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ORIGINAL ARTICLE

Larvicidal and histological effects of *Melia azedarach* extract on *Culex quinquefasciatus* Say larvae (Diptera: Culicidae)

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Abstract Extracts from *Melia azedarach* L. (Meliaceae) were effective against third instar larvae of *Culex quinquefasciatus* (Say) in Saudi Arabia, using crude extract obtained in ethanolic solution from King Saud University. Toxicity was varied according to the concentration and period of exposure. We investigated the effect of the LC_{50} on midgut and gastric caecae of 3rd larval instar of *Cx. quinquefasciatus*, the plant extract causing serious damage to the epithelial columnar cells. Light and electron microscopic observations revealed, by time 6, 12, 24 and 48 h, increasing damages of the larvae midgut epithelium. The most characteristic effects were midgut columnar cell vacuolization, microvilli damages, epithelium cell contents passing into the midgut lumen and finally the cell death. This article is the first report of the histopathological effects of the *M. azedarach* as a bioinsecticide in the midgut of *Cx. quinquefasciatus* larvae and the data obtained may contribute to a better understanding of the mode of action of this plant extract used as a bioinsecticide against *Cx. quinquefasciatus* larvae.

1. Introduction

The biological control of immature stages now appears to be the most powerful means of reducing target populations of Culicidae and other dipteran pests. As a result of continuous application of chemical pesticides, resistance was a squired by insect pests besides residue contamination of human foods, mammalian toxicity and pollution of the environment. Numerous secondary compounds from plants are being studied for use as new effective, eco-friendly biopesticides (Pathak and Dixit, 1988; Chockalingam et al., 1990; Govindachari et al., 1996; Jayaprakasha et al., 1997). Muthukrishnan and Puspalatha (2001) evaluated the larvicidal activity of extracts from *Calophyllum inophyllum* (Clusiaceae), *Rhinacanthus nasutus*

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(Acanthaceae), *Solanum suratense* (Solanaceae) and *Samadera indica* (Simaroubaceae), *Myriophyllum spicatum* (Haloragaceae) against *Anopheles stephensi*. Several indigenous plants viz., *Ocimum basilicum*, *Ocimum santum*, *Azadirachta indica*, *Lantana camera*, *Vitex negundo* and *Cleome viscosa* were studied for their larvicidal action on the field which collected fourth instar larva of *Cx. quinquefasciatus* (Kalyanasundaram and Dos, 1985). Studies have shown the potential of plants for use in *Cx. quinquefasciatus* control, such as *Piper nigrum* (Chahad and Boof 1994), *Azadirachta indica*, *Rhazya sticta* and *Syzygium aromaticum* (Mishra et al., 1995; Su and Mulla, 1998; Das et al., 1999; El-Hag et al., 1999), *Agave americana* and *Kaempferia galanga* (Dharmshaktu et al., 1987; Choochote et al., 1999, *Atriplex halimus* (El-Gougary, 1998; Massoud and Labib, 2000) and *Commiphora molmol* (Pitasawat et al., 1998). Persian lilac tree and also known as 'cinnamon' is related to Meliaceae trees. Effects of *M. azedarach* extracts on many insects have been already reported (Saxena et al., 1984; Schmidt et al., 1998; Juan et al., 2000; Carpinella et al., 2003). The Meliaceae plant family has been known as a potential source for insecticide properties. Extracts from the neem and other plants seeds and leaves have shown excellent insecticidal properties against fecundity and fertility of mosquito vector and were at the same time very eco-friendly (Schmutterer, 1990; Senthil Nathan et al., 2005). The efficacy of these neem products on mosquitoes was established (Zebitz, 1984; Schmutterer, 1990; Murugan et al., 1996). The histopathological changes in treated insects with alternative Insect control as a toxic action were previously investigated (Charles, 1987; Davidson and Titus,1987; Singh and Gil, 1988; Silva-Filha and Peixoto, 2003), and with botanical insecticides were also studied (Nasiruddin and Mordue, 1993).

The present study was conducted to evaluate the mosquito larvicidal activity of *Melia azedarach* ethanol extracts against *Cx. quinquefasciatus* larvae. Larval susceptibility to *M. azedarach* was also compared at the histopathological level to elucidate the effects of this potential bioinsecticide.

2. Materials and methods

2.1. Rearing technique

Laboratory sensitive strain of *Cx. quinquefasciatus* mosquitoes was obtained from susceptible reared strain in College of Food and Agricultural Science, King Saud University, the larvae used in the tests were reared in a plastic cup with water from the public water supply, under the standard techniques at 27 ± 2 °C and 70 ± 5% R.H. and 12 h photoperiod. Larvae were reared in dechlorinated water and fed daily on tetramine (tropical fish food). Adults were maintained on a 10% sugar solution and females were also fed on chicken. Third instar was used in the bioassay tests.

2.2. Preparation of stock solution

Ethanolic extract of whole plant of *M. azedarach* was obtained from Pharmacy College, King Saud University, as a dark green color crude extract. This crude extract was used to prepare stock solution. The known amount (10 mg/l) of filtered crude extract obtained from the above-mentioned source was serially diluted to obtain the desired concentration. The stock solution was serially diluted to prepare the test solutions of 0.50, 1.00, 1.25, 1.50, 1.75 and 2.00 mg/l. One drop of emulsifier (Tween 20, Sigma Chemical Company) was added with the extract to ensure complete solubility of the solutions in water.

2.3. Bioassays and larval mortality

Ten larvae were pipette into each 20 ml volume (in four replicates) and observed for a maximum of 48 h, when mortality was recorded. Larvae were considered dead or moribund if they stopped moving for a prolonged period even after gentle probing with a small spatula, as described in the World Health Organization's technical report series. A minimum of 40 larvae/concentration was used for all the experiments (WHO/CDS/WHOPES/GCDPP/2005.13). Larvae maintained in distilled water were used as a control. The LC_{50} and LC_{90} were calculated using probit analysis (Finney, 1971). Percentage mortality in the treatments was corrected when necessary for mortality in the controls using Abbot's (1925) formula.

2.4. Histological studies

For the Histological tests, treated and untreated newly ecdysed third instar larvae of *Cx. quinquefasciatus* were isolated from the standard laboratory colony reared on fed which were incorporated with the LC_{50} of *M. azedarach* after 24 h = (1.035 mg/l). Only live larvae were examined. Then they were fixed in bouins solution (after 6, 12, 24 and 48 h from exposures) for 24 h. After dehydration in a graded ethanol series, the material was embedded and cut with glass knives in a rotary microtome. The sections were stained with haematoxylin-eosin, analyzed, and photographed with a photomicroscope. After 24 and 48 h for electron microscopic studies, the midgut was fixed in a solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3), post fixed in 1% osmium tetroxide solution in the same buffer, dehydrated in a graded acetone solution, and embedded. Ultra-thin sections were stained with uranyl acetate and lead citrate before examination.

3. Results

Effect of *M. azedarach* extract on the 3rd instar larvae increased with an increase of concentration. The LC_{50} after 24 h was 1.035 mg/l. The LC_{50} after 48 h was 0.75 mg/l. Regression analysis showed a concentration-dependent significant correlation of the plant extract with larval mortality. Strong correlation between concentration and mortality (r) was 0.963 and 0.97 after 24 and 48 h, respectively (Table 1) (see Fig. 1).

The first aspect investigated in this work was the histological structure of midgut epithelial cells from untreated larvae. The midgut of dipteran larvae has been subdivided into two different regions, each including one characteristic cell type (Rey et al., 1999). The anterior midgut included flatter cells with clear cytoplasm (clear cells: Clements, 1992), extending along one-third of the midgut. Depending on their stage of development, clear cells displayed different degrees of apical swelling into the gut lumen, reducing intercellular contacts with the neighboring cells and degeneration of the nuclei and brush border, as shown, for example, in control *Cx. quinque-*

Table 1 Comparing of efficacy of *M. azedarach* extract on 3rd larval instar of *Cx. quinquefasciatus* at different period of exposure.

No. larvae treated	Exposure time (h)	LC50 (mg/l)	Lower limit	Upper limit	Index	RR	Slope ± SE	Chi-square	LC_{90}
40	*M. azedarach* after 48 h	0.754	0.646	0.861	100.0	1.00	3.236 ± 0.372	5.15	1.876
40	*M. azedarach* after 24 h	1.035	0.935	1.124	72.85	1.373	5.477 ± 00.60	6.01	1.773

Index compare with *M. azedarach* after 48 h.
Resistance Ratio (RR) compared with *M. azedarach* after 48 h.

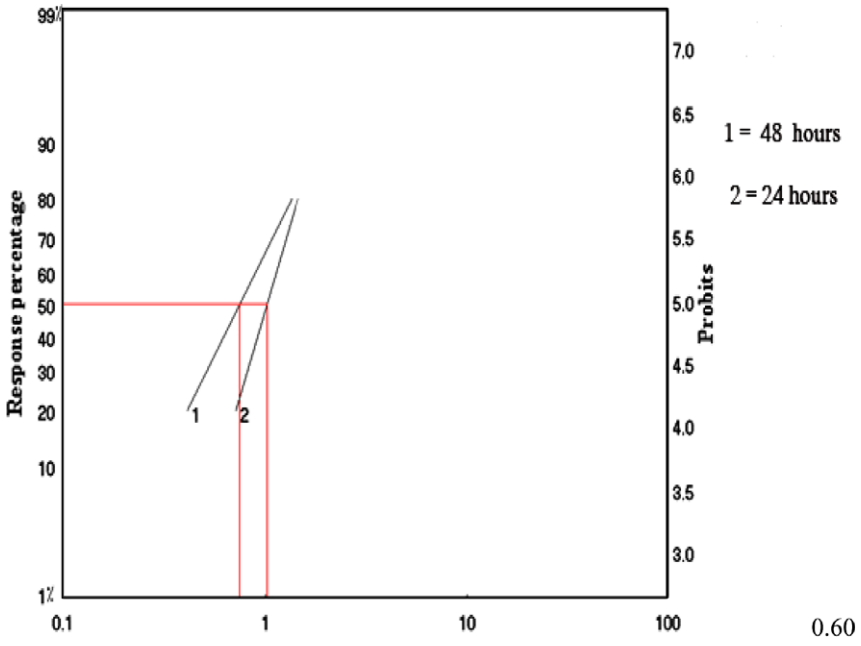


Figure 1 Efficacy of *M. azedarach*.extracts on 3rd instar larvae of *Cx. quinquefasciatus* mosquitoes at different periods of exposure.

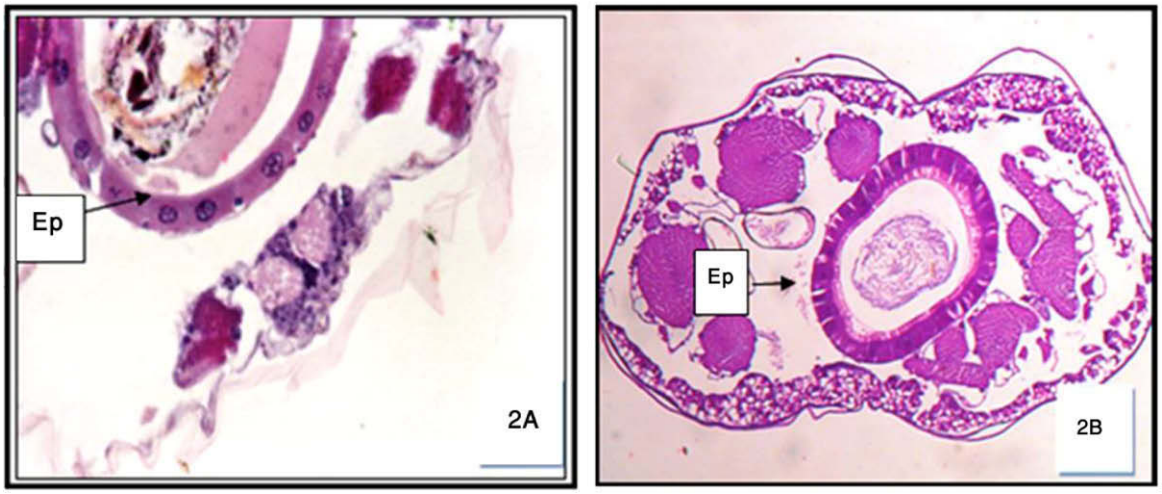


Figure 2 Cross-section in the anterior (A) and posterior (B) regions of normal untreated *Cx. quinquefasciatus* 3rd instar larva showing the foregut-epithelia cells (Ep.). 200×.

fasciatus (Fig. 2A). The posterior midgut was characterized by tall epithelial cells (dark cells: Clements, 1992). Dark cells show normal intercellular contacts along the whole lateral plasma membranes, normal nuclei, a well-developed brush border, and a normal adhesive basement membrane, as observed in control sections (Fig. 2B). When treated with *M. azedarach* extract, all larvae developed dramatic lesions, affecting mainly the midgut epithelium and secondarily the caeca.

Histopathological effects differed qualitatively according to their localization along the midgut and quantitatively according to the duration of the treatment.

3.1. After 6 h of treatment

Cells of the anterior midgut show hardly any morphological deviation from normal ones, through slightly apical degenerated (lysis) of clear cells and little disruption of the lateral junctional complexes. Compared to controls, acceleration of the lysis of clear cells was perceptible at the level of their brush border, basal membrane, nucleus, and cytoplasmic organelles, before their bursting into the gut lumen. At the same time, partial lysis of the posterior midgut began through local detachment among small groups of dark cells bearing a dilated basal membrane, destruction of the peritrophic membrane; the cells were elongated (Figs. 3 and 4A and B).

3.2. After 12 h of treatment

The lysis of the anterior midgut progressed through swollen clear cells, vacuoles, nuclear degenerated (Fig. 5). In the posterior midgut, disruption of the junctional complexes among dark cells progressed apically, together with their cytoplasmic and nuclear lysis, their local detachment from the basal lamina, and the degeneration of the microvilli (Fig. 6).

3.3. After 24 h of treatment

The anterior midgut was almost globular structure bulging from their free surface. The other kinds of cell appeared short and polygonal; few cells of the epithelium exhibited a brush border, some with normal nuclei, a well-developed brush border. As shown in ultrastructure the midgut of non-treated

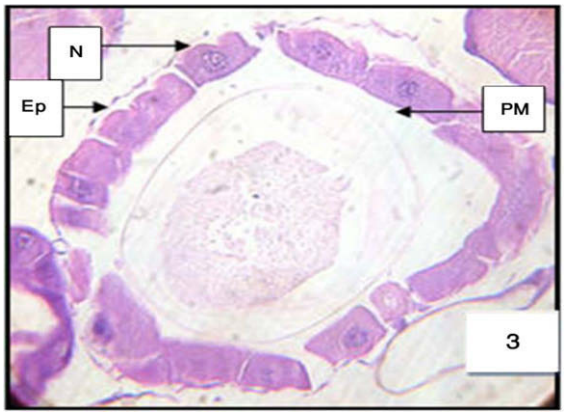


Figure 3 Anterior midgut of 3rd instar larvae of *Cx. quinquefasciatus* treated with LC_{50} *of M. azedarach* extract, showing the effect after 6 h of exposure. Epithelial cells (EP), peritrophic membrane (PM) and nucleus (N). 400×.

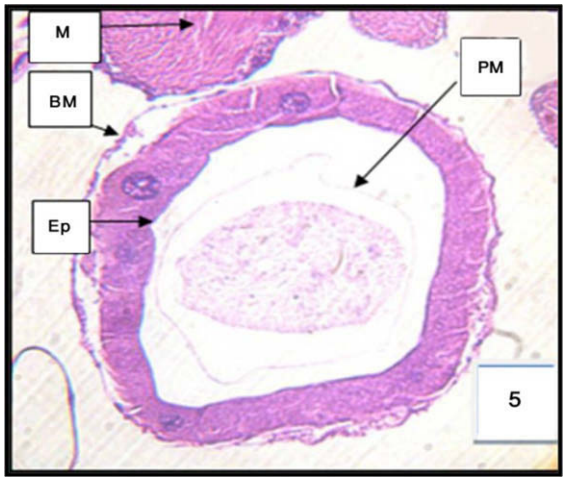


Figure 5 Cross section through the anterior midgut of 3rd instar larvae of *Cx. quinquefasciatus* treated with LC_{50} *of M. azedarach* extract, showing the effect after 12 h of exposure. 400×. 1-Epithelial cells of anterior region (EP.), muscles (M), basement membrane (BM), and peritrophic membrane (PM).

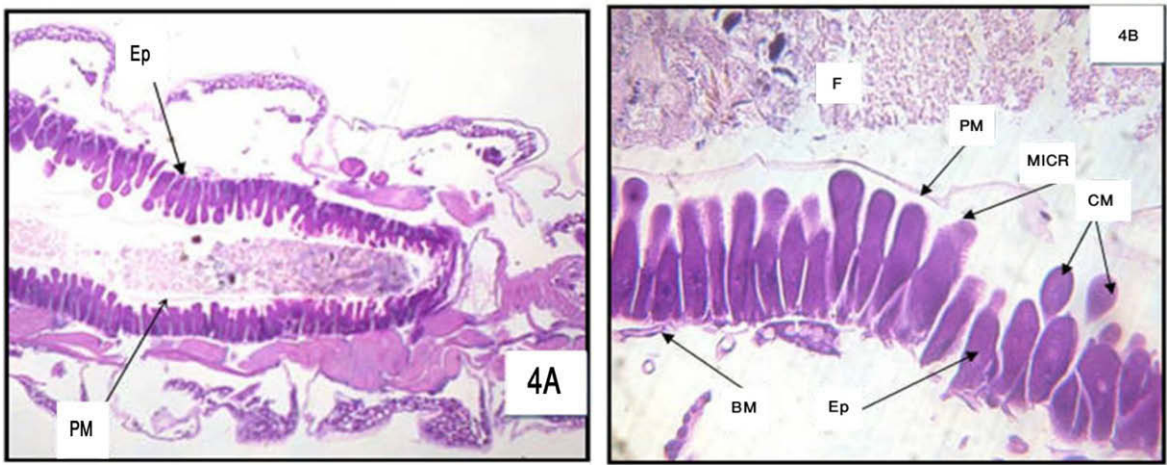


Figure 4 (A) and (B) Longitudinal section in the posterior region of midgut of 3rd larval instar of *Cx. quinquefasciatus treated* with LC_{50} of *M. azedarach* after 6 h (A = 100× and B = 400×) Epithelial cells (Ep), peritrophic membrane (PM), food bolus (FB), basement membrane (BM), microvilli (MICR) and cytoplasm masses (CM).

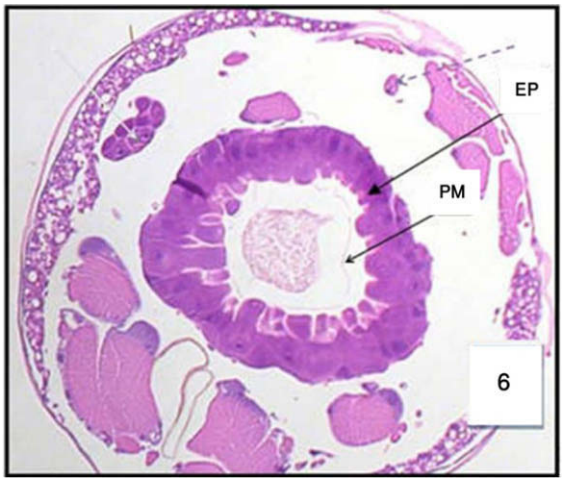


Figure 6 Cross section in the posterior region of midgut in 3rd *Cx. quinquefasciatus* larval instar treated with LC_{50} *of M. azedarach* extract, showing the effect after 12 h of exposure. Epithelial cells (EP) and peritrophic membrane (PM). 400×.

larvae possesses a well-preserved layer of epithelial cells, the surrounding basal lamina was underneath the basal labyrinth. The gut lumen was lined by a brush border membrane composed of numerous and regularly placed microvilli (Figs. 16 and 17), as observed in control sections of the posterior midgut progressed both extracellular, through detachment of dark cells from each other, and from the basal lamina (Figs. 7 and 8 and ultrastructural sections Figs. 18 and 19), and intracellular, through cell bursting.

3.4. After 48 h of treatment

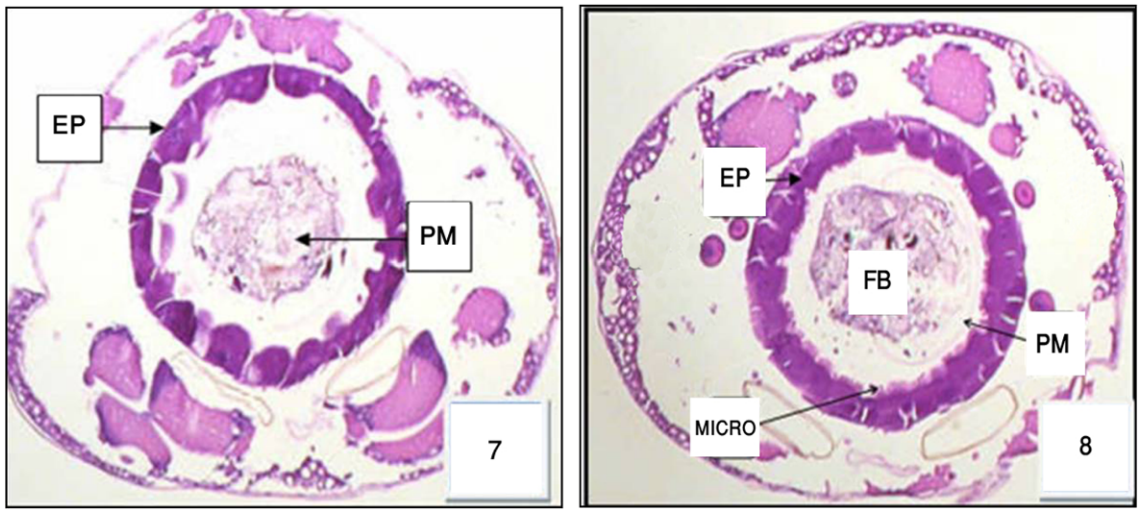
Most of the epithelial cells degenerated and vacuolated. Histopathological data reveal differential toxic effects of *M. azedarach* between the two different regions of the midgut epithelium according to the period of exposure. The major cytopathological alteration was large vacuoles of different sizes and containing broken membranes, at the apical side of the epithelial cells, and extensive cellular microvillar disruption. (Figs. 9 and 10 and ultrastructural sections Figs. 20 and 21).

3.4.1. Gastric caecae

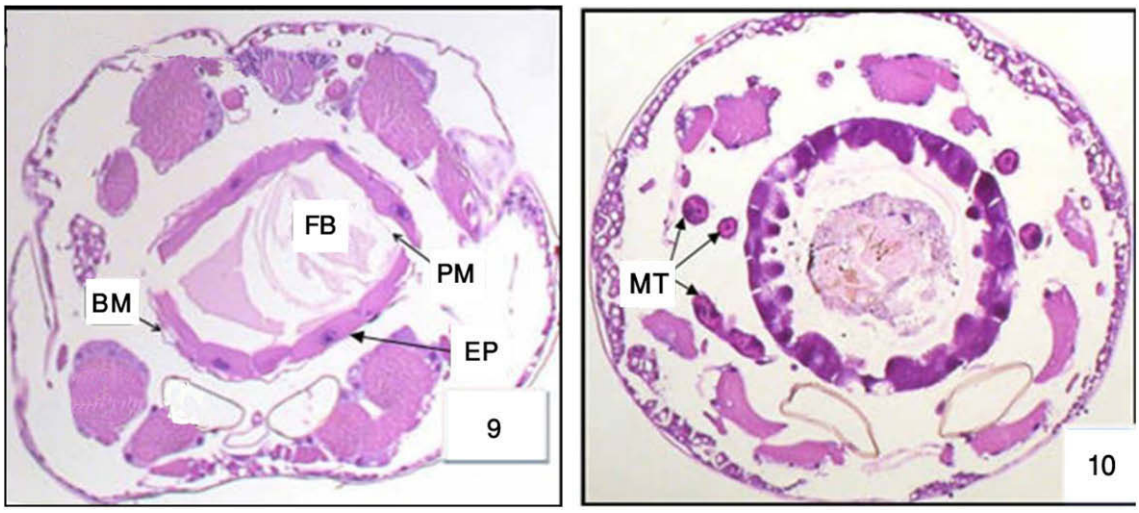
The histopathological effect of Melia in gastric caeca region was studied. The choice of this region is justified by the fact that it is directly in contact with toxic element (azadirachtin) of melia compounds. The untreated larvae of *Cx. quinquefasqiatus* gastric caecum showed a well-preserved layer of epithelial cells. The ovoid nuclei are located in the center of the cell. One observes a regularly microvilli border in the gastric lumen (Fig. 11) for the treated larvae. After 6 h the signs of intoxication began on the level of caecum gastric. The histology of *Cx. quinquefasciatus* larvae showed on the level of this region morphological and serious damage of the epithelial columnar cells (Fig. 12). After 12 h some cells appear slightly hypertrophied with a perceptible beginning of vacuolization at the apical level. These vacuoles invaded the cells. Sometimes, we noted an enlargement of intercellular spaces (Fig. 13**)**. After 24 h, the Epithelial cells of the gastric ceacae start to burst and we noted a cytoplasmic rejection of cells material that mixed with food column (Fig. 14). After 48 h, some cells degenerated and showed beaches of lysis (Fig. 15). These cells are very advanced in their infection.

4. Discussion

It is clearly proved that crude or partially purified plant extracts are less expensive and highly efficacious for the control of mosquitoes rather than the purified compounds or extracts (Jang et al., 2002; Cavalcanti et al., 2004). The plant tested in the present study is reported to be eco-friendly and are not toxic to vertebrates (Al-Sharook et al., 1991). In this study, the results showed that the ethanolic extract of *M. azedarach* exhibited hig degree of blocking the development by induction of great mortality of larvae. Concerning the insectiostatic or



Figures 7 and 8 Cross section in the anterior and posterior region of midgut in 3rd *Cx. quinquefasciatus* larval instar treated with LC_{50} *of M. azedarach* extract, showing the effect after 24 h of exposure. Epithelial cells (EP) and food bolous (FB) and basement membrane (PM). 400×.



Figures 9 and 10 Cross section in the anterior and posterior regions of midgut in 3rd *Cx. quinquefasciatus* larval instar treated with LC_{50} *of M. azedarach* extract, showing the effect after 48 h of exposure. Epithelial cells (EP), peritrophic membrane (PM), food bolous (FB), malpighian tubules (MT) and basement membrane (BM). 400×.

insecticidal action of *M. azedarach*, its leaves and fruits significantly reduced the growth of *Spodoptera littoralis* (Lepidoptera: Noctuidae) (Schmidt et al., 1998). *Lymnaea cubensis* (Pulmonata: Lymnaeidae), a Cuban intermediate host for fasciolosis, is lethally affected by the extracts of *M. azedarach* seeds (Perez et al., 1998). Anonymous (1999) described that leaves of *M. azederach* caused 54%, 62% and 75% %mortality of *Callosobruchus chinensis* at the rate of 0.5%, 1.0% and 2.0% (w/w) on the 12th day. But in this experiment *M. azederach* showed strong effective results. It showed high mortality after 24 and 48 h of treatment. The results of this study indicate the plant-based compounds such as limonoids (compounds present in the Meliaceae plant family seed) may be effective alternative to conventional synthetic insecticides for the control of *Cx. quinquefasciatus*. Undoubtedly, plant-derived toxicants are a valuable source of potential insecticides. These and other naturally occurring insecticides may play a more prominent role in mosquito control programs in the future (Mordue and Blackwell, 1993). The results of this study will contribute to a great reduction in the application of synthetic insecticides, which in turn increase the opportunity for natural control of various medicinally important pests by botanical pesticides. Since these are often active against a limited number of species including specific target insects, less expensive, easily biodegradable to non-toxic products, and potentially suitable for use in mosquito control programme (Alkofahi et al., 1989), they could lead to the development of new classes of possible safer insect control agents. Changes were seen in the anterior and posterior regions of the midgut, included separation of the epithelial cells from the basement membrane with damage of the peritrophic membrane. The mixing of the gut contents with the haemolymph caused the larval mortality. The border of midgut showed a striated appearance due to the presence of the microvilli which line the inner edge of the epithelial cells. These microvilli enhanced the rate of absorption (DeRobertis et al., 1965). The gut apical portion of columnar cells was swollen and sometimes, distinct elongations protruded into its lumen as a bulbous eversion section showed vacuolated

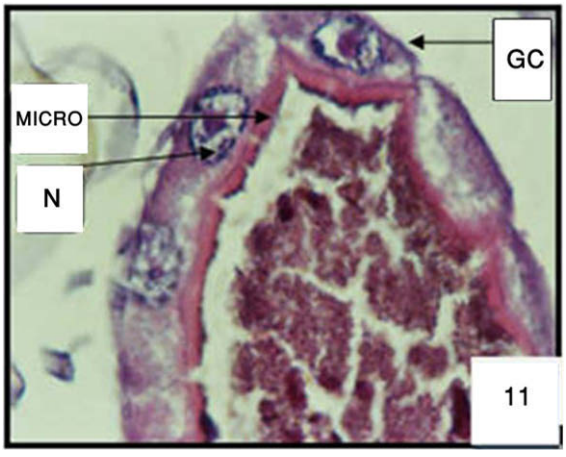
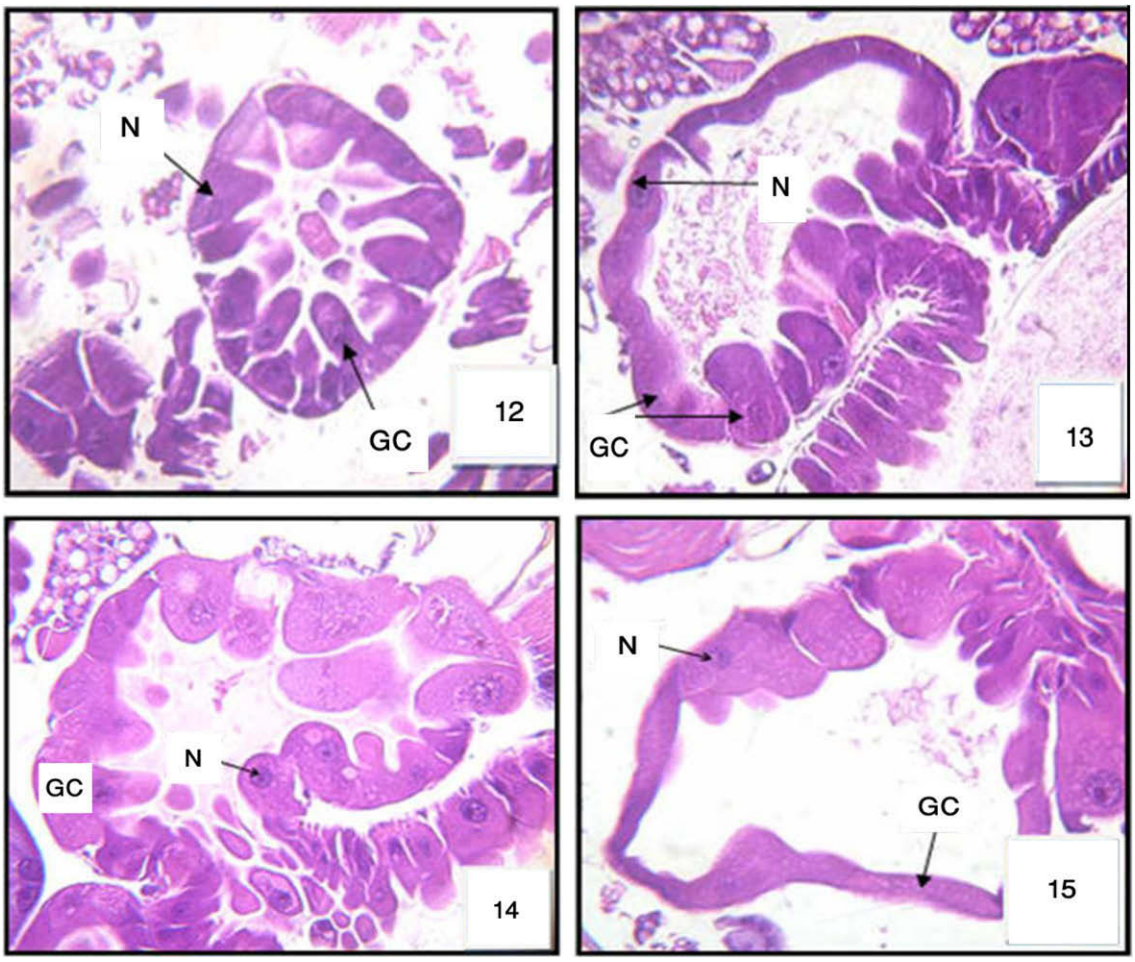


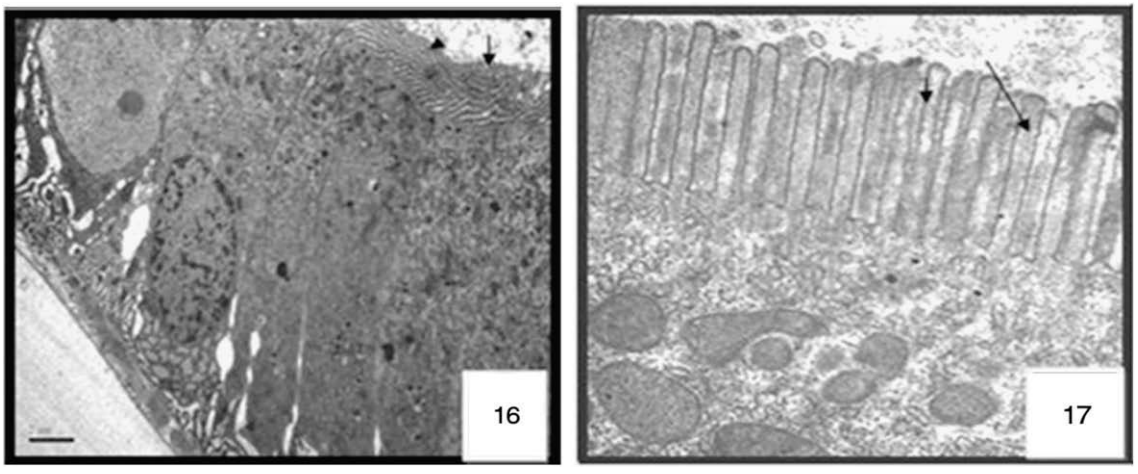
Figure 11 Cross section in gastric caeca (GC) of 3rd larval instar of *Cx. quinquefasciatus* untreated. Microvilli (MICRO) and nucleus (N). 400×.

cytoplasm, cells were dislodged, sloughed and detached from each other. The most obvious ultra structural change noted in the epithelium of the midgut was the disruption of the microvilli, thus giving the midgut a vacuolization appearance, this coincides with the observations of Sutter and Raun (1967) in *Ostrinia nubilalis* who suggested that the enzymatic activity of the vegetative rods is responsible for the disruption of microvilli.

The observed histopathological effects of the ethanolic extract of *M. azedarach* on the midgut of *Cx. quinquefasciatus* larvae were in agreement with the results obtained by Hamouda et al. (1996), Hussin and Shoukry(1997) and Assar and El-Sobky (2003) on *Cx. pipiens*. Hamouda et al. (1996) stated that the midgut of *Cx. pipiens* treated with *Artemisia judaica* was affected, the epithelial layer was vacuolated, swollen cells, masses of cellular material appeared in the lumen and finally the

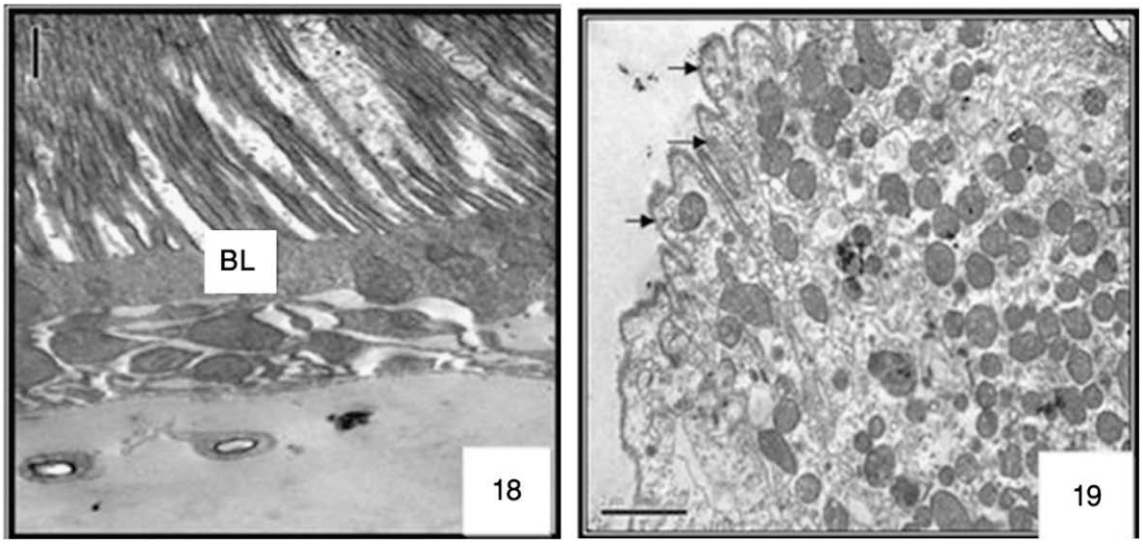


Figures 12–15 Cross section in gastric caeca (GC) of 3rd larval instar of *Cx. quinquefasciatus* treated with LC_{50} of *M. azedarach* after 6 h, after 12 h: Fig. 13, after 24 h: Fig. 14, and after 48 h: Fig. 15). Nucleus (N). 400×.

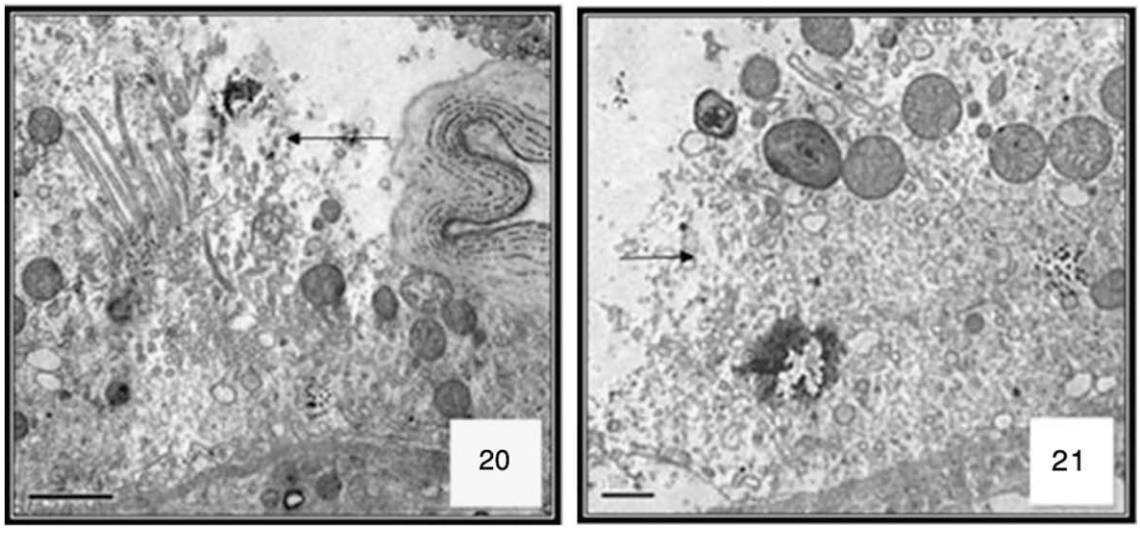


Figures 16 and 17 Longitudinal section of a columnar cell of the midgut in control larvae of *Cx. quinquefasciatus*. Transmission electron micrograph, microvilli ARROW. 4000×.

epithelium lost their normal appearance. Also, they found that larvae treated with *Anagallis arvensis* showed a rupture of the cell wall and destruction of the peritrophic membrane. Assar and El-Sobky (2003) observed that the water extract of



Figures 18 and 19 Ultrastructure of cells at 24 h after *M. azedarach* treatment larvae of *Cx. quinquefasciatus*. Transmission electron micrograph, destroyed MICROVILLI ARROW. 4000×.



Figures 20 and 21 Ultrastructure of cells at 48 h after *M. azedarach* treatment larvae of *Cx. quinquefasciatus*. Transmission electron micrograph. 4000×. Cell disruption arrow.

Eichhornia crassipes, revealed drastic effect on larval midgut as the brush border and some of the epithelial cells were apically degenerated after 48 h and after 72 h, most of the epithelial cells completely degenerated and vacuolated.

The results of the present study may encourage further researches on using simple and inexpensive application methods for controlling mosquitoes in their breeding sites.

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