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Ultra-structural Changes of *Aedes aegypti* Larvae Submitted to Treatment with Formulated Product

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***Abstract*—** *Bioactive products have been used against Aedes aegypti larvae. This study aimed to evaluate the ultrastructural changes of Aedes aegypti larvae submitted to treatment with a product formulated containing oils of Azadirachta indica, Melaleuca alternifolia, Carapa guianensis, and fermented extract of Carica papaya. The larvicidal assay was performed with third-stage larvae of A. aegypti, in triplicate (n = 900) for 24h. The experimental groups were G1 (50%), G2 (25%), and G3 (12.5%). Positive controls with industrial larvicide (Bacillus thuringiensis Israeli variety) at 0.37ppm (CP1) and 0.06ppm (CP2) concentrations. Finally, negative controls were water (CN1) and dimethyl sulfoxide (CN2). Specimens were collected from all groups for ultrastructural scanning by the end of the experiment, fixed in 2.5% buffered glutaraldehyde, and post-fixed in 2% osmium tetroxide. Then, all samples were routinely processed by scanning electron microscopy. The larvae treated with 12.5% (G3) of the formulated product showed wrinkling, skin damage, head, and chest rupture with exposure of the underlying musculature. We conclude that the formulated product containing oils of Azadirachta indica, Melaleuca alternifolia, Carapa guianensis, and fermented extract of Carica papaya promoted ultrastructural changes and death of A. aegypti third-stage larvae, being effective to control diseases propagated by this vector*.

***Keywords*—** *Tegument; respiratory siphon; anal papillae; scanning electron microscopy*.

I. Introduction

Aedes aegypti found in the world favorable conditions for its rapid spread [1] becoming the most important vector of several viruses and heartworms to men and animals [2]. The development of *A. aegypti* in the larval phase depends on several morphophysiological changes in the exoskeleton during ecdysis [3]. The cuticle corresponds to a structure rich in scleroproteins that gives the appearance of slightly rigid and limits growth, which must be replaced for the insect's continued development [4].

Growth regulating insecticides (GRI), such as diflubenzuron and methoprene, interferes with the development and changes the exoskeleton, being used in the control of *Culex spp.*, *Aedes spp*., and *Anopheles spp*. [5]. The control of the larvae forms of this Diptera is necessary. It can be performed using natural larvicides, which is an excellent alternative to synthetic insecticides due to the variety of active compounds obtained from renewable sources, as well as the rapid degradation without accumulation of toxic residues in food or the environment [6,7].

Several authors have reported plant extracts' action against mosquito larvae [8,9,10]. However, ultrastructural studies on *A. aegypti* larvae's cuticle are quite scarce [11]. This type of study is important to understand the action of bioactive products to later be used in the development of commercial insecticides [12,13,11]. The goal of this work was to evaluate the ultrastructural changes of *A. aegypti* (Linnaeus, 1762) larvae (Diptera: Culicidae) submitted to treatment with a product formulated with oils of *Azadirachta indica*, *Melaleuca alternifolia*, *Carapa guianensis* and fermented extract of *Carica papaya*.

II. Material and Methods

A. Obtaining the Formulated Product

The oil formulated product and the fermented extract were obtained from Gued's Biotecnologia®. They had the following composition: 1% of the seed oil of *Azadirachta indica*, 0.3% of the fruit oil of *Melaleuca alternifolia*, 1% of the essential oil of *Carapa guianensis*, and 5% of the fruit's fermented bacterial extract of *Carica papaya*.

B. Larvicide Assay

The toxicological test followed the methodology described by Jang et al. [14], using third-stage larvae. The test was performed in triplicate with 300 larvae for each experimental group, totalizing 2100 specimens. The larvae were exposed to the solutions for 24 hours and monitored every hour. The experimental groups were treated with formulated oil products in concentrations of 50% (G1), 25% (G2), and 12.5% (G3). Industrial larvicide (*Bacillus thuringiensis Israeli* variety) with 0.37ppm (CP1) and 0.06ppm (CP2) CL90 concentrations were used as positive controls. Water (CN1) and dimethyl sulfoxide (CN2) were used as negative controls.

C. Ultrastructural Analysis

Sample processing was performed at Immunopathology Laboratory Keizo Asami of the Federal University of Pernambuco (LIKA), both in Recife, PE, Brazil. For the ultrastructural analysis (SEM) of the third-stage larvae's body structure, samples were collected from all experimental

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groups, alive and dead, and fixed overnight in a 2.5% glutaraldehyde fixing solution in 0.1M phosphate buffer, pH 7.2, and post-fixed in 2% osmium tetroxide (OsO4). Subsequently, the samples were dried through the critical point method, metalized with gold, and analyzed in a JEOL-5600LV scanning microscope [15].

III. Results and Discussion

The results of the scanning electron microscopy (SEM) analysis of third-stage larvae of *A. aegypti* from groups G1 and G2 treated with the product formulated from *Azadirachta indica*, *Melaleuca alternifolia*, and *Carapa guianensis* oils and *Carica papaya* fermented extract presented body morphology and tegumentary skin, except for the generalized dehydration aspect (Figure 1).

The ultrastructural assessment of the G3 group's larvae showed a rupture of the head and thorax with muscular exposure. Also, a wrinkled appearance and cuticle injuries are also present (Figure 2). According to Borges et al. [16,11], third-stage larvae of *A. aegypti* submitted to diflubenzuron present injuries similar to those found in groups G2 and G3, such as rupture of the cephalic capsule, mummification, and rough appearance due to overlapping cuticles. A study using *Copaifera reticulata* oil on *A. aegypti* larvae showed the same findings described caused by cuticle folds [17].

The injuries in groups CP1 and CP2 are shown in figure 3. In both groups, there were extensive injuries to the cuticle of the entire abdominal segment of the larva, causing exposure of the underlying musculature. In the anal region, there is an absence of anal papillae, with prolapse of Malpighi tubules. No change in the respiratory siphon was observed. Structurally, BTI's action has been studied only focusing on the digestive tract and the changes caused by its endotoxins in the crystal formation [18,19]. However, no study has shown the injuries caused by BTI on the cuticle, being it reported for the first time in the present study.

In figure 4, we can see the *A. aegypti* larvae of the negative control groups showing typical aspects of the tegument and external structures. In CN1 and CN2, larvae presented intact abdominal segments, the absence of tegumentary injury, preservation of anal papillae, and respiratory siphon.

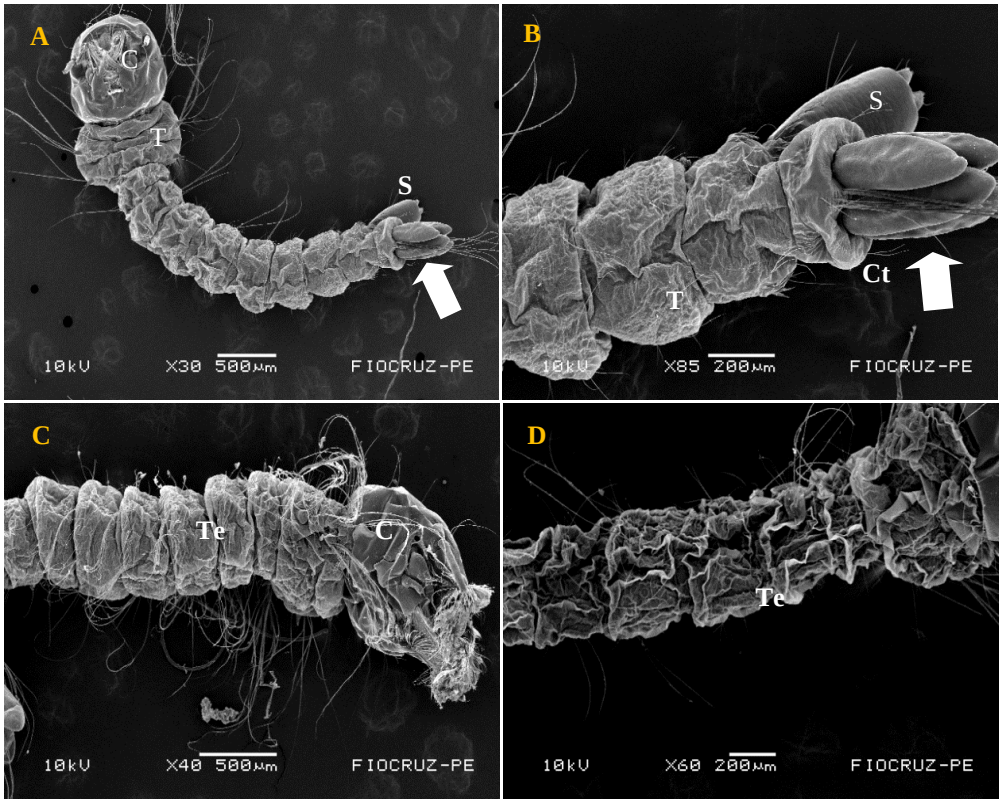

Fig. 1. Electromicrography of third-stage larvae of *Aedes aegypti* treated with a formulated product with 50% (G1) and 25% (G2) concentration of *Azadirachta indica*, *Melaleuca alternifolia*, and *Carapa guianensis* oils and *Carica papaya* fermented extract. (A): Larva morphology with a dehydrating aspect of the head (C) and chest (T), integument (Te) of the abdominal segments, and respiratory siphon (S). The anal papillae (broad arrow) remain intact.

Sandra Maria de Torres, Rafael José Ribeiro Padilha, Fábio André Brayner, Luiz Carlos Alves, Lêucio Câmara Alves, and Valdemiro Amaro da Silva Júnior, "Ultra-structural Changes of *Aedes aegypti* Larvae Submitted to Treatment with Formulated Product," *International Research Journal of Pharmacy and Medical Sciences (IRJPMS)*, Volume 3, Issue 6, pp. 11-14, 2020.

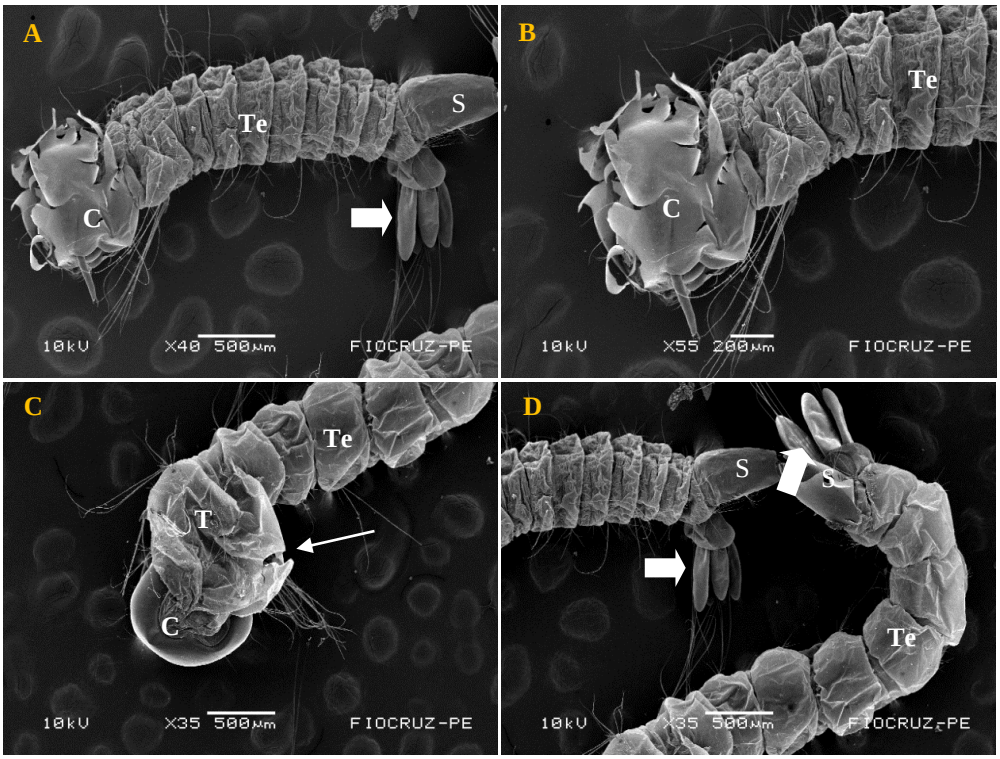

Fig. 2. Electromicrography of third-stage larvae of *Aedes aegypti* treated with a formulated product with 12.5% concentration of *Azadirachta indica, Melaleuca alternifolia,* and *Carapa guianensis* oils and *Carica papaya* fermented extract (G3). Larva with a ruptured head (C) and thorax (T), exposing the underlying musculature. Some focal injuries (thin arrow) are observed in the tegument's (Te) chest. The anal papillae (broad arrow) and the respiratory siphon (S) have their morphology preserved.

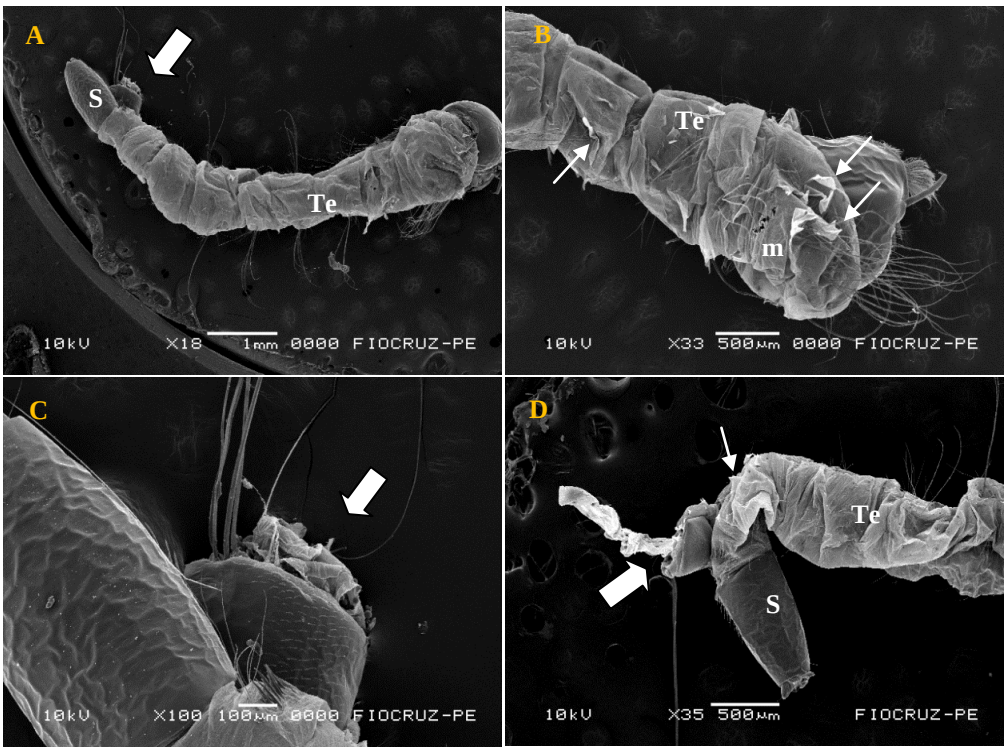

Fig. 3. Electromicrography of *Aedes aegypti* third-stage larvae treated with BTI CL90 0.37ppm (CP1) and CL50 0.06ppm (CP2). Multifocal injuries in the Tegument (Te), with flaking of the superficial layer (thin arrow) and exposure of the underlying musculature (m) in the chest. In the anal region, there was the loss of anal papillae (large arrow) with prolapse of the Malpighi Tubules and morphological integrity of the respiratory siphon (S).

Sandra Maria de Torres, Rafael José Ribeiro Padilha, Fábio André Brayner, Luiz Carlos Alves, Lêucio Câmara Alves, and Valdemiro Amaro da Silva Júnior, "Ultra-structural Changes of *Aedes aegypti* Larvae Submitted to Treatment with Formulated Product," *International Research Journal of Pharmacy and Medical Sciences (IRJPMS)*, Volume 3, Issue 6, pp. 11-14, 2020.

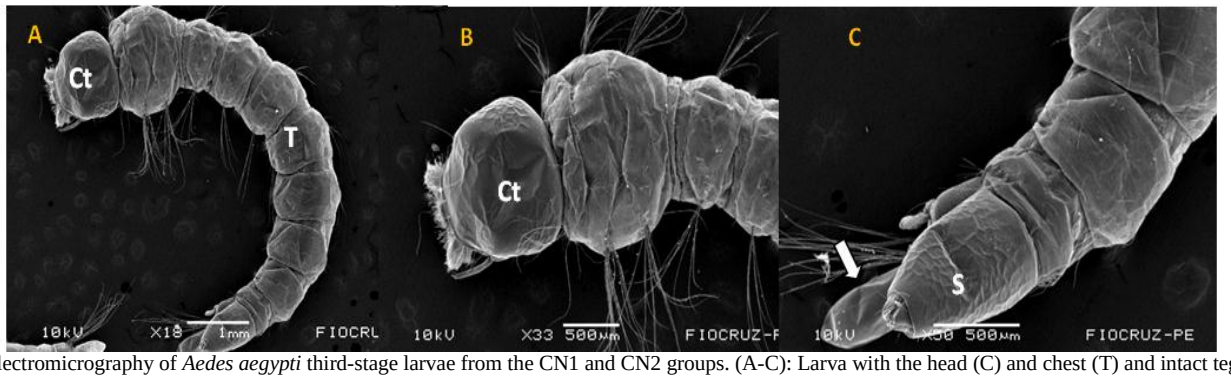

Fig. 4. Electromicrography of *Aedes aegypti* third-stage larvae from the CN1 and CN2 groups. (A-C): Larva with the head (C) and chest (T) and intact tegument (T) and abdominal segments, with preservation of the anal papillae (broad arrow) and respiratory siphon (S).

Barreto et al. [20], studied the extract of *Sapindus saponaria* on *A. aegypti* larvae. They found that the extract leads the larvae to wrinkle with the development of large cuticle folds, promoting a darkening by overlapping the cuticles of the abdominal segments.

Salvador [21] also visualized abdominal darkening and a decrease of up to 50% in the length of *A. aegypti* larvae treated with temephos. Larvae of the same mosquito exposed to the growth inhibitor diflubenzuron also decreased in size and morphological appearance due to the accumulation of incomplete seedlings [16]. The wrinkling aspect observed in our treated groups (G1, G2, and G3) is like the findings described by these studies.

IV. Conclusion

The association of *A. indica*, *M. alternifolia*, and *C. guianensis* oils and fermented extract of *C. papaya* caused ultrastructural changes in the cuticle and the death of *A. aegypti* (Liverpool lineage) third-stage larvae, indicating its larvicide potential. However, further studies are needed using field populations and different larval stages of *Aedes aegypti*.

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