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Cymbopogon citratus essential oil-induced ultrastructural & morphological changes in the midgut, cuticle & Haller's organ of the tick *Haemaphysalis longicornis* (Acari: Ixodidae)

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Abstract

Haemaphysalis longicornis Neumann is a widely distributed species known for its capacity to transmit pathogens of medical, public health, and veterinary importance. Due to the environmental contamination caused by synthetic acaricides, plant essential oils have emerged as a promising alternative to manage tick infestation. This study evaluated the ultrastructural and morphological changes induced by *Cymbopogon citratus* Stapf (lemongrass) essential oil on adult *H. longicornis* tick. The concentrations of lemongrass essential oil used for the treatment via the adult immersion test were from 10 to 40 mg/mL. The most significant alterations 24 h post-exposure include the proliferation of lysosomes, reduction in the number of the rough endoplasmic reticulum, disorganization of the microvilli, the disappearance of lipid droplets and hematin granules, and cytoplasmic vacuolization (midgut); a thinner endocuticle and epicuticle, the deletion of the subdivision's demarcation of the procuticle, disfiguration of the lamellar arrangements of the exocuticle, and cytoplasmic vacuolization of the epithelial cells (integument); cracks on the sensilla sockets, the detachment of one or two sensilla in the anterior pit, and partial disfiguration of part of the slit's edge (Haller's organ). These results reflect the acaricidal properties of the essential oil and could be an alternative means of tick control.

Keywords: Acaricidal effect, essential oil, *Haemaphysalis longicornis*, lemongrass, transmission and scanning electron microscopy

Introduction

Haemaphysalis longicornis Neumann, sometimes, referred to as Asian long-horned tick, scrub tick, and bush tick is considered a serious pest of livestock in the Australasian and Western Pacific Regions (Jiang *et al*. 2018; USDA 2020). Although they are mainly found on medium to large size domestic and wild animals, humans are an accidental host (Jiang *et al*. 2018). Among the known pathogens vectored by *H. longicornis* include the causative agents (*Theileria sergeni* and *T. buffeli*) of hemolytic anemia in wild and domestic cattle (Chen *et al*. 2014), the causative agent (*T. orientalis*) of bovine theileriosis (Heath 2016), and the causative agent (*Babesia* spp.) of babesiosis that affect domestic livestock and canine population (Ikadai *et al*. 2007; Jongejan *et al*. 2018). Recently, *H. longicornis* has been implicated as a potential vector of severe fever with thrombocytopenia syndrome virus (SFTSV) that has been considered an emerging hemorrhagic fever (Luo *et al*. 2015; Zhuang *et al*. 2018) and cases of infected ticks were reported in China, Japan, and the Republic of Korea (ROK) (Yun *et al*. 2014; Yu *et al*. 2015). Another medical condition associated with *H. longicornis* as a vector is spotted fever group rickettsiae (SFGR) which include *R. raoultii* and

Candidatus Rickettsia tarasevichiae identified in *H. longicornis* from Japan (Uchida *et al*. 1995; Tabara *et al*. 2011), China (Liu *et al*. 2016) and the ROK (Lee *et al*. 2003; Noh *et al*. 2017). *H. longicornis* distribution cuts across Japan, China, Korea, New Caledonia, Fiji, Vanuatu, Tonga, Western Samoa, Hawaii, Australia, and New Zealand (Hoogstraal *et al*. 1968; Steele 1977). In 2017, *H. longicornis* was first identified on a sheep at New Jersey in the United States of America (Rainey *et al*. 2018), but since then, until recently, it has been reported in 11 more states which include New York, North Carolina, Connecticut, Kentucky, Pennsylvania, Tennessee, Delaware, Virginia, West Virginia, Maryland, and Arkansas (Duncan *et al*. 2020; USDA 2020). Therefore, new ways of controlling the populations of ticks are needed.

The conventional means of tick control has been the use of synthetic acaricide but the environmental implication has been massive coupled with the development of resistance by ticks against these chemicals (De Meneghi *et al*. 2016; Higa *et al*. 2019). Therefore recent research endeavors have focused on eco-friendly alternatives such as plant essential oil. One of the plant essential oils with acaricidal properties is that of *Cymbopogon citratus* Stapf (lemongrass). It as an aromatic grass whose acaricidal properties have been attributed to the presence of monoterpenes such as citronellal, geraniol, limonene, etc. (Chagas *et al*. 2014; Rodriguez-Vivas *et al*. 2018).

Lemongrass essential oil has shown pesticidal properties against mosquitoes, flies, and the larvae and adults of *Rhipicephalus microplus* (Chauhan *et al*. 2016; Manh *et al*. 2020; Sheziyna *et al*. 2020). Additionally, our previous investigation demonstrated the acaricidal effects of lemongrass essential oil on the mortality and external morphology of *H. longicornis* and revealed lethal concentrations (LC_{50}) of 29.21, 28.18, 28.06 mg/mL for the adults, nymphs, and larvae, respectively (Agwunobi *et al*. 2020a), and also its effect on the expression profiles of genes of the detoxification enzyme glutathione s-transferases in *H. longicornis* (Agwunobi *et al*. 2020b). The present study evaluated the ultrastructural and morphological alterations induced by the essential oil of lemongrass on the midgut, integument, and the Haller's organ of the unfed adult *H. longicornis* tick with the purpose to aid the further understanding of the acaricidal potentials of lemongrass essential oil.

Materials and methods

Lemongrass (Cymbopogon citratus)

The essential oil of lemongrass used for this study was purchased from the online shop of the Oshadhi brand in Tabao (a Chinese website for online shopping) (Hangzhou, China; https://oshadhi.tmall.com/). The essential oil was diluted with 50% ethanol to 10, 20, 30, and 40 mg/mL for the unfed adult tick treatment.

Sample collection and tick rearing

The ticks used for this study were collected from the vegetation of Xiaowutai National Natural Reserve Area (114°47'–115°28'E, 39°50'– 40°6'N) of Hebei Province, China, using the flag dragging method. The ticks were brought back to the laboratory and were morphologically identified according to (Teng & Jiang 1991). The ticks were reared on domestic rabbits, *Oryctolagus cuniculus* at 20–25 °C with relative humidity (RH) of 50% under natural daylight cycles. All the stages of the unfed ticks were incubated at 80±5% RH and 16/8 h of L/D cycle at 27±2°C in the environmental chambers (Liu *et al*. 2005). Ethical guidelines of the institution were observed and all procedures were conducted according to the China Guide for the Care and Use of Laboratory Animals (Protocol Number: IACUC-157031).

Concentrations of lemongrass essential oil

The concentrations of lemongrass essential oil used for the treated groups via the AIT were based on the lethal concentration LC_{50} (29.21 mg/mL) for adult *H. longicornis* as previously determined during the preliminary test by our previous investigation (Agwunobi *et al*. 2020a). Numerous concentrations were evaluated by dilution of the essential oil with an aqueous solution containing 50% ethanol. For the present study, the concentrations of the treated groups used were 10, 20, 30, and 40 mg/mL, and the control group of 50% ethanol. The bioassay of the adult immersion test was employed. The groups of ticks were immersed in 5 mL of the respective concentrations for 5 min by directly placing them into Eppendorf tubes. The essential oil solution was decanted and the ticks were transferred to the tissue paper towel for drying and evaporation of the solution. The ticks were then kept separately in tubes and sealed with a muslin cloth. Three replicates of each group of 20 individuals were performed for the concentrations. The treated ticks were kept in an environmental chamber with an optimum temperature of 27±2 °C and 80±5% RH. The ticks were brought out 24 h later for dissection and preparation for transmission and scanning electron microscopy.

Transmission electron microscopy (*TEM*)

About 10 ticks from each group (10-30 mg/mL) were dissected in 0.1 mol/L PBS (phosphate-buffered saline) at pH 7.2 and the midguts and integuments were collected. The samples were fixed in 2.5% glutaraldehyde (GLA) for 2 h, rinsed with 0.1M phosphate buffer for 15 min (3 times), and then in 1% osmium tetroxide fixing solution for 2 h, before rinsing again with 0.1M PBS for 15 min for 3 times. The samples were passed twice each through series of 30%, 50%, 70%, 80%, 90%, and 100% acetone for 15 min, and infiltrated twice with a mixture of Epon-812 and acetone (1:1 for 1 h and 3:1 for 3 h). After they have dried, the materials were embedded in pure Epon-812 for at least 5 h. Ultrathin sections of 60 nm were collected on copper grids, stained with 3% uranyl acetate and lead citrate, and observed in H-7650 transmission electron microscope at 80KV (Hitachi, Tokyo, Japan).

Scanning electron microscopy (*SEM*)

Unfed adult ticks of treated and control groups were preserved in GLA (GLA 2.5% in 0.1 mol/L PBS, pH 7.4) for at least 2 h, and were rinsed 3 times in 0.1 mol/L PBS at pH 7.2 for 15 min. The samples were fixed in 1% osmium tetroxide for 90 min and then were rewashed 3 times in PBS for 15 min. They were passed through a graded series of acetone as aforementioned for dehydration before being transferred to isoamyl acetate twice for 20 min. The samples were dried using the critical point drying method (HCP-2 Critical Point Dryer; Hitachi, Tokyo, Japan). Thereafter, they were mounted and sputter-coated with platinum for at least 60 s using an E-1020 ion sputter coater (Hitachi). The samples were examined and their images were captured with an S-4800 scanning electron microscope. All three mentioned machines were purchased from Hitachi, Tokyo, Japan. This study was approved by the Animal Ethics Committee of Hebei Normal University as complying with the Animal protection law of the People's Republic of China (Protocol Number: IACUC-157031).

Results

Midgut

Group I (*Control*)

The TEM was employed to unravel the ultrastructure of the midgut of unfed adult *H. longicornis*. The unfed midgut structure is distended in nature and thus can have rounded or flat nuclei, with a predominance of decondensed chromatin and nucleoli (Fig. 1A–I). The basal lamina

could be thick or thin and sometimes folded due to the distensions of the intestine (Fig. 1C–D, F). The microvilli and basal lamina line up the walls of the lumen (Fig. 1C–D). The digestive cells are composed of drops of lipid material (Fig. 1A–B) and some moderately electrodense vesicles such as protein granules (Fig. 1E–H). The cytoplasm has moderate electron density with few organelles which include mitochondria of various shapes ranging from round to elliptical and rough endoplasmic reticulum (Fig. 1A–I). Sometimes, there is the presence of sperocrystals, endosomes, and electrodense hematin residues (Fig. 1E, G–H). Generally, most cytoplasmic structures and organelles of the control group were undisturbed and intact.

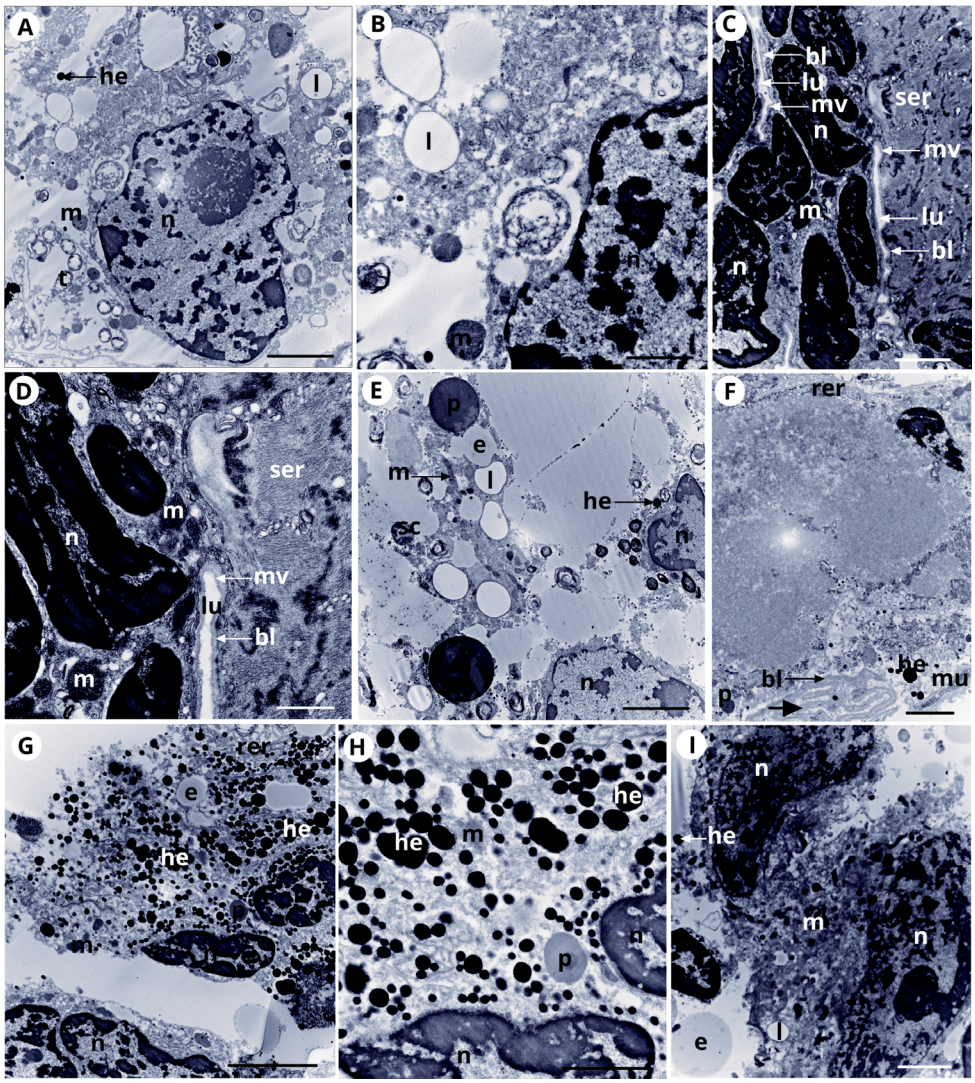

FIGURE 1. Transmission electron microscopy image of the midgut of the unfed adult *Haemaphysalis longicornis* (Control). (**A)** Digestive cell showing undisturbed organelles (**B)** Higher magnification of undisturbed cytoplasm of the digestive cell. (**C)** View from the basal region (**D**) Higher magnification of the basal region showing lumen (lu), microvilli (mv), and basal lamina (bl). (**E**) Digestive cells with the presence of protein granules (p), endosomes (e), and spherocrystals (sc). (**F**) The peripheral area of the generative cells showing hematin residue (he), folded basal lamina (arrow), and muscle layer (mu). (**G–I**) General view of digestive cells with organelles intact and undisturbed with the presence of hematin residue (he). n: nucleus; l: lipid; ser: smooth endoplasmic reticulum; m: mitochondria; t: trachea. Scale bars: 2 µm (**A, C, E, F, G,** and **I**), 500 nm (**B, D,** and **H**).

Group II

The midgut of the unfed adult tick exposed to 20 mg/mL essential oil of lemongrass revealed some ultrastructural alterations when compared to the control group samples. The digestive cells present circular and oval electron-dense protein granules of various sizes within the cytoplasm (Fig. 2A–B, E–H). Disorganized microvilli and electron-dense hematin residues were observed in some digestive cells (Fig. 2G–H). Vacuolated areas appear within the basal regions of some digestive and muscle cells (Fig. 2C–E). Endosomes were observed in some of the cells while small-sized vacuoles appeared in all the cells (Fig. 2A–H). However, not many alterations were observed within the nucleus as most heterochromatin adhered to the internal region of the nuclear membrane.

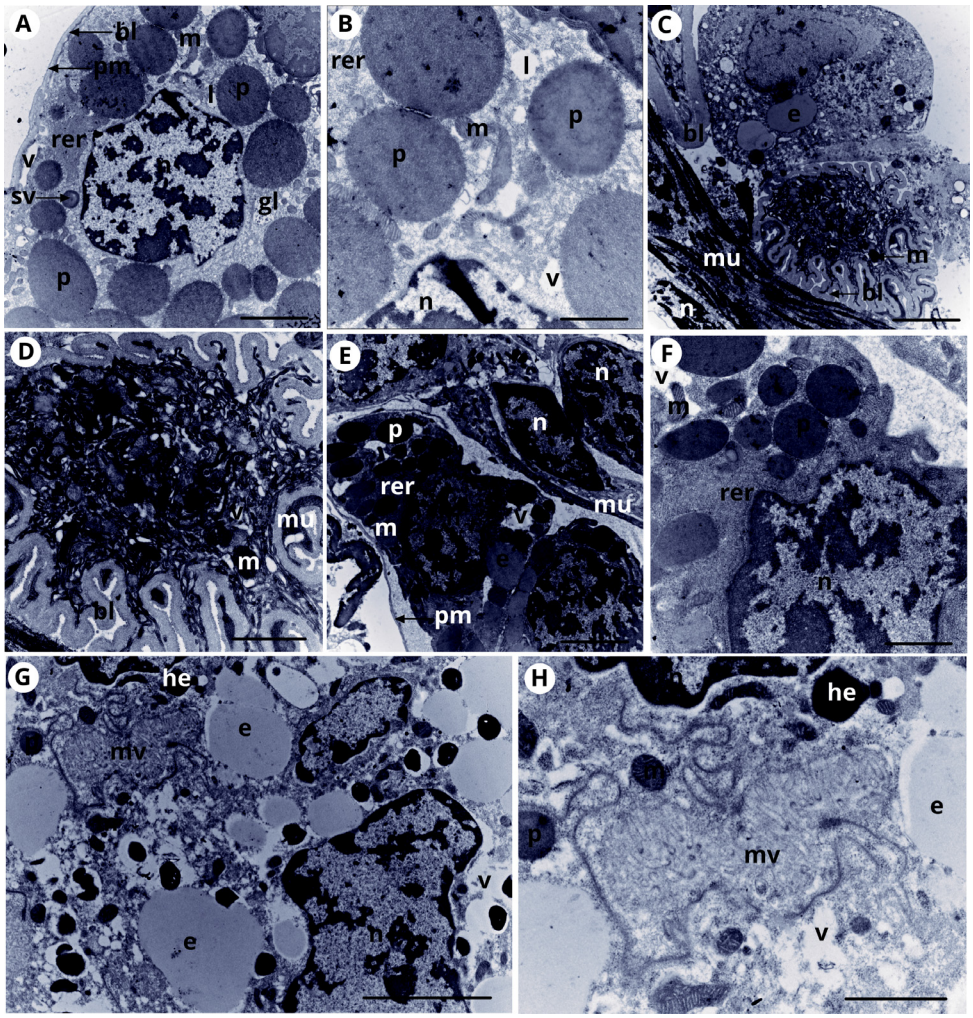

FIGURE 2. Transmission electron microscopy image of the midgut of 20 mg/mL essential oil-treated unfed adult *Haemaphysalis longicornis*. (**A)** Digestive cell with an increased number of protein granules (p) (**B)** Higher magnification of the cytoplasm of the digestive cell. (**C**) View from the basal region showing folded basal lamina (bl) and muscle layer (mu). (**D**) Higher magnification of the folded basal lamina. (**E**) General view from muscle cells and digestive cells separated by the plasma membrane (pm). (**F**) Higher magnification of partially dispersed nucleus (n) and cytoplasm of the digestive cell with the increased number of protein granules. (**G)** Digestive cell showing endosomes (e) and disorganized microvilli (mv). (**H**) Higher magnification of the cytoplasm of the digestive cell showing disorganized microvilli. m: mitochondria; e: endosomes; he: hematin residue; rer: rough endoplasmic reticulum; v: vacuole; mu: muscle layer. Scale bars: 2 µm (**A, C, E,** and **G**), 500 nm (**B, D, F,** and **H**).

Group III

The midgut of unfed *H. longicornis* treated with 30 mg/mL lemongrass essential oil showed remarkable ultrastructural changes when compared to the two previous groups. The midgut cells present increased vacuolization (Fig. 3A–I). Lysosomes break down protein granules in some digestive cells (Fig. 3E–F). Most of the cells have expanded vacuolated areas (Fig. 3A–E, I). Rough endoplasmic reticulum reduced sharply and many ribosomes were shredded off from their respective rough endoplasmic reticulum (Fig. 3G–H). Most of the cytoplasm appeared to be less electron-dense and eroded. Lipid droplets and hematin residues were scarce (Fig. 3A–I).

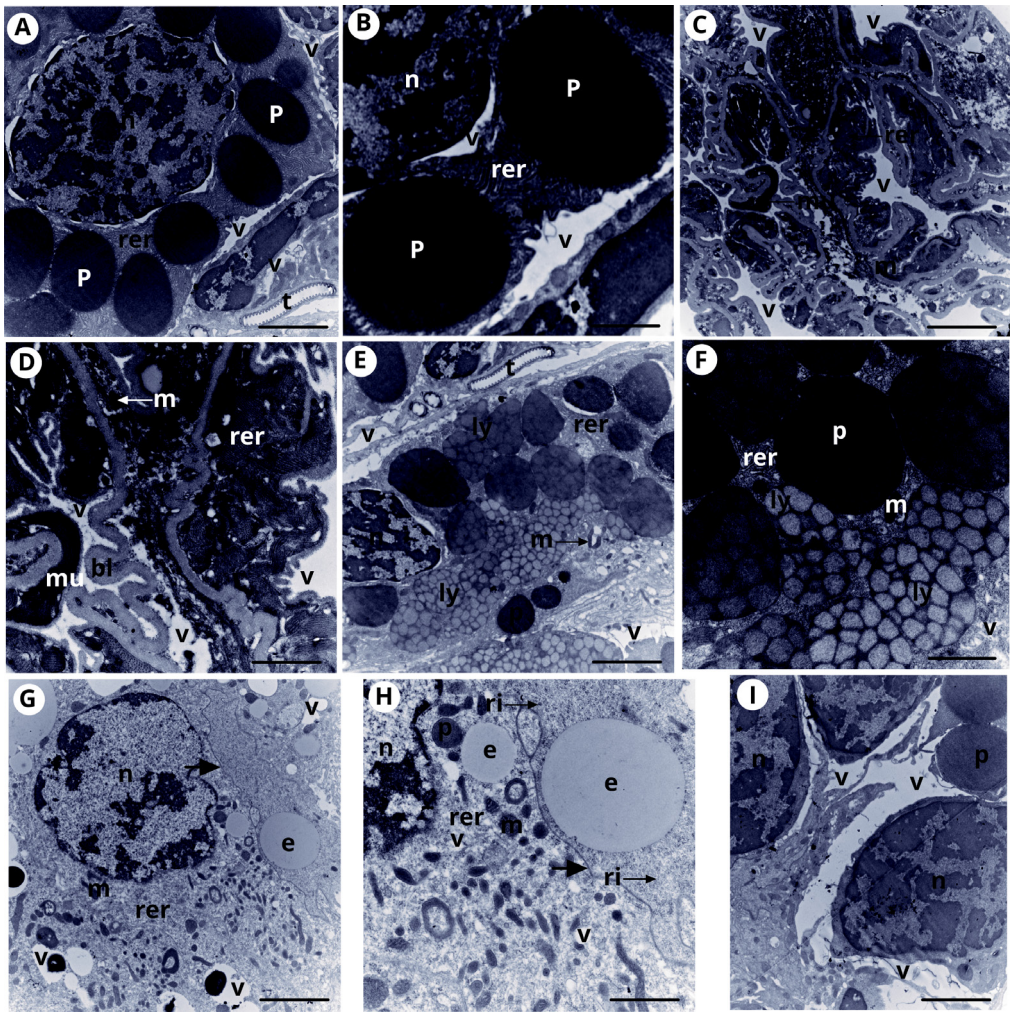

FIGURE 3. Transmission electron microscopy of the midgut of 30 mg/mL essential oil-treated unfed adult *Haemaphysalis longicornis*. (**A**) Digestive cells with an increased number of protein granules (p) and vacuole (v). (**B**) Higher magnification of the cytoplasm of the digestive cell with expanding vacuoles. (**C**) View from the basal region showing folded basal lamina and muscle layers (mu) interwoven with more vacuoles. (**D**) Higher magnification of the folded basal lamina (bl) interwoven with more vacuoles and patches of the rough endoplasmic reticulum (rer), mitochondria (m), and muscle layer (mu). (**E**) Digestive cell with lysosomes (ly). (**F**) Higher magnification of the cytoplasm with lysosomes. (**G**) Generative cell with scanty heterochromatin in the nucleus (n), and the development of more vacuoles. (**H**) Higher magnification of the cytoplasm of the generative cell showing ribosomes (ri) separated from the rough endoplasmic reticulum. The ribosomes separated from the rough endoplasmic reticulum. (**I**) Digestive cells with expanding vacuoles. Thick arrow: interdigitation; e: endosomes; m: mitochondria. Scale bars: 2 µm (**A, C, E, G,** and **I**), 500 nm (**B, D, F,** and **H**).

Integument

Group I (*Control*)

The ultrastructural results obtained showed that the integument of the unfed *H. longicornis* adult is composed of a cuticle and an epithelial cell layer below it (Fig. 4A–E). The cuticle has two main subdivisions: the outer epicuticle (thinner region), and the inner procuticle (thicker region). The procuticle is further subdivided into two distinct areas: the exocuticle, which is close to the epicuticle, and the endocuticle, which is adjacent to the epithelial layer (Fig. 4A–E). The appearance of the exocuticle and endocuticle depict some lamellar arrangements. The epicuticle and the cuticular folds found on the alloscutum are neatly intact with an organized arrangement. (Fig. 4D–E).

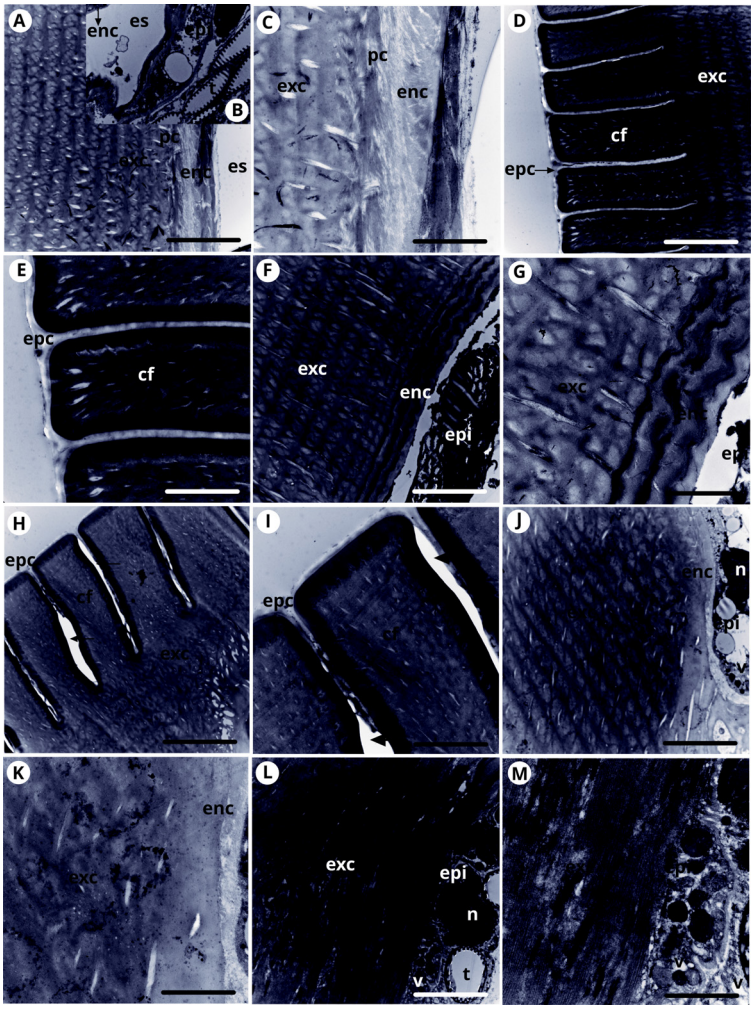

FIGURE 4. Transmission electron microscopy of the integument of the unfed adult *Haemaphysalis longicornis*. (**A**–**E**) Control group showing the normal separation of three distinct layers of the tick's integument and the epithelial layer. (**C**) Higher magnification of the control group showing the procuticle (pc) subdivided into exocuticle (exc) and endocuticle (enc). (**D**–**E**) The integument showing alloscutum with intact epicuticle (epc) and cuticular folds (cf). (**F**–**I**) 10 mg/mL treatment group. (**G**) Higher magnification of the integument showing the exocuticle and moderately electron-dense endocuticle. (**H**–**I**) The integument showing alloscutum with structurally altered cuticular folds (arrows) and epicuticle. (**J**–**K**) 20 mg/mL treatment group. (**K**) Higher magnification of the integument showing eroded exocuticle and endocuticle. (**L-M**) 30 mg/mL treatment group. (**M**) Higher magnification of the integument showing only eroded exocuticle with the absence of endocuticle. epi: epithelium; es: exuvial space. Scale bars: 2 µm (**A, B, D, F, H, J,** and **L**), 500 nm (**C, E, G, I, K,** and **M**).

Group II

Below the endocuticle is the epithelial layer which is made up of a simple layer of cells supported by a basal membrane. The epithelial cells present flat and electron-dense nuclei with little or no heterochromatin (Fig. 4B). There are some regions where the separation between the cuticle and epithelial cells is quite evident to the extent that space (exuvial space) develops between these layers (Fig. 4B).

Some ultrastructural alterations were observed on the integuments of the unfed ticks treated with 10 mg/mL essential oil of lemongrass in comparison with the control group. A smaller, thinner, and moderately electron-dense endocuticle was observed as compared to the ones found in the control group (Fig. 4F–G). There were some minor changes in the lamellar arrangements found in the exocuticle and endocuticle (Fig. 4A and F). The alloscutum presents a thinner epicuticle, and less organized and structurally altered cuticular folds (Fig. 4H–I). No significant changes were observed in the epithelial layer.

Group III

The integuments of the unfed adult of *H. longicornis* treated with 20 mg/mL present more remarkable ultrastructural alterations than the previous groups. The demarcations between the subdivisions were more disorganized and difficult to distinguish (Fig. 4J–K).

The exocuticle and endocuticle are eroded and less electron-dense. The boundary that demarcates the exocuticle and endocuticle is no longer distinct. The lamellar arrangements found within the exocuticle and endocuticle were eroded and disorganized (Fig. 4J–K). The epithelial cells below the endocuticle show signs of cytoplasmic vacuolization (Fig. 4J, L–M).

Group IV

The treatment of the integument of unfed adult tick *H. longicornis* with 30 mg/mL lemongrass essential oil presents more significant ultrastructural alterations than all the previous groups including the control group. The deletion of the subdivision's demarcation of the procuticle was observed (Fig. 4L–M).

The disappearance of the endocuticle was observed which denotes the deletion of the subdivision's demarcation of the procuticle. Only an eroded exocuticle was present. The lamellar structures of the exocuticle were disfigured and thus cannot be recognized (Fig. 4L–M). Cytoplasmic vacuolization was observed in the epithelial cells adjacent to the cuticle.

The Haller's organ

Group I

The scanning electron microscopy was employed to examine the external morphology of the Haller's organ of the unfed adult *H. longicornis*. The Haller's organ of *H. longicornis* is located dorsally on the surface of the first pair tarsus. Two distinct regions can be observed, the anterior pit and the proximal capsule (Fig. 5A). The anterior pit possesses seven sensilla in adults. Out of the seven sensilla, one is distinctively longer and thicker than the others and is known as the olfactory sensillum; about five are of medium size while the remaining one is comparatively very short than others. (Fig. 5A). A couple of long sensilla are found around the outside of the proximal capsule. The Haller's organ of *H. longicornis* has a single thick slit (Fig. 5B).

Group II and III

Group II and III *H. longicornis* ticks were treated with 2 mg/mL and 3 mg/mL respectively. The scanning electron microscopy of the Haller's organ revealed some similar minor alterations in both groups when compared to the control group. Unlike the control group, there were cracks on the sockets of the sensilla at the base (Fig. 5C and E). Also, the surface of the Haller's organ of both

groups seems disturbed and rough, and one or two sensilla in the anterior pit were detached. However, no form of abnormality was observed on their respective slits (Fig. 5D and F).

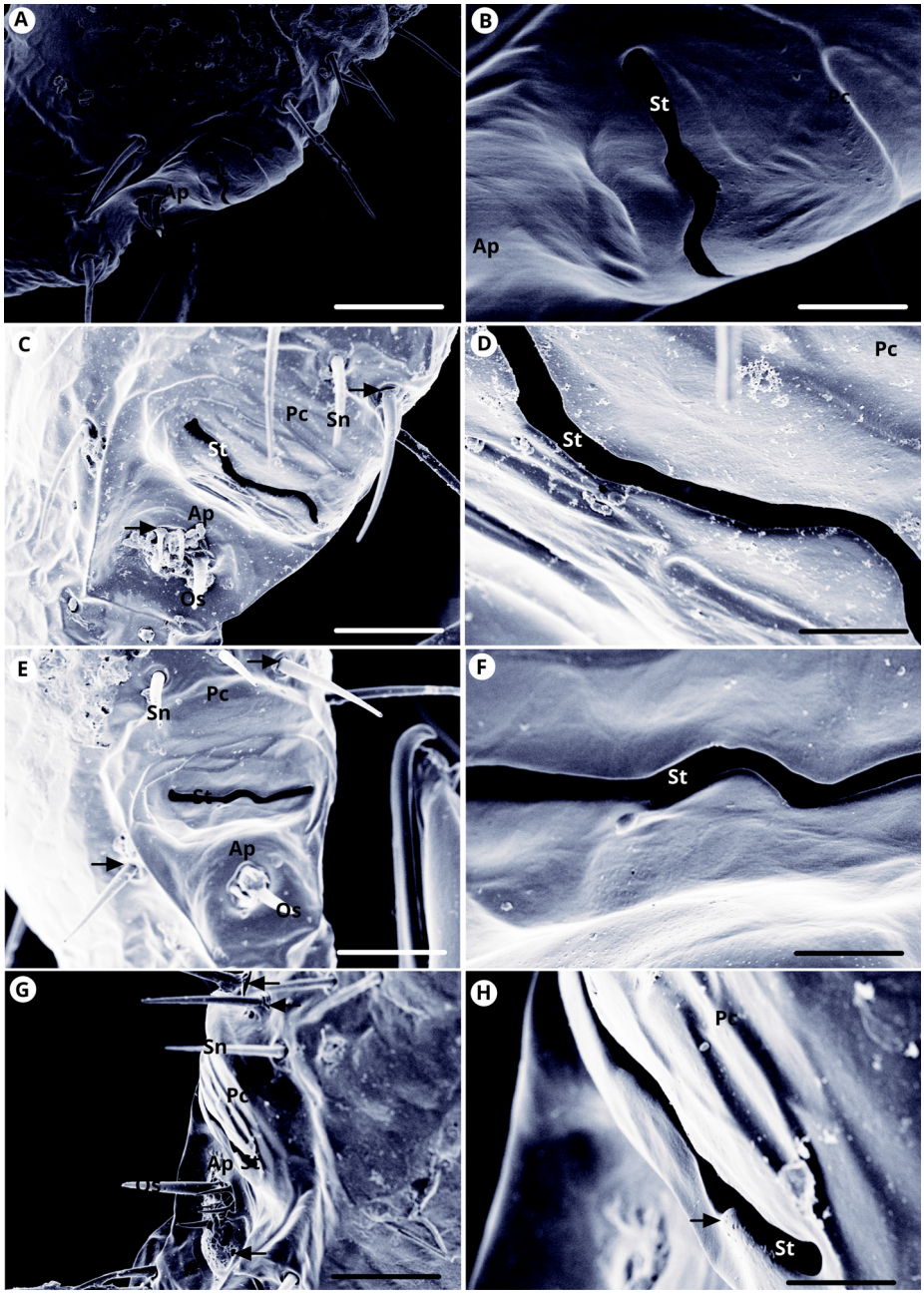

FIGURE 5. The scanning electron microscopy image of the Haller's organ of *Haemaphysalis longicornis*. (**A**–**B**) Control group showing undisturbed Haller's organ with smooth sensilla sockets. (**B**) Higher magnification of the slit (St) and proximal capsule (Pc). (**C**–**D**) 20 mg/mL treatment group showing partially disturbed Haller's organ with rough sensilla sockets (thick arrow). (**D**) Higher magnification of the slit and proximal capsule. (**E**–**F**) 30 mg/mL treatment group showing partially disturbed Haller's organ with rough sensilla sockets (thick arrow). (**F**) Higher magnification of the slit and proximal capsule. (**G**–**H**) 40 mg/mL treatment group with disturbed Haller's organ with rough and cracked sensilla sockets (thick arrow). (**H**) Higher magnification of the slit and proximal capsule with abnormal slit's edge (arrow). Ap: anterior pit; Pc: proximal capsule; Sn: sensilla; Os: olfactory sensillum. Scale bars: 50 µm (**A, C, E, and G**), 10 µm (**B, D, F, and H**).

Group IV

The scanning electron microscopy of the Haller's organ of *H. longicornis* treated with 40 mg/mL essential oil of lemongrass revealed some remarkable changes when compared to the control group. Like those of groups II and III, there were cracks at the sensilla sockets which was even more significant than the previous groups (Fig. 5G). Also, the surface of the Haller's organ appeared more disturbed and rough, and one or two sensilla in the anterior pit were detached. The base of the olfactory sensilla and other sensilla within the anterior pit presents cracks and swellings (Fig. 5G). Unlike the previous groups, part of the slit's edge was partially disfigured (Fig. 5H). The ultrastructural and morphological alterations that occurred at different concentrations of essential oil treatment were summarized in Table 1.

TABLE 1. Summary of the main ultrastructural and morphological alterations that occurred in the midgut, integument and Haller's organ of *Haemaphysalis longicornis* after treatment with lemongrass essential oil at different concentrations.

Organ	Microscopy	Concentration (mg/mL)	Ultrastructural/Morphological alterations
Midgut	Transmission electron microscopy	20	Circular and oval electron-dense protein granules of various sizes appear within the cytoplasm; disorganized microvilli and electron-dense hematin residues; vacuolated areas within the basal regions; endosomes were observed.
		30	Lysosomes break down protein granules; expansion of vacuolated areas; reduction of the rough endoplasmic reticulum; shredding off of ribosomes from the rough endoplasmic reticulum; less electron-dense and eroded cytoplasm; scarcity of lipid droplets and hematin residues.
Integument	Transmission electron microscopy	10	A smaller, thinner, and moderately electron-dense endocuticle; minor changes in the lamellar arrangements of procuticle; the alloscutum presents a thinner epicuticle, and less organized and structurally altered cuticular folds.
		20	Demarcations between subdivisions were difficult to distinguish; exocuticle and endocuticle were eroded and less electron-dense; erosion and disorganization of the lamellar arrangements found in the procuticle; signs of cytoplasmic vacuolization in the epithelial cells below the endocuticle.
		30	The deletion of the subdivision's demarcation of the procuticle; the disappearance of the endocuticle; the lamellar structures of the exocuticle were disfigured and unrecognizable; Cytoplasmic vacuolization was observed in the epithelial cells adjacent to the cuticle.
Haller's Organ	Scanning electron microscopy	20 and 30	Cracks on the sockets of the sensilla at the base; the surface of the Haller's organ was disturbed and rough; the detachment of one or two sensilla in the anterior pit.
		40	More significant cracks at the sensilla sockets; the surface of the Haller's organ was more disturbed and rough; cracks and swellings at the base of the olfactory sensilla and other sensilla within the anterior pit; the detachment of one or two sensilla in the anterior pit; partial disfiguration of part of the slit's edge.

Discussion

For several decades, the use of chemical acaricides for the control of ticks has been the norm. However, recent studies have demonstrated the acaricidal effects of some phytochemicals on pests with the advantage of little or no environmental contamination (Ghosh & Nagar 2014). The use of plant essential oils for tick control is an interesting prospect that has been verified in several studies (Nong *et al*. 2013; Godara *et al*. 2014; Muyobela *et al*. 2016), but more research is still required to fully understand the ramifications of their acaricidal effects. In the present study, the effects of the essential oil of lemongrass on the midgut, integument, and the Haller's organ of unfed adult *H. longicornis* ticks were substantiated by some ultrastructural and morphological alterations after observation via TEM and SEM. The midgut was used because it is one of the main organs for toxicological studies and it has enzymes such as glutathione S-transferases (GSTs) that play role in the metabolic detoxification process (Claudianos *et al*. 2006; Catae *et al*. 2014). Secondly, the integument was investigated for morphological alteration because it plays a significant role in

penetration resistance to acaricides (Schnitzerling *et al*. 1983), and also the Haller's organ because it is the main organ for the detection of olfactory information which is only found in ticks (Foelix & Axtell 1971; Hess & Vlimant 1982).

To the best of our knowledge, no work has been carried out to examine the ultrastructural and morphological effects of lemongrass essential oil on the midgut, integument, and Haller's organ of the tick *H. longicornis*. In the present study, the presence of several protein granules in the digestive cells of the treated midgut, the resultant proliferation of lysosomes, and reduction in the number of the rough endoplasmic reticulum were corroborated by the study that treated the soldiers of red fire ant with periplocoside X (a plant derivative) where it was observed that the number and volume of lysosomes increased sharply with the corresponding reduction in the number of rough endoplasmic reticulum and mitochondria (Li & Zeng 2013). Lysosomes are the site of catabolism of organic molecules and a wide range of cellular material which includes proteins and other organelles are broken down there via the process of autophagy (Cuervo 2011). The organelles of most midgut cells are eventually destroyed by lysosomes and many vacuoles are formed. This is followed by the disruption of the lysosomes and the leakage of the enzymes into the cytoplasm which marks the commencement of cytolysis. At this stage, the midgut epithelium is incapacitated to perform its normal digestive and absorptive functions. It should be noted that more investigations should be carried out in future studies to fully unravel the details of the biochemical mechanisms of how lemongrass essential oil functions.

The disorganization of the microvilli was observed in the midgut of the adult ticks from the treated group. Note that the plasma membrane of the microvilli has receptors that enable it to play the absorptive role in the digestive cells and the absorbed essential oil of lemongrass must have inflicted some physiological damages on them (Harrison & Foelix 1999). The absence of receptors will stall the obtention of the stored blood in the midgut lumen which will, in turn, obstruct the posterior internalization and formation of endosomes. Similarly, other studies by Borges *et al*. (2008) and Barreto *et al*. (2007) observed the destruction of the microvilli in the epithelial cells of *Aedes aegypti* larvae after treatment with diflubenzuron and the *Sapindus saponaria Lin* raw ethanolic extract, respectively; while another study detected a complete retraction of the microvilli in the plasma membrane of the digestive cells after the exposure of *R. sanguineus* to fluazuron (de Oliveira *et al*. 2014). This disorganization could be caused by citral, one of the major components of *C. citratus* essential oil, which inflicted irreversible damage to the cell membrane and organelles of the hyphae of *Trichophyton mentagrophytes* (Park *et al.* 2009).

The presence of little or no lipid droplets and hematin residues in the treated group was corroborated by a similar observation made in the midgut of *R. sanguineus* treated with fluazuron (de Oliveira *et al*. 2014). A possible explanation for the decrease or absent of lipid droplets could be that the digestive processes which guarantee the release of nutrients to be converted to lipid have been massively reduced or stalled (Sawby *et al*. 1992). Also, the absence of hematin residues could suggest that the essential oil of lemongrass have interfered with the digestive enzymes responsible for the formation of the digestive vacuoles where the hemolysis of the ingested blood cells take place (Sonenshine 1991; Harrison & Foelix 1999) which results to a significant reduction in the number of digestive/hematin residues produced that usually accumulates in the cytoplasm of digestive cells. There was a progressive increase of cytoplasmic vacuolization in the midgut cells of the treated groups. This could be an indication that higher concentrations of lemongrass essential oil inflicts physiological damage on the cell organelles and thus induced more intense autophagic processes in order to eliminate more damaged elements from the midgut cells. Vacuolization could be attributed to the effect of α-terpineol, a monoterpenoid component of most essentials oils (including *C. citratus*), that caused high vacuolization for the exposed hyphae of *T. mentagrophytes* (Park *et al.* 2009). A similar occurrence was reported in the digestive cells of *R. sanguineus* exposed to

dinotefuran (de Oliveira *et al*. 2016), in *A. aegypti* larvae treated with ethanolic extract of *Sapindus saponaria* Lin (Sapindaceae) (Barreto *et al*. 2007), in *Ceraeochrysa claveri* larvae exposed to neem oil (Scudeler & dos Santos 2013), and in *A. aegypti* larvae treated with the natural compound obtained from *Magonia pubescens* (Sapindaceae) (Valotto *et al*. 2011).

In the present study, the ultrastructural alterations of the treated cuticle (thinner procuticle and epicuticle with less organized and structurally altered cuticular folds, and the deletion of the subdivision's demarcation of the procuticle with the disfiguration of its lamellar) occurred as the concentration increased, denoting a dose-dependent effect. Similarly, the nymphs of *R. sanguineus* treated with 20 mg/kg of fluazuron presented a less thick and less organized procuticle and epicuticle, and as the concentration increased to 80 mg/kg, all the cuticular subdivisions disappeared (de Oliveira *et al*. 2014), indicating that lemongrass essential oil can have similar effects as chemical acaricides. A possible explanation for these cuticular alterations could be that lemongrass essential oil inhibited the synthesis and deposition of chitin. Chitin plays a vital role in the polymerization of the new cuticle and its deficiency implies the stalling of the cuticular growth which adversely affects ecdysis and the ability of the integument to accommodate large volumes of blood during engorgement (Sonenshine 1991; Harrison & Foelix 1999), and also leads to disorganization and incorrect deposition of chitin (Gangishetti *et al*. 2009). Other studies that corroborate these alterations observed thinness and disappearance of procuticle subdivisions after exposure to lufenuron (Mommaerts *et al*. 2006; Sáenz-de-Cabezón *et al*. 2006). The cuticular alterations could be caused by the monoterpene content of essential oils which caused bubble lesions over the entire body of treated *Schistosoma mansoni* (Matos-Rocha *et al.* 2017). In the epithelial cells, the cytoplasmic vacuolization that was observed with an increase in the concentration of lemongrass essential oil could be attributed to the effect of α-terpineol—one of the monoterpene compounds in *C. citratus* essential oils, which also caused high vacuolization in the cells of *T. mentagrophytes* (Park *et al.* 2009). This suggests the commencement of the damage of cell organelles which will be packed and lysed in the vacuoles. A similar formation of vacuoles in the epithelial cells was observed after the treatment of *R. sanguineus* nymph with fluazuron (de Oliveira *et al*. 2014).

The scanning electron microscopy revealed some morphological alterations on the Haller's organ of the adult *H. longicornis* treated with different concentrations of lemongrass essential oil. The rough surfaces with cracks at some sensilla sockets within and around the Haller's organ and the detachment of one or two sensilla in the anterior pit of the treated groups were corroborated by the partial disjoint of the antennal sensilla of aphids from its socket after treatment with the extract of *Eupatorium adenophorum* (Dey *et al*. 2005). The cracks at the sensilla socket became more prominent at the concentration of 40 mg/mL with partial disfiguration of part of the slit's edge. Similarly, drastic sensilla disturbance and cuticular disruptions in the form of cracks were observed in the treated group of *H. longicornis* treated with lemongrass essential oil (Agwunobi *et al*. 2020a). Significant distortion in the sensilla with crack-like marks on the sensory placode and campaniform were observed across the antennae segments of honey bees treated with pesticides (Chakrabarti *et al*. 2015).

Additionally, some studies have attributed these induced ultrastructural and morphological alterations to the lipophilic monoterpene constituents of essential oils (Bakkali *et al.* 2008; Matos-Rocha *et al.* 2017). The passage of these lipophilic compounds through the integument, cell wall, and cell membranes may damage the structure of the cellular membrane which may rsult in cellular lysis (Bakkali *et al.* 2008). It is also suggested that the activities of some essential oil could be a function of the ion loss, membrane potential reduction, proton pump collapse, and ATP pool depletion (Matos-Rocha *et al.* 2017).

Conclusion

The ultrastructural and morphological alteration caused by the essential oil of *C. citratus* on the midgut, integument, and Haller's organ of the tick *H. longicornis* demonstrated the acaricidal properties of *C. citratus* essential oil. This is a prospect that could offer a sound eco-friendly alternative as part of an integrated control strategy against ticks especially *H. longicornis*. However, more investigations are required to further understand its mode of action.

Abbreviations. GC-MS: Gas chromatography-mass spectrometry; L/D: Light/Darkness; RH: Relative humidity; GLA: Glutaraldehyde.

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Conflict of Interests

The authors declare no conflicts of interest.

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