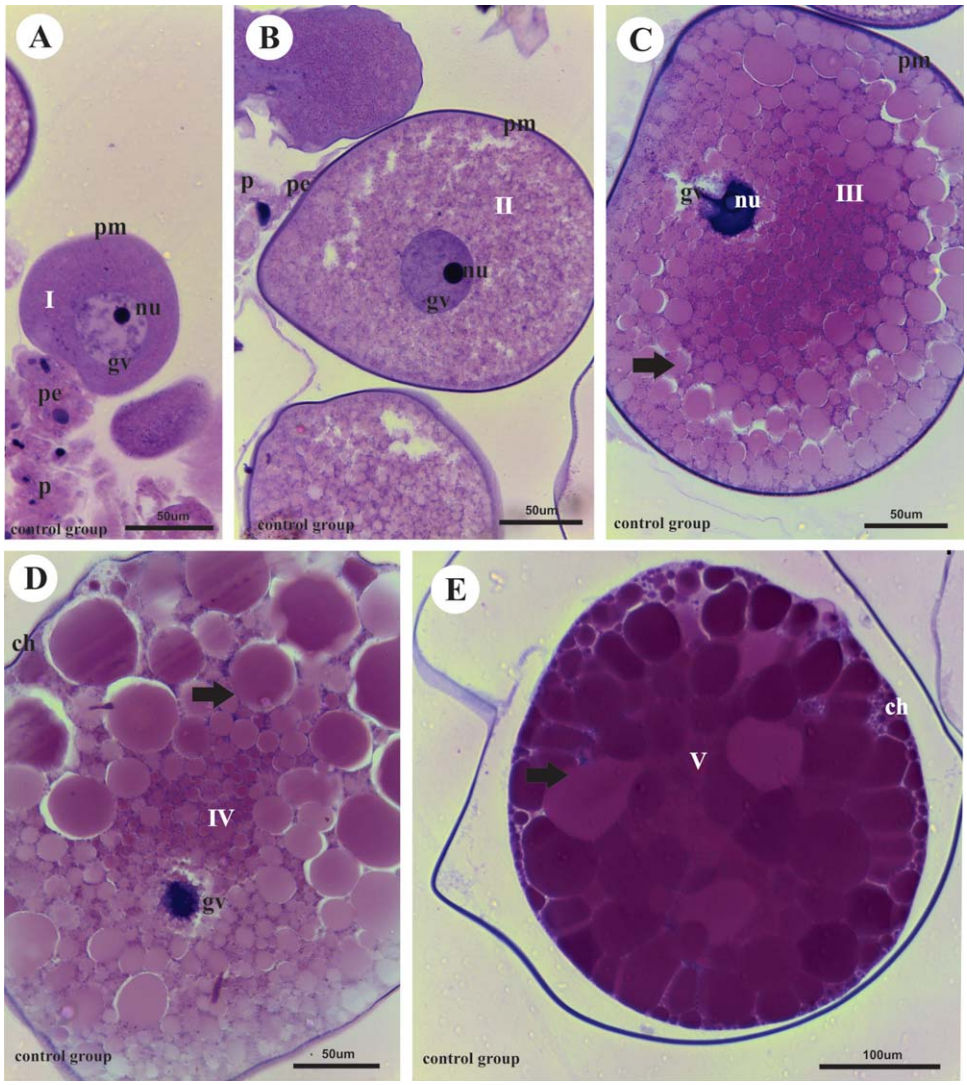

Fig. 1. Histological sections of extract-treated Rhipicephalus microplus ovary. A–E. Control Group I and II (Group I and II). I = Oocyte I; II = Oocyte II; III = Oocyte III; IV = Oocyte IV; V = Oocyte V; ch = chorium; p = ovary ephitelium; gv = germ vesicle; nu = nucleolus; pe = pedicel; pm = plasmic membrane; arrow = yolk granules. Bars: A–D = 50 μm, E = 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

rounded, the germinal vesicle is no longer observed and the chorium (membrane) is thick and completely deposited (Fig. 1E) (Oliveira et al., 2005; Saito et al., 2005). In this species of ticks, small vacuoles can be seen among the yolk granules in the oocytes II, III, IV, and V (Saito et al., 2005).

Group III. Semi-engorged females exposed to 12.5 mg/mL of the *Acmela oleracea* extract showed oocytes with significant morphological alterations when compared with the control group (Table 1).

The oocytes I show the cytoplasm with large rounded vacuoles next to the plasmic membrane. These vacuoles dislocate the germinal vesicle towards the cell poles. In some oocytes I, the germinal vesicle showed vacuoles or even a ring-shaped nucleolus (Figs. 2A–2D).

The oocytes II show several rounded vacuoles of medium size occupying the region next to the plasmic membrane. The germinal vesicle is surrounded by vacuoles, mainly in the region in contact with the nuclear membrane. The plasmic membrane also shows alterations, in some regions it appears to be divided, or the basal lamina might have detached. Some oocytes II show extensive vacuolation throughout the cytoplasm, alterations in the plasmic membrane and absence of germinal vesicle (Figs. 2E and 2F).

The oocytes III show a vacuolated region surrounding half of the cell, pointing to the pedicel (Figs. 2G and 2H).

The oocytes IV show the cytoplasm with larger vacuoles around the yolk granules (Fig. 2I).

The oocytes V showed no alterations, except for few folds in the membrane (chorium and plasmic membrane) and few and small cytoplasmic vacuoles in the periphery of the cell (Fig. 2J).

Group IV. The ovary of *R. microplus* semi-engorged females from treatment group IV showed a larger number of modified oocytes and morphological

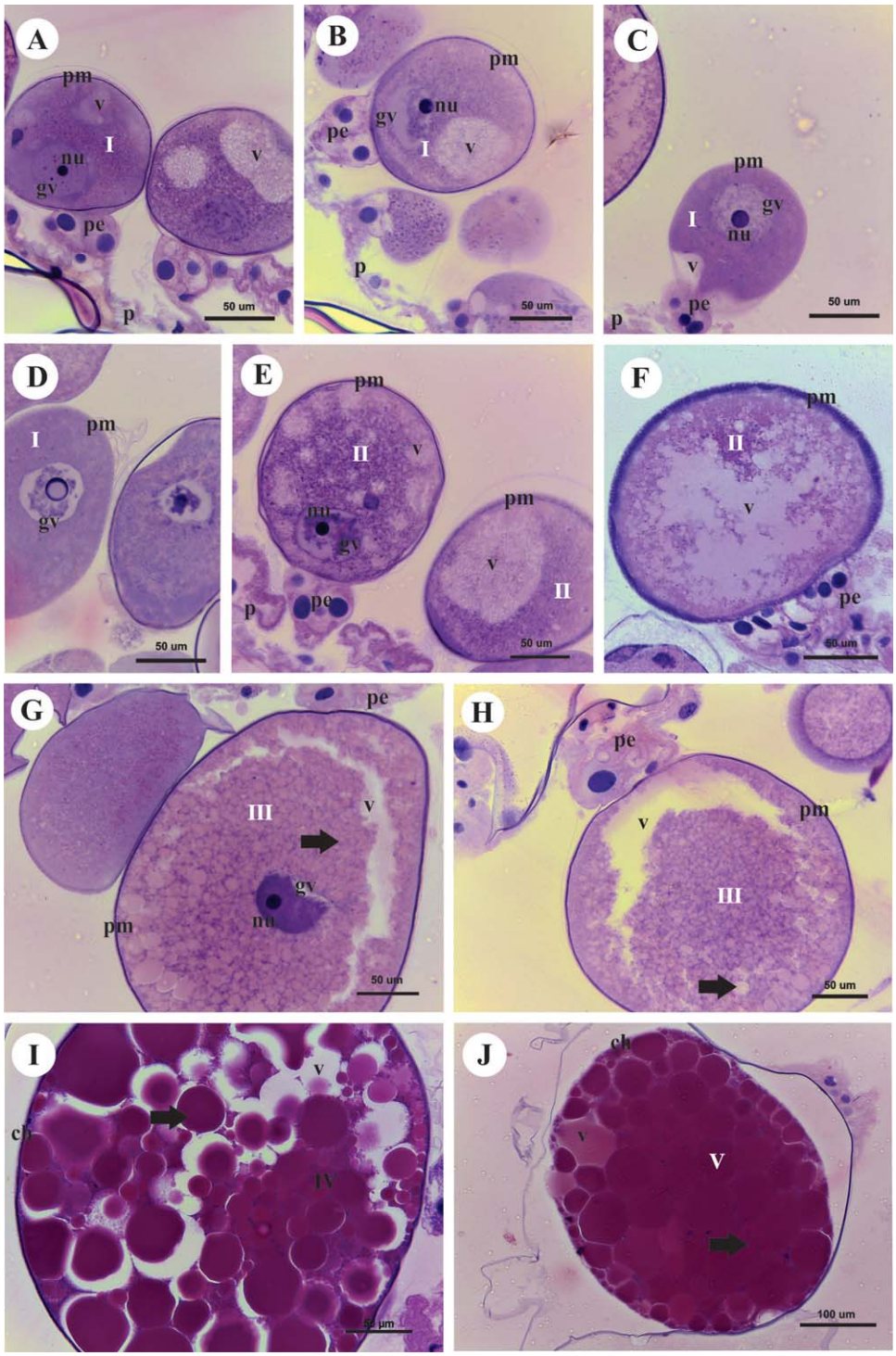

Fig. 2. Histological sections of extract-treated Rhipicephalus microplus ovary. A–J. Group III (12.5 mg/mL). I = Oocyte I; II = Oocyte II; III = Oocyte III; IV = Oocyte IV; V = Oocyte V; ch = chorium; p = ovary ephitelium; gv = germ vesicle; nu = nucleolus; pe = pedicel; pm = plasmic membrane; arrow = yolk granules, v = vacuoles. Bars: A–I = 50 µm, J = 100 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

alterations when compared with those from the previous group (Table 1).

The oocytes I have lost their original shape and are totally deformed. The plasmic membrane shows several folds, some of them long and with ramifications. The germinal vesicle is not observed and the cytoplasm is scarce and no longer homogeneous (Figs. 3A and 3B).

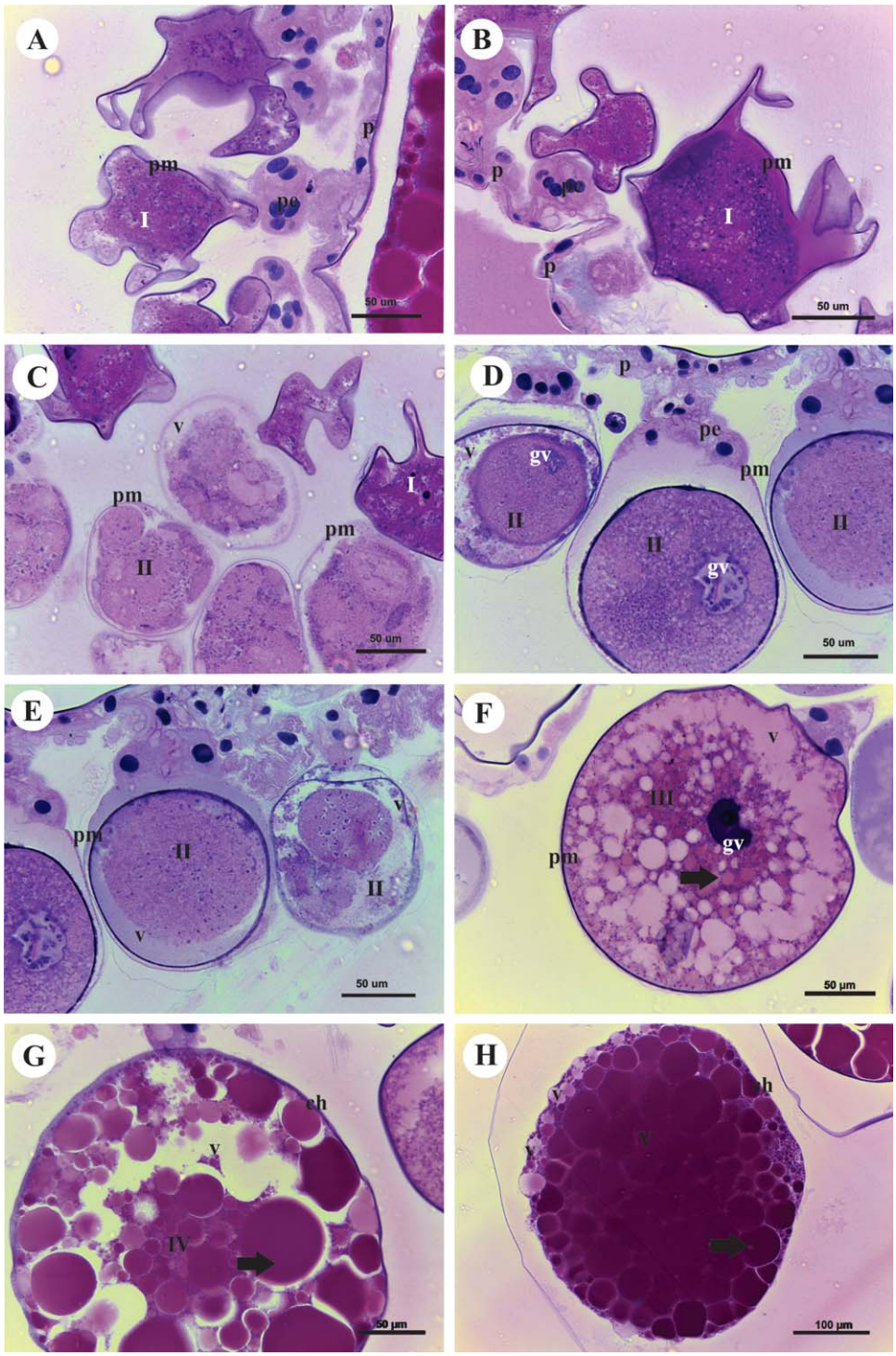

Fig. 3. Histological sections of extract-treated Rhipicephalus microplus ovary. A–H. Group IV (25 mg/mL). I = Oocyte I; II = Oocyte II; III = Oocyte III; IV = Oocyte IV; V = Oocyte V; ch = chorium; p = ovary ephitelium; gv = germ vesicle; nu = nucleolus; pe = pedicel; pm = plasmic membrane; arrow = yolk granules, v = vacuoles. Bars: A–I = 50 µm, H = 100 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The oocytes II show some folds in the plasmic membrane, cytoplasm with several rounded vacuoles and disorganized regions, which limits the area occupied by yolk granules. However, the germinal vesicle is rarely observed, and when present, it shows irregular morphology with the membrane full of folds. In some oocytes II, large vacuoles forming a halo surrounding the whole or half periphery of the cell are observed (Figs. 3C–3E).

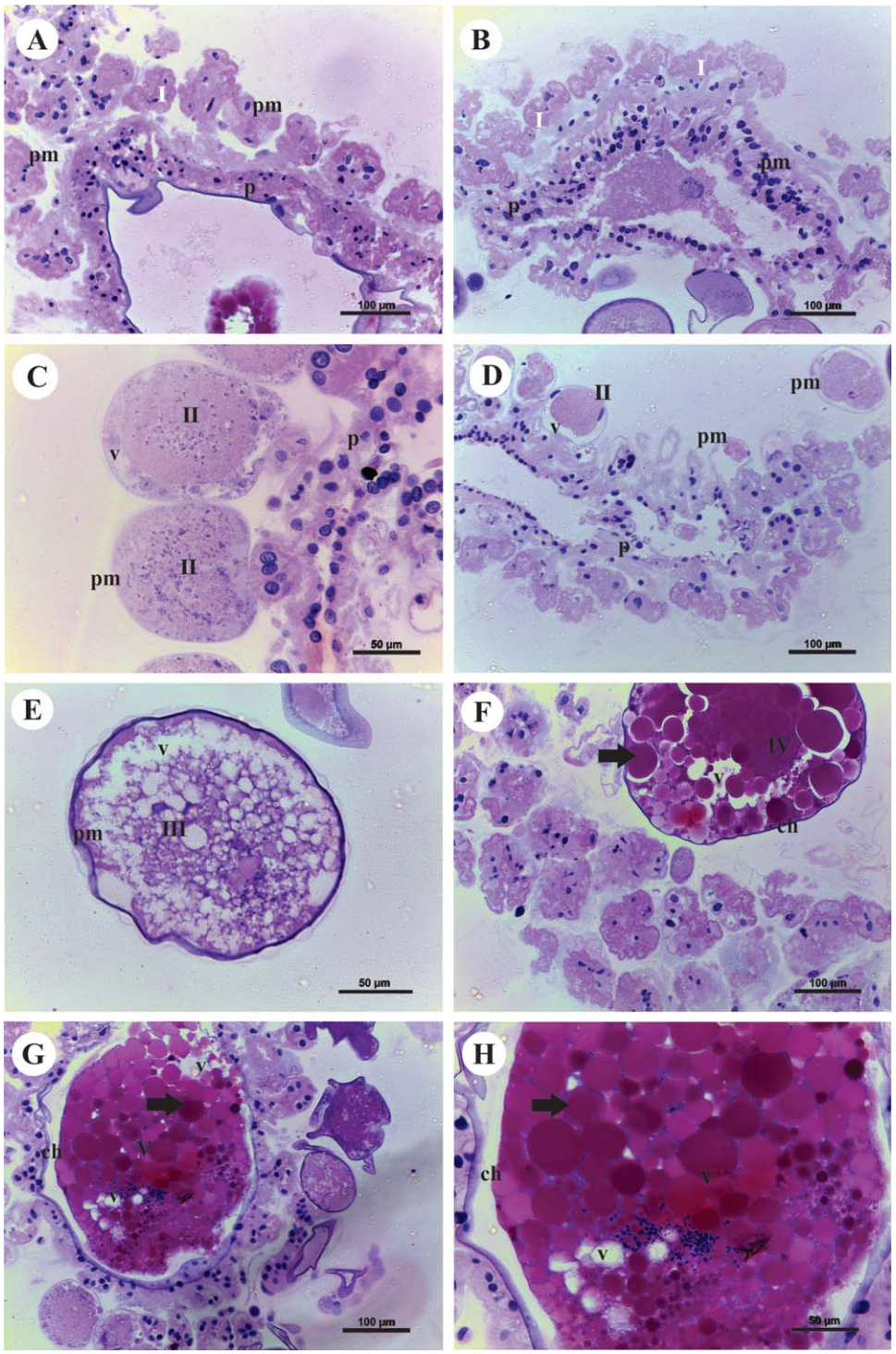

Fig. 4. Histological sections of extract-treated Rhipicephalus microplus ovary. A–H. Group V (50 mg/mL). I = Oocyte I; II = Oocyte II; III = Oocyte III; IV = Oocyte IV; V = Oocyte V; ch = chorium; p = ovary ephitelium; gv = germ vesicle; nu = nucleolus; pe = pedicel; pm = plasmic membrane; arrow = yolk granules, v = vacuoles. Bars: A-B = 100 µm, C = 50 µm, D = 100 µm, E = 50 µm, F = 100 µm, G = 100 µm, H = 50 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The oocytes III show alterations in their original shape, with folds in the membrane. Numerous and large vacuoles (especially rounded) are distributed throughout the cytoplasm, among the yolk granules and next to the peripheral region. The yolk granules previously found are no longer observed (Fig. 3F).

The oocytes IV show slight alterations in their shape and the cytoplasm with cytoplasmic vacuoles, more

frequent than those found in oocytes IV from the previous groups. These vacuoles occupy the region around the yolk granules, among these granules and towards the pedicel (Fig. 3G).

The oocytes **V** show numerous folds in the membrane and small rounded vacuoles in the peripheral region around the cell (Fig. 3H).

Group V. The ovaries of the females from group V showed numerous oocytes with significant histological alterations when compared with those from the control groups (I and II), groups III and IV (Table 1).

The oocytes I were no longer observed, having become a heterogeneous mass (Figs. 4A and 4B).

The oocytes II are rarely observed, and when present they show an irregular morphology, absence of germinal vesicle, numerous disorganized areas and vacuolated region distributed throughout the cytoplasm. Some oocytes II also showed large vacuoles forming a halo around the cell and yolk granules located in the central region (Figs. 4C and 4D).

The oocytes III also suffered severe alterations. Their shape is altered due to the presence of folds in the membrane, and the plasmic membrane appears to be divided, or the basal lamina detached, or be "delaminating." The germinal vesicle is rarely observed. The cytoplasm shows extensive vacuolated regions in more than 50% of the cell, showing large empty spaces mainly next to the peripheral region. The few yolk granules occupy mainly the central region (Fig. 4E).

The oocytes IV show slight deformations in the membrane and the cytoplasm with vacuolation around the yolk granules, among these granules and towards the pedicel. The yolk granules are smaller than those observed in the previous groups (Fig. 4F).

The oocytes V show irregular morphology with the presence of many folds in the membrane and large yolk granules, but smaller and less dense in comparison with the previous ones. Cytoplasmic vacuoles are observed among the large yolk granules and in the periphery around the whole cell. In the central region, ruptured yolk granules releasing their content into the cytoplasm are observed. Some oocytes V are ruptured (Figs. 4G and 4H)

The ovary wall was affected in this treatment group. The epithelium is now composed by vacuolated cells with flat morphology and pyknotic nucleus (Figs. 4A, 4B, and 4D).

DISCUSSION

The physical and economic losses caused by the infestation and infections by ectoparasites can be minimized through chemical control, by the use of synthetic acaricides with a great variety of active principles existing in the market. However, it is not possible to prevent the emergence of new resistant ectoparasite populations (Furlong et al., 2007; Nolan, 1985). This chemical control causes serious problems, such the contamination of the environment and non target organisms with residues (Nolan, 1985; Oliveira et al., 2009, 2008; Pruett, 1999).

The use of natural substances, obtained from plant extracts, is a promising alternative to control these ectoparasites. Plants from the family Meliaceae, including the species *Melia* (Oelrichs et al., 1983), *Trichilia*, *Toona*, *Aglaia* and *Azadirachta indica* (Martinez, 2002), from the family Asteraceae, as the species *Acmella oleracea*, commonly known as Jambu, have been recognized as promising tools to control pest populations (Castro et al., 2014).

The individuals from the treatment groups III, IV and V subjected to the concentrations of 12.5 mg/mL, 25 mg/mL and 50 mg/mL of the HEAO demonstrated the action of this extract through alterations in the germ cells in the females from different groups when compared with groups I and II (control groups). These alterations occurred mainly in relation to the size and shape of the oocytes; number of yolk granules in the oocytes; presence, number and location of vacuoles in the cytoplasm of all the germ cells and the presence of nuclear alterations in these cells.

The histological analysis of the semi-engorged *Rhipicephalus microplus* females from the control groups I and II (groups I and II), showed that the ovary of these individuals is constituted by a large number of oocytes in different developmental stages attached to an epithelial wall through a pedicel, corroborating data obtained by Balashov (1983) for *Hyalomma asiaticum*, Sonenshine (1991) for *Dermarcentor andersoni* and *Dermarcentor variabilis*, Harrison and Foelix (1999) for *Ornithodoros moubata*, Denardi et al. (2004) for *Amblyomma cajennense*, Oliveira et al. (2005) for *R. sanguineus*, Saito et al. (2005) for *R. microplus* and Oliveira et al. (2006) for *A. triste*.

Comparing the ovaries of the females from groups I and II (groups I and II), no differences were observed. Both showed oocytes in five developmental stages (I-V) with typical morphological characteristics, widely described for several tick species (Denardi et al., 2004; Oliveira et al., 2006, 2005; Saito et al., 2005).

The oocytes I and II of the females subjected to 12.5 mg/mL of the HEAO show the cytoplasm and germinal vesicle with large vacuoles, which indicates that the extract in this concentration is able to damage the germ cells. When exposed to 25.0 mg/mL of the extract, these cells showed even more significant alterations, such as the presence of folds in the membranes and broad restriction in the cytoplasmic area. In the females treated with 50 mg/kg of the extract, the oocytes I are no longer observed and the oocytes II are rarely verified, demonstrating that as the concentration of the extract increased, more damage in these germ cells were observed. These data can be explained by the absence of the chorium (protective membrane that is not completely deposited). This situation allowed the entrance of the extract components, and consequently the occurrence of more damage in the interior of the cells. The deposition of this membrane begins in oocytes III (Denardi et al., 2004), finishes in oocytes V, (Oliveira et al., 2005) and finally results in an extra protection to the entrance of foreign substances in the oocytes (Oliveira et al., 2008). Similar results were found by Oliveira et al. (2008, 2009) and Roma et al. (2010) for *Rhipicephalus sanguineus* ticks exposed to synthetic acaricides such as fipronil and permethrin, respectively.

The oocytes I and II, as mentioned above, showed autophagic vacuoles, structures that would probably have the function of eliminating the cell organelles damaged by the extract, conditioning them in small

TABLE 1. Morpho-histological differences observed in the germ cells of semi-engorged Rhipicephalus microplus female ticks treated with different concentrations of the extract

Oocytes	Control group	Group III	Group IV	Group V
Oocytes I	Small and elliptical cells, prominent nucleus, homogeneous cytoplasm no yolk granules, covered by a thin plasmic membrane (Fig. 1A).	Cytoplasm with large rounded vacuoles. In some oocytes I, the germinal vesicle showed vacuoles or even a ring-shaped nucleolus (Figs. 2A–2D).	Totally deformed. The plasmic membrane shows several folds. The germinal vesicle is not observed and the cytoplasm is scarce (Figs. 3A and 4B).	Oocytes I were no longer observed, having become a heterogeneous mass (Figs. 4A and 4B).
Oocytes II	Larger compared with oocytes I, elliptical, central germinal vesicle and thin and homogeneous cytoplasmic granulation (Fig. 1B).	Several vacuoles of medium size in the cytoplasm. Germinal vesicle is surrounded by vacuoles. Plasmic membrane appears to be divided. Some oocytes II show extensive vacuolation, alterations in the plasmic membrane and absence of germinal vesicle (Figs. 2E and 2F).	Folds in the plasmic membrane, cytoplasm with several rounded vacuoles and disorganized regions, germinal vesicle rarely observed. In some oocytes II, large vacuoles forming a halo (Figs. 3C–3E).	Rarely observed, and when present they show an irregular morphology, absence of germinal vesicle, numerous disorganized areas and vacuolated region. Some oocytes II also showed large vacuoles forming a halo (Figs. 4C and 4D).
Oocytes III	Intermediate size, round shape, germinal vesicle occupy the pole turned to the pedicel, granules of different sizes (the smaller are located in the central region and the larger ones are seen in the periphery) (Fig. 1C).	Vacuolated region surrounding half of the cell, pointing to the pedicel (Figs. 2G and 2H).	Folds in the membrane, numerous and large vacuoles are distributed throughout the cytoplasm (Fig. 3F).	Suffered severe alterations. Folds in the membrane. The germinal vesicle is rarely observed. The cytoplasm shows extensive vacuolated regions in more than 50% of the cell (Fig. 4E).
Oocytes IV	Larger than oocytes III, rounded, germinal vesicle is rarely seen, occupying the oocyte pole, many yolk granules of different sizes distributed, beginning of the chorium deposition (Fig. 1D).	Cytoplasm with larger vacuoles around the yolk granules (Fig. 2I).	Slight alterations in their shape and the cytoplasm with many cytoplasmic vacuoles (Fig. 3G).	Slight deformations in the membrane and the cytoplasm with vacuolation. The yolk granules are smaller than those observed in the previous groups (Fig. 4F).
Oocytes V	The largest germ cells, rounded, germinal vesicle is no longer observed, large yolk granules, the chorium (membrane) is thick and completely deposited (Fig. 1E)	No alterations, except for few folds in the membrane and few and small cytoplasmic vacuoles in the periphery of the cell (Fig. 2J).	Numerous folds in the membrane and small rounded vacuoles in the peripheral region around the cell (Fig. 3H).	Presence of many folds in the membrane and yolk granules smaller and less dense. Cytoplasmic vacuoles and ruptured yolk granules are observed. Some oocytes V are ruptured (Figs. 4G and 4H)

compartments that would posteriorly undergo degradation processes (autophagic), in order to maintain the viability of the cell (Carvalho and Recco-Pimentel, 2012; Junqueira and Carneiro, 2013). As the concentration of the extract increased, more damage was caused to the cells, and there was a consequent increase in the number and size of the vacuoles in the oocytes treated.

The oocytes I and II exposed to the extract in the concentration of 12.5 mg/mL, showed vacuoles in the germinal vesicle and even a ring-shaped nucleolus. In higher concentrations of the extract (25 and 50 mg/mL), the germinal vesicle was not detected. These characteristics indicate the occurrence of degenerative processes in the nuclear material (Cruz-Landim and Höfling, 1979; Roma et al., 2010). Such alterations allow us to infer that the degeneration of the oocytes genetic material is occurring after the treatment, causing irreversible damage that will impair the development of these cells and culminate in the death of germ cells. Thus, the extract concentrations applied would be affecting the fertility of these ectoparasites. Similar finding were obtained by Denardi et al. (2010) for *R. sanguineus* treated with neem leaves extract, Roma et al. (2010) for *R. sanguineus* treated with permethrin and Vendramini et al. (2012) for *R. sanguineus* treated with andiroba oil.

The oocytes III of the females subjected to 12.5 mg/mL of the HEAO showed vacuolated region around half of the cell. In the oocytes of group V, significant alterations in shape, in the membrane, in the germinal vesicle and in the cytoplasm were observed. These severe modifications confirm the occurrence of damage caused by the extract and its direct action on the reproductive system. The extract has probably crossed the tegument of the ectoparasite, was transported through the hemolymph until reaching the reproductive system and was absorbed to the interior of the oocytes. Finally, the extract affected the germ cells, originating damaged organelles that were captured and digested by the vacuoles, trying to preserve the integrity of the cell. Studies conducted by Oliveira et al. (2009) on *R. sanguineus* ticks exposed to the chemical fipronil also demonstrated extensive alterations in the germ cells after treatment. Remedio et al. (2015), studying *R. sanguineus* ticks exposed to neem oil, also reported

that the oocyte wall is the main entrance route of the compounds to the interior of these cells.

The massive presence of vacuoles occupying extensive areas of the cytoplasm in oocytes III from group V would bring significant damage to the cell, as the decrease in the number of yolk granules, dislocation and atrophy of the germinal vesicle. Such alterations could interfere in the activities of the geminal vesicle and impair or prevent these oocytes from advancing to more advanced developmental stages, conclude the vitellogenesis and form the embryo, impairing the development of new individuals. The occurrence of this process was also observed by Friesen et al. (2003), in *A. hebraeum* ticks treated with the chemical avermectin.

Friesen and Kaufman (2003) studied the *A. hebraeum* tick and reported that the chemical cypermethrin inhibited the vitellogenin (main yolk granules protein) uptake by the oocytes in these individuals. In the present study, this process can be occurring in the oocytes of the *R. microplus* exposed to the extract, once smaller and fewer yolk granules were found in this oocytes after the treatment.

The oocytes in advanced developmental stages (IV and V) did not show alterations when treated with 12.5 mg/Kg of *A. oleraceae* hexane extract, except for small vacuoles around the yolk granules of oocytes IV, few folds in the membrane and vacuoles in the periphery of oocytes V. In the group treated with 25 mg/Kg of the extract, the oocytes IV showed deformation in the membrane and cytoplasm with cytoplasmic vacuoles. Oocytes V showed several folds in the membranes and small rounded vacuoles in the peripheral region around the cell. In the group treated with the highest concentration of the extract (50 mg/Kg), the oocytes IV showed slight deformations in the membrane and the cytoplasm with and more intense and frequent vacuolation. The oocytes V showed irregular morphology, smaller, less dense and sometimes ruptured yolk granules, in addition to vacuoles. These data indicate the action of the extract on the cell components and that the chorium (thick protective membrane, totally deposited in the developed oocytes) obstructs the entrance of the *A. oleraceae* extract, probably for reducing the contact surface between the oocyte membrane and the hemolymph. However, this is not enough to completely prevent the entrance of the extract, once alterations were observed in the oocytes IV and V treated, as observed by Vendramini et al. (2012) in *R. sanguineus* treated with andiroba oil.

The damages observed in this study demonstrated that the components of the HEAO (Jambu) caused direct effects on the oocytes. The Jambu effects are mainly caused by the spilanthol (Pandey et al., 2007; Ramsewak et al., 1999; Saraf and Dixit, 2002). Further studies are needed to elucidate action mechanisms of each compound found in *A. oleracea*.

The high *in vitro* efficiency of the HEAO on engorged *R. microplus* females has been recently proved, and a significant reduction in oviposition and hatchability has been observed only in high concentrations (over 150.0 mg mL^{-1}) (Castro et al., 2014). According to these authors, females exposed to a concentration sex times lower (25mg/mL) would only suffer a moderate action on their reproduction capacity, represented by a slight reduction of oviposition (14.7 ± 9.4), maintenance of the hatchability in higher levels (91.6 ± 1.5) and reduced efficacy (20.7 ± 9.3). However, in this study the ovaries of females from group IV exposed to 25mg/mL of extract showed numerous oocytes with histological alterations when compared with the control groups (I and II) and group III.

This apparent contradiction occurred because the females used in the reproductive performance study were completely engorged (Castro et al., 2014), showing a larger number of oocytes in more advanced developmental stages, and these oocytes are more resistant to the action of the compounds (Oliveira et al., 2009, 2008; Roma et al., 2010). As these oocytes are more resistant, probably fewer alterations occurred in these cells after the treatment with the extract. Thus, it can be suggested that, despite the damages suffered by the germ cells, they would be trying to promote the genesis of new individuals, continuing the life cycle. Simultaneously, the preservation of a small number of germ cells could be contributing to maintain the reproductive rates close to normal levels. The presence of a thicker cuticle in the engorged females (which occurs during the engorgement process (Harrison and Foelix, 1999; Sonenshine, 1991), could also have obstructed the penetration of the active principle, reducing its action on the reproductive system of these females, limiting the damages caused by higher concentrations. In addition, the treatment of engorged females with the extract can interfere in the development of larva, which can have presented some deficiencies. Therefore, even if the larva have hatched, their formation would not be satisfactory in order to allow them to climb the grass and be able to cause infestation, being unable to complete their cycle and evolve to the next instar.

In this study, the *A. oleraceae* extract was applied in semi-engorged *R. microplus* females. In this engorgement phase, the females show a large number oocytes in the initial developmental phases, when they are less resistant to the compounds (Oliveira et al., 2009, 2008; Roma et al., 2010). These cells were damaged by the extract concentrations used in this study, decreasing their capacity of producing viable eggs and form a new individual; i.e., the treatment in females in the beginning or in the middle of the engorgement process is more efficient in the control of this species.

Thus, the present study demonstrated that the concentrations of HEAO applied in *R. microplus* ticks affect their germ cells. These effects are similar to those caused by renowned and efficient chemical products used to control ticks, and is also efficient in reducing the fertility of these ectoparasites, with the advantage of being natural.

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REFERENCES

Agnolin CA, Olivo CJ, Leal MLR, Beck RCR, Meinerz GR, Parra CLC, Machado PR, Foletto V, Bem CM, Nicolodi PRS. 2010. Eficácia do óleo de citronela [*Cymbopogon nardus* (L.) Rendle] no

controle de ectoparasitas de bovinos. Rev Bras Pl Med Botucatu 12: 482–487.
Balashov, Y. 1983. The female reproductive system. In: Balashov YS, editor. An atlas of ixodid tick ultrastructure. Russia: Entomological Society of America. pp. 98–128.
Carvalho HF, Recco-Pimentel SM. 2012. A Célula, 3rd ed. São Paulo: Editora Manole.
Castro KNC, Lima DF, Vasconcelos LC, Leite JRSA, Santos RC, Paz Neto AA, Costa-Júnior LM. 2014. Acaricide activity in vitro of *Acmella oleracea* against *Rhipicephalus microplus*. Parasitol Res 113:3697–3701.
Cruz-Landim C, Höfling MA. 1979. Comportamento dos nucléolos e mitocôndrias durante a ovogênese de peixes teleósteos de água doce. Acta Amaz 9:723–728.
Denardi SE, Bechara GH, Oliveira PR, de Nunes ET, Saito KC Mathias MIC. 2004. Morphological characterization of the ovary and vitellogenesis dynamics in the tick *Amblyomma cajennense* (Acari: Ixodidae). Vet Parasitol 125:379–395.
Denardi SE, Bechara GH, Oliveira PR, de Camargo-Mathias MI. 2010. *Azadirachta indica* A. Juss (neem) induced morphological changes on oocytes of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) tick females. Exp Parasitol 126:462–470.
Friesen KJ, Kaufman WR. 2003. Cypermethrin inhibits egg development in the ixodid tick, *Amblyomma hebraeum*. Pestic Biochem Physiol 76:25–35.
Friesen KJ, Suri R, Kaufman WR. 2003. Effects of the avermectin, MK-243, on ovary development and salivary gland degeneration in the ixodid tick, *Amblyomma hebraeum*. Pestic Biochem Physiol 76: 82–90.
Furlong J, Prata MCA, Martins JR. 2007. O carrapato dos bovinos e a resistência: Temos o que comemorar? A Hora Veterinária 159:1–7.
Garcia MV, Matias J, Barros JC, de Lima DP, Lopes R, da S, Andreotti R. 2012. Chemical identification of *Tagetes minuta* Linnaeus (Asteraceae) essential oil and its acaricidal effect on ticks. Brazilian J Vet Parasitol 21:405–411.
Ghosh S, Azhahianambi P, de la Fuente J. 2006. Control of ticks of ruminants, with special emphasis on livestock farming systems in India: Present and future possibilities for integrated control—A review. Exp Appl Acarol 40:49–66.
Graf JF, Gogolewski R, Leach-Bing N, Sabatini GA, Molento MB, Bordin EL, Arantes GJ. 2004. Tick control: An industry point of view. Parasitology 129 Suppl:S427–S442.
Harrison W, Foelix R. 1999. Chelicerata: Arthropoda. In: Harrison W, Foelix R, editors. Microscopic anatomy of invertebrates. New York: Wiley-Liss. pp. 202–252.
Junqueira LC, Carneiro J. 2013. Histologia Básica. Rio de Janeiro: Guanabara Koogan.
Martinez SS. 2002. O Nim—Azadirachta indica: Natureza, Usos Múltiplos. Londrina: IAPAR.
Mendes MC, Lima CKP, Nogueira AHC, Yoshihara E, Chiebao DP, Gabriel FHL, Ueno TEH, Namindome A, Klafke GM. 2011. Resistance to cypermethrin, deltamethrin and chlorpyriphos in populations of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) from small farms of the State of São Paulo, Brazil. Vet Parasitol 178:383–388.
Nolan J. 1985. Mechanisms of resistance to chemicals in arthropod parasites of veterinary importance. Vet Parasitol 18:155–166.
Oelrichs PB, Hill MW, Vallely PJ, MacLeod JK, Molinski TF. 1983. Toxic tetranortriterpenes of the fruit of *Melia azedarach*. Phytochemistry 22:531–534.
Oliveira PR, De Bechara GH, Morales MAM Mathias MIC. 2009. Action of the chemical agent fipronil on the reproductive process of semi-engorged females of the tick *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae). Ultrastructural evaluation of ovary cells. Food Chem Toxicol 47:1255–1264.
Oliveira PR, Bechara GH, Denardi SE, Nunes ET, Camargo-Mathias MI. 2005. Morphological characterization of the ovary and oocytes vitellogenesis of the tick *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae). Exp Parasitol 110:146–156.
Oliveira PR, Camargo-Mathias MI, Bechara GH. 2006. *Amblyomma triste* (Koch, 1844) (Acari: Ixodidae): Morphological description of the ovary and of vitellogenesis. Exp Parasitol 113:179–185.
Oliveira PR, Bechara GH, Camargo-Mathias MI. 2008. Evaluation of cytotoxic effects of fipronil on ovaries of semi-engorged *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) tick female. Food Chem Toxicol 46:2459–2465.
Oliver JH. 1989. Biology and systematics of ticks (Acari:Ixodida). Annu Rev Ecol Syst 20:397–430.
Pandey V, Agrawal V, Raghavendra K, Dash AP. 2007. Strong larvicidal activity of three species of *Spilanthes (Akarkara)* against malaria (*Anopheles stephensi Liston, Anopheles culicifacies*, species C) and filaria vector (*Culex quinquefasciatus Say*). Parasitol Res 102:171–174.
Prachayasittikul V, Prachayasittikul S, Ruchirawat S, Prachayasittikul V. 2013. High therapeutic potential of *Spilanthes acmella*: A review. Excli J 12:291–312.
Pruett JH. 1999. Immunological control of arthropod ectoparasites—A review. Int J Parasitol 29:25–32.
Ramsewak RS, Erickson AJ, Nair MG. 1999. Bioactive N-isobutylamides from the flower buds of *Spilanthes acmella*. Phytochemistry 51:729–732.
Remedio RN, Nunes PH, Anholeto LA, Oliveira PR, Camargo-Mathias MI. 2015. Morphological effects of neem (*Azadirachta indica* A. Juss) seed oil with known azadirachtin concentrations on the oocytes of semi-engorged *Rhipicephalus sanguineus* ticks. Parasitol Res 114:431–444.
Roel AR. 2001. Utilização de plantas com propriedades inseticidas: Uma contribuição para o desenvolvimento rural sustentável. Rev Int Desenvolv Local 1:43–50.
Roma GC, Furquim KCS, Bechara GH, Camargo-Mathias MI. 2010. Permethrin-induced morphological changes in oocytes of *Rhipicephalus sanguineus* (Acari: Ixodidae) semi-engorged females. Food Chem Toxicol 48:825–830.
Saito KC, Bechara GH, Nunes ET, Oliveira PR, Denardi SE, Camargo-Mathias MI. 2005. Morphological, histological, and ultrastructural studies of the ovary of the cattle-tick *Boophilus microplus* (Canestrini, 1887) (Acari: Ixodidae). Vet Parasitol 129:299–311.
Saraf VK, Dixit VK. 2002. *Spilanthes acmella Murr.*: Study on its extract spilanthol as larvicidal compound. Asian J Exp Sci 16:9–19.
Singh M, Chaturvedi R. 2012a. Evaluation of nutrient uptake and physical parameters on cell biomass growth and production of spilanthol in suspension cultures of *Spilanthes acmella* Murr. Bioprocess Biosyst Eng 35:943–951.
Singh M, Chaturvedi R. 2012b. Screening and quantification of an antiseptic alkylamide, spilanthol from in vitro cell and tissue cultures of *Spilanthes acmella* Murr. Ind Crops Prod 36:321–328.
Sonenshine, D.E. 1991. The female reproductive system In: Sonenshine DE, editor. Biology of ticks. New York: Oxford University Press. pp. 280–304.
Vendramini MCR, Camargo-Mathias MI, Faria AU, Bechara GH, Oliveira PR, Roma GC. 2012. Cytotoxic effects of andiroba oil (*Carapa guianensis*) in reproductive system of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) semi-engorged females. Parasitol Res 111:1885–1894.