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Histopathological Effects of Azadirachtin on *Dacus ciliatus* Loew (Diptera: Tephritidae)

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ABSTRACT

Insecticidal activities of Azadirachtin (15% EC) were investigated on larval and pupal stages of lesser pumpkin fly, *Dacus ciliatus*. The mortality was recorded after 24h post-treatment. The third instar larvae more susceptible to this Azadirachtin formulation (LC_{50} = 23.74 ppm) than the pupal stage (LC_{50} = 49.97 ppm). Insects emerged with crumpled, distorted wings also female insects show swollen abdomen and deformed ovipositor after treatment of pupae with median lethal concentration. Treatment of third larval instar with median lethal concentration induced histological alterations in the cuticle and digestive system. Complete disintegration in epicuticle in some regions. The appearance of vacuoles between cuticle and epidermis has been observed. Destruction of muscular cells and fragmentation of muscular layers observed. The gastric caecal cells show serious damage, some cells appear hypertrophied, some cells have vacuoles and the lumen and intercellular spaces enlarged. Treatment-induced severe effect on the mid-gut where destruction of muscular cells and detachment of the basement membrane and muscle layers observed. The brush border of Malpighian tubules showed coagulation and detachment from epithelial cells.

INTRODUCTION

Dacus ciliatus commonly known as the lesser pumpkin fly, this fly is belonging to the Family Tephritidae. It is one of the most invasive of all the Tephritidae. It is an oligophagous serious pest of Cucurbitae in temperate and tropical regions (El Nahal *et al.*, 1971). This fruit fly was recorded in Africa and Asia in 1914 (Weems, 2002). Azab and Kira, (1954) collected *D. ciliatus* from Kom-Ombo. Adult females infest a wide range of cultivated and wild cucurbits where larvae of *D. ciliatus* develop in the fruits. Infestations were recorded on marrow, grounds, squash, cucumber, and cantaloupe fruits at Sharkia, Ismailia, Giza, and Aswan (Fetoh, 2003 and 2006). Few studies on *D. ciliatus* control with particular concerns on histopathology have been investigated. Females oviposit about 210 eggs during the life span and the eggs are laid in groups of 5-15 (El Nahal *et al.*, 1971).

After eggs hatching, the young larvae feed on the host plant, causing damage to the ripe fruit. The economic damage caused by this fruit fly is a result of the reduction in yield and its control using expensive insecticides.

Multiple uses of synthetic chemical pesticides have led to many environmental problems as pest resistance and toxicological effects on non-target organisms. In many cases, synthetic pesticides caused poisoning to farmers (Bag, 2000). It is necessary to search for environmentally safe methods for insect control. The use of plant extracts and their derivatives have evidenced to be effective against many insect pests. Azadirachtin is the most isolated constituent from neem leaves, stems, and seeds. Azadirachtin is a complex tetranortriterpenoid limonoid with pesticidal properties. Many scientists reported that it has insecticidal effects. It acts as a feeding deterrent, repellent, delay molting process, interferes with oogenesis, and affects spermiogenesis (Chaudhary *et al.,* 2017). It is considered as a botanical pesticide with exceptional growth-regulating and biocidal efficacy along with deterrent effects on the oviposition and feeding of insects (Morgan, 2009). Azadirachtin can act against many insects including thrips, aphids, caterpillars, beetles, lace bugs, whiteflies, leafhoppers, leaf miners, mealy bugs, and psyllids (Schmutter, 1990). The main goal of this research is to investigate the potency of Azadirachtin on larvae and pupae of the lesser pumpkin fruit fly and insight the histopathological effects induced in larvae after 24h of treatment.

MATERIAL AND METHODS

Rearing Technique:

The stock colony of, *Dacus ciliatus* was established from infested squash in February 2016. All colonies were reared in the laboratory at 25 ± 2^{o}C and 65 ± 5 RH. *Cucurbita pepo* was used as a rearing medium in aplastic jars (8x10) cm containing wheat bran. After pupation, the pupae were transferred to wooden cages (30 x 30 x 30) cm for adult emergence. The cages were supplies with dried sugar and cotton pads soaked with a solution of equal volumes of milk and water (1:1) for adult feeding (source of protein) and egg-laying. Eggs laid on the cotton pads were transferred to the rearing medium with wheat bran. The pupae could be separated by sieving after about seven days. The flies usually emerged about 15th day after pupation.

Bioassay:

The early third instar larvae of *D. ciliatus* were exposed to five different concentrations of the selected compound under investigation 250, 125, 62.5, 31.1and 15.6 ppm, while for pupae concentrations 500, 250, 125, 62.5and 31.5 ppm were used. The procedures were replicated five times for each concentration. Twenty larvae and pupae were used for each replicate. Ten milliliters of each concentration were used in the dipping technique in a glass jar (2.5 x 5 cm) for 2 minutes, after the exposure period, larvae transferred to rearing media. Water only used in the control experiments. Experiments were carried out under laboratory conditions at 25±2^{o} C, 65±5 %R H, and 12:12 light: dark cycle. Larval mortality was recorded 24 h after treatments.

Histological Methods:

The effect of median lethal concentrations LC_{50} of the Azadirachtin on histology of early third instar larvae were investigated. Some larvae were immediately placed in aqueous Bouin's fixative after 24h treatment from either control and treated experiments and placed in the refrigerator at 5C^{o} for 2h. After dehydration in a graded series of ethanol, the larvae were embedded in liquid paraffin. Histological sections were prepared and stained with hematoxylin and eosin stains.

Statistical Analysis:

The average larval mortality rates were subjected to probit analysis for calculating LC_{25}, LC_{50}, LC_{90} and other statistics at 95% confidence limits of the upper confidence limit (U.C.L.) and lower confidence limit (L.C.L.), and Chi-square values were calculated. (Reddy *et al*., 1992).

RESULTS

Toxicological Studies:

The susceptibility of pumpkin fly, *D. ciliatus* larvae, and pupae to the Azadirachtin was recorded after 24h treatment (Table 1). There is zero mortality recorded in the control experiments. The results presented in Figures (1A & 1B) showed that both larvae and pupae are susceptible to Azadirachtin. The results showed that the percentage of mortality increased with increasing concentration. The calculated LC_{50} after larval treatments is 23.74 ppm and the slope function equals 1.84 $\pm$ 0.18. Median lethal concentration, LC_{50} is determined by 49.97 ppm and the slope function is 0.99±0.14 for pupae treatment. Based on these results the early third instar larvae more susceptible to this Azadirachtin formulation than the pupal stage.

The adult of *D. ciliatus* emerged from pupae treated with median lethal concentrations of Azadirachtin show various degrees of morphological abnormalities. Insects with crumpled, distorted wings had observed also female insects show swollen abdomen and the deformed ovipositor (Fig. 2).

Table 1: Lower and Upper confidence limits of Azadirachtin (15% EC) treatments against *D. ciliatus* larvae and pupae after 24h treatments.

Stage	LC25 (L.C.L-U.C.L)	LC50 (L.C.L-U.C.L)	LC90 (L.C.L-U.C.L)	Slope$\pm$ SE	Chi square (df=4)
Larvae	10.22 (6.94- 13.48)	23.74 (18.91- 28.48)	117.71 (94.20-158.89)	1.84±0.18	2.554
Pupae	10.53 (3.96- 18.65)	49.97 (32.14- 67.66))	961.68 (565.21-2375.82)	0.99$\pm$ 0.14	0.175

L.C.L, lower confidence limits; U.C.L, upper confidence limits; df, degree of freedom.

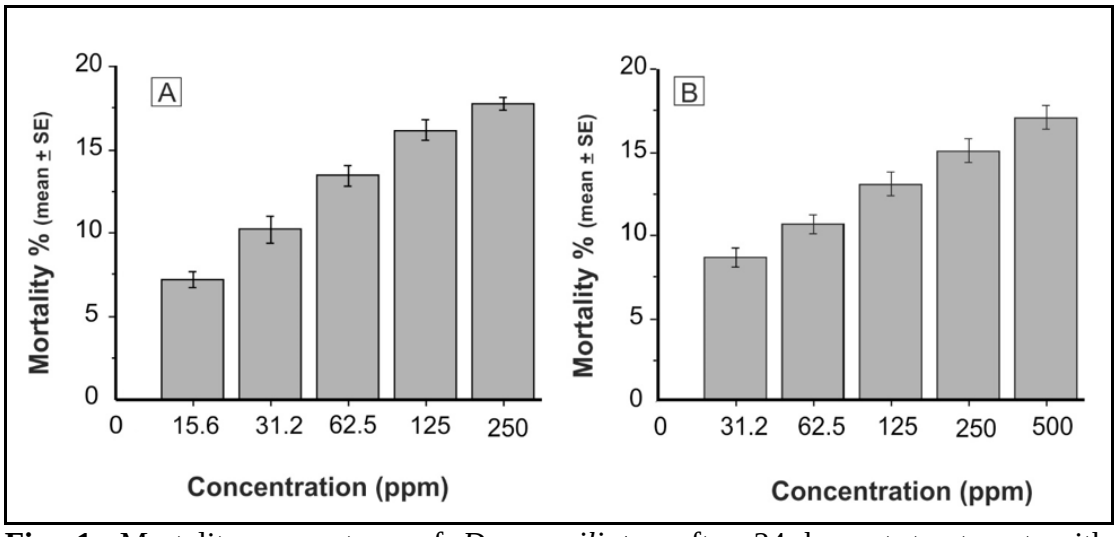

Fig. 1: Mortality percentage of *Dacus ciliatus* after 24 h post treatment with Azadirachtin (15% EC). A, larvae; B, pupae.

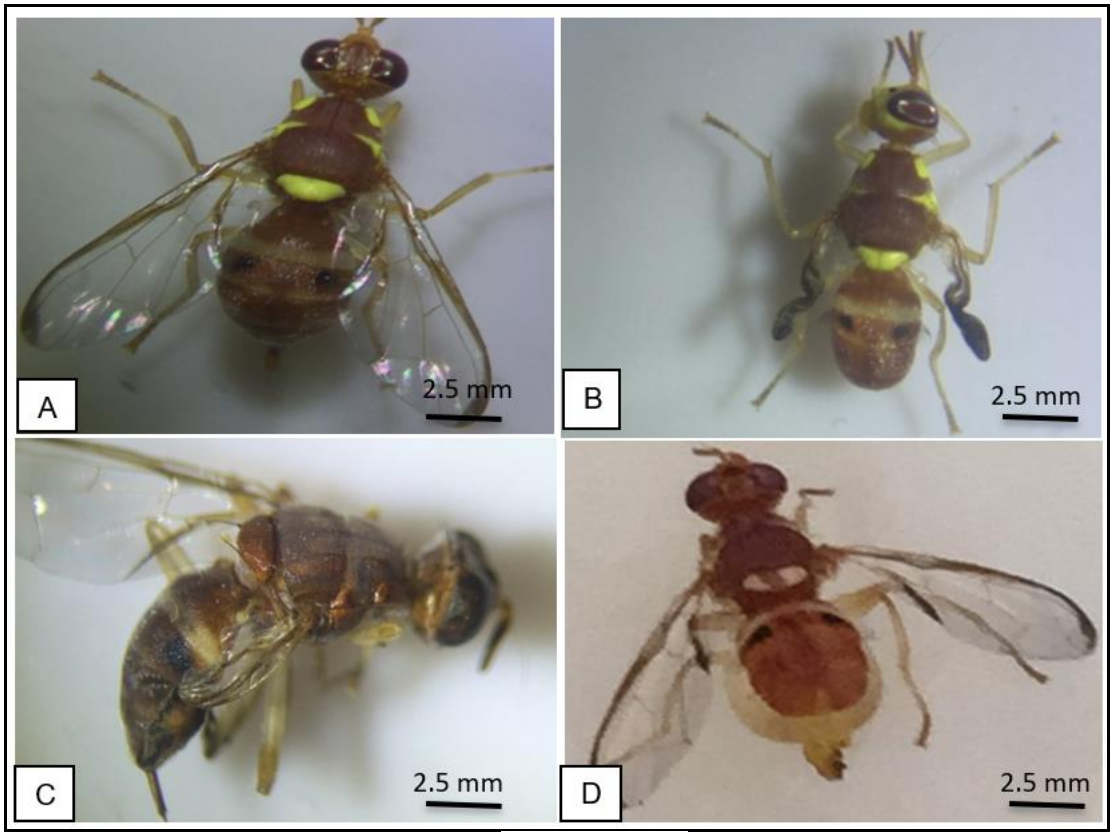

Fig. 2: Normal & deformed adults of *Dacus ciliatus* resulted from pupa treated with LC_{50} of Azadirachtin, (15% EC). A) Normal adult, B&C) Adult with crumpled wings and D) Adult with swollen abdomen and deformed oviposter.

Histological Studies:

The histological studies were carried out for normal and treated early third larval instar of *D. ciliatus* after treatment with median lethal concentration LC_{50} of Azadirachtin. The integument of normal third larval instar of *D. ciliatus* consists of the cuticle, epidermis, and basement membrane. The cuticle consists of three sublayers, epicuticle, exocuticle, and endocuticle (Fig.3A). Beneath the cuticle, the muscle layer is present. The muscle layer is composed of bundles of striated fibers. Each muscle fiber consists of a number of myofibrils, occupying the whole of the cross-section of the fiber (Fig. 3A). The normal fat bodies composed of two layers, an outer layer which constitutes ribbons underlying the body wall and visceral layer lies between the various organs (Fig.3A& C).

The alimentary canal of *D. ciliatus* larvae consists of foregut, midgut, and hindgut. The histological sections in the foregut region showed that the cardia and crop of larvae consisted of a single layer of cubical cells with a clear rounded nucleus and were covered by a very strong muscular layer that consisted of circular muscle and longitudinal muscle (Fig.4A). The larval midgut strongly convoluted in the body cavity. The larval midgut is the longest and most efficient portion of the alimentary canal where digestion and absorption take place. The midgut consisted of a single layer of law columnar epithelial cells rested on the basement membrane and surrounded by a thin muscle layer. (Fig.4A). There are numbers of gastric caeca and Malpighian tubules associated with the alimentary canal. Generally, four gastric caeca are located between foregut and midgut

meanwhile, the Malpighian tubules located at posterior midgut. like most dipterans, the control or untreated larvae of *D. ciliatus* larvae has four gastric caecum which showed a single layer of epithelial cells. Each cell has an ovoid centrally located nucleus. The free border of epithelial cells of gastric caeca has numerous, thin microvilli (Fig.4E). The Malpighian tubule consists of a single layer of flattened epithelial cells without clear borders in between. The epithelial cells of Malpighian tubules characterized by possessing large nuclei, granular cytoplasm, and brush border are projected to the lumen of the tubule (Fig. 5A).

Severe damage has occurred on the integument of *D. ciliatus* third instar larvae when they were treated by Azadirachtin. Complete disintegration in epicuticle in some regions. Lack of differentiation between epicuticle and endocuticle and disorganization in the epithelial cells of the epidermis (Fig. 3D). The appearance of vacuoles between cuticle and epidermis has been observed (Fig.3B). Irregular thickening of the cuticle (Fig.5C) and degeneration of epidermal cells (Fig.3D) were detected. Noticeable destruction and vacuolization on the fat bodies were detected (Fig.3B, D& E). The histopathological effect of Azadirachtin on muscles was ranged from slight degeneration by the occurrence of fissures (Fig.3B), to fragmentation of the whole muscular tissue (Fig.3C). The foregut especially cardia and crop do not show any change after treatment with Azadirachtin (Fig. 4A). The gastric caecal cells show serious damage, some cells appear hypertrophied or become degenerated in some regions, some cells have vacuoles, the intercellular spaces enlarged and loss of microvilli of gastric caeca cells observed (Fig.4F). Treatment-induced severe effect on the mid-gut, where shrinkage in some epithelial cells and swelling of other cells were noticed. The boundaries between epithelial cells were damaged. Destruction of muscular tissue and detachment of the basement membrane and muscle layers observed and loss of microvilli of midgut cells from treated larvae observed (Fig. 4C& D).

The histopathological changes in Malpighian tubules include separation of microvilli from the cells (Fig.5B) and clumping or coagulation of the microvilli at many specimens (Fig.5C).

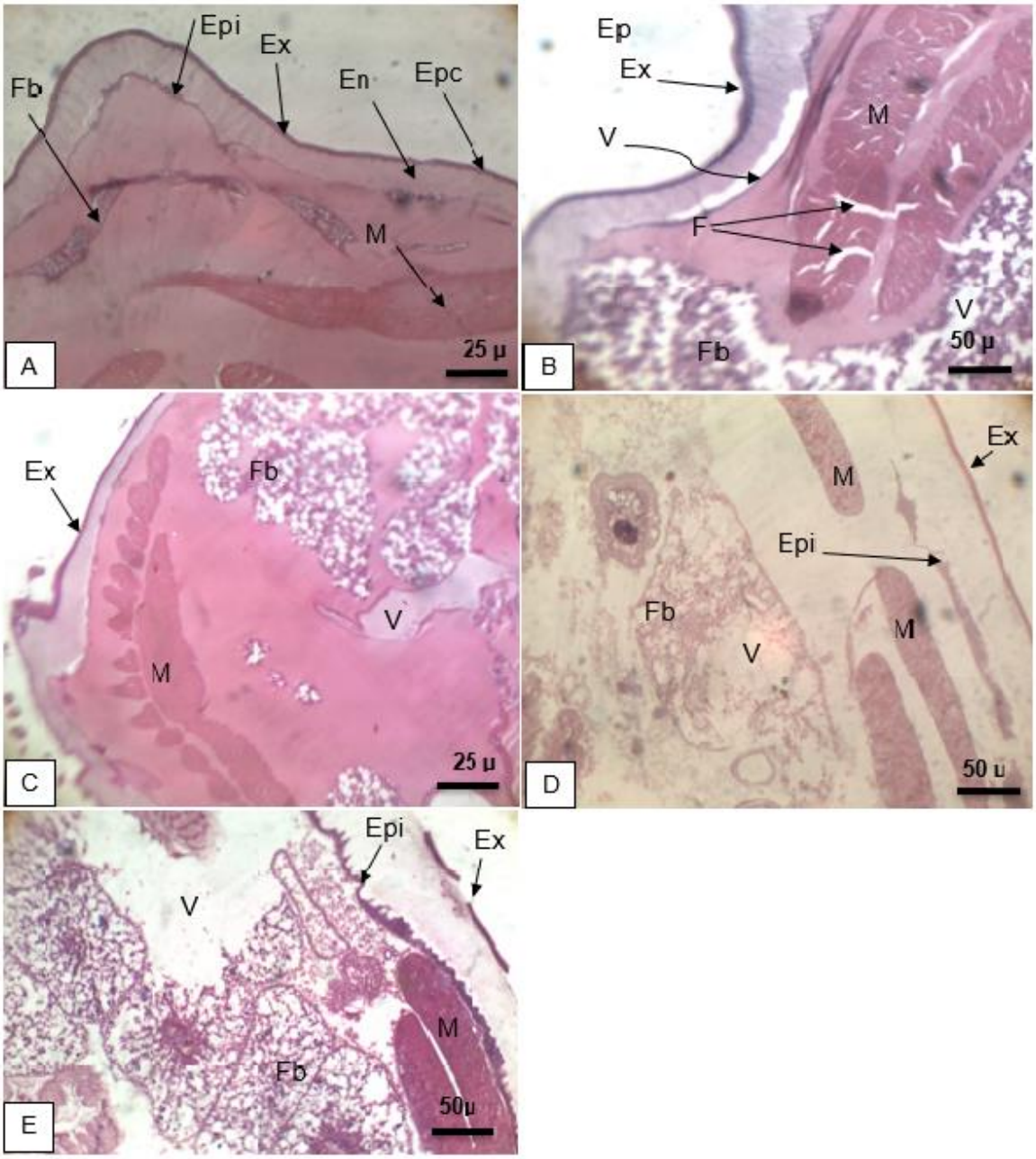

Fig. 3: Photomicrographs of T.S. of *D. ciliates* larvae showing, A) normal cuticle, muscles and fat bodies; B-E) T.S. of *D. ciliates* larvae treated with LC50 of Azadirachtin; B) vacuoles between cuticle and epidermis, fissures in muscles and vacuoles in fat bodies; C) fragmentation in muscular tissue and lack of differentiation between epicuticle and endocuticle; D) degenerations of epidermal cells, vacuolation in fat bodies; E) degeneration in exocuticle and epicuticle and disorganization of epidermal cells. (Ex, exocuticle; Ep, epicuticle; Epi, epidermis; En, endocuticle; F, fissures; Fb, fat bodies; M, muscles and V, vacuole).

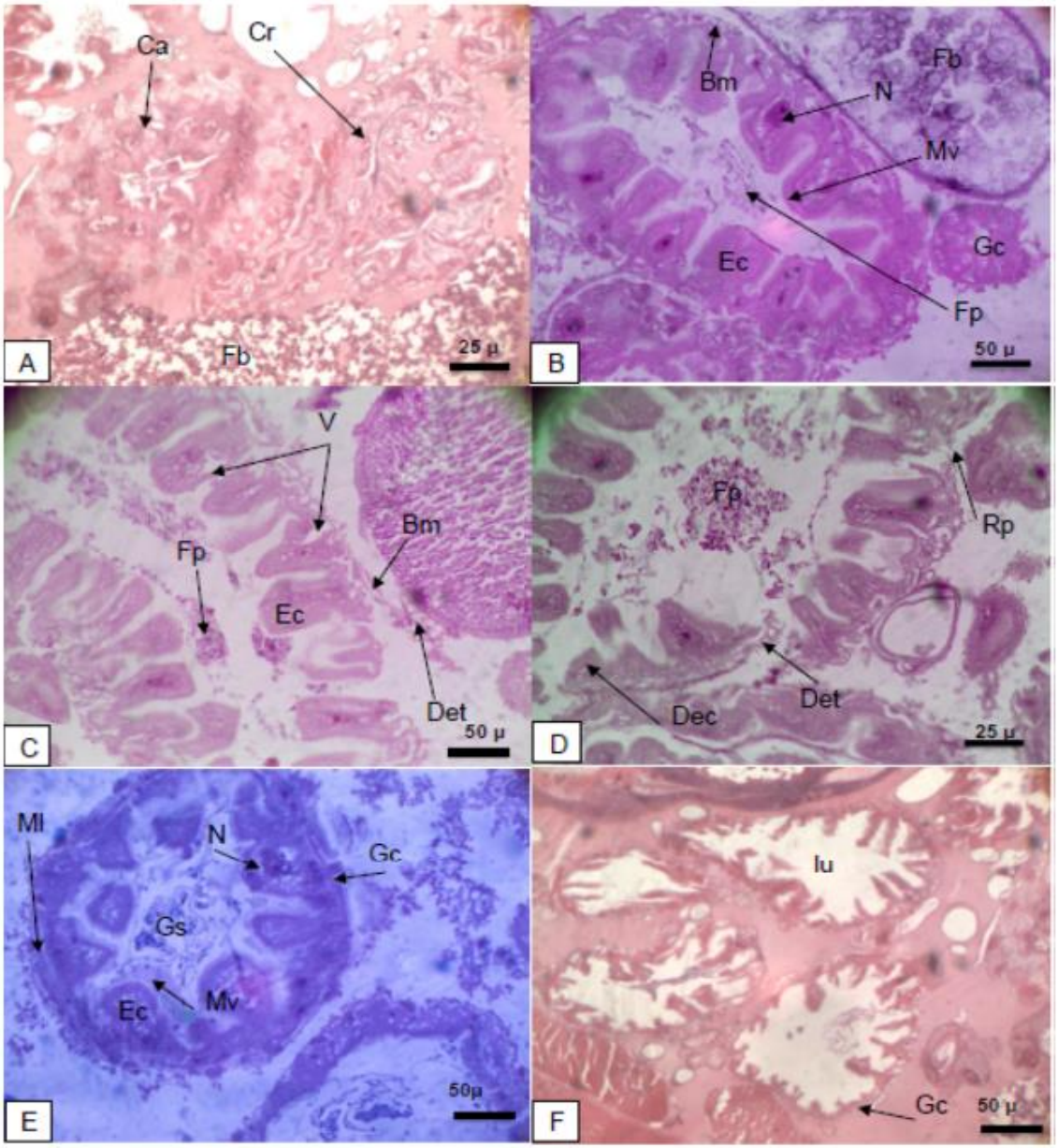

Fig. 4: Photomicrographs of T.S. of *D. ciliates* larvae showing, A) normal cardia and crop of fore gut; B) normal mid gut; C&D) Photomicrographs of T.S. of midgut of *D. ciliates* larvae treated with Azadirachtin showing appearance vacuoles, degenerated epithelial cells and detachment of basement membrane of; E) normal gastric ceaca; F) treated gastric ceaca showing enlargement of gastric ceaca lumen and hypotrophy of gastric ceaca cells . (Bm, basement membrane; Ca, cardia; Cr, crop; Dec, degenerated epithelial cells; Det, detachment; Ec, epithelial cells; Fb, fat bodies; Fp, food particles, Gc, gastric ceacae; Gs, gastric secretions; Ml, longitudinal layer; Mv, microvilli; N, nucleus and Rp, rupture).

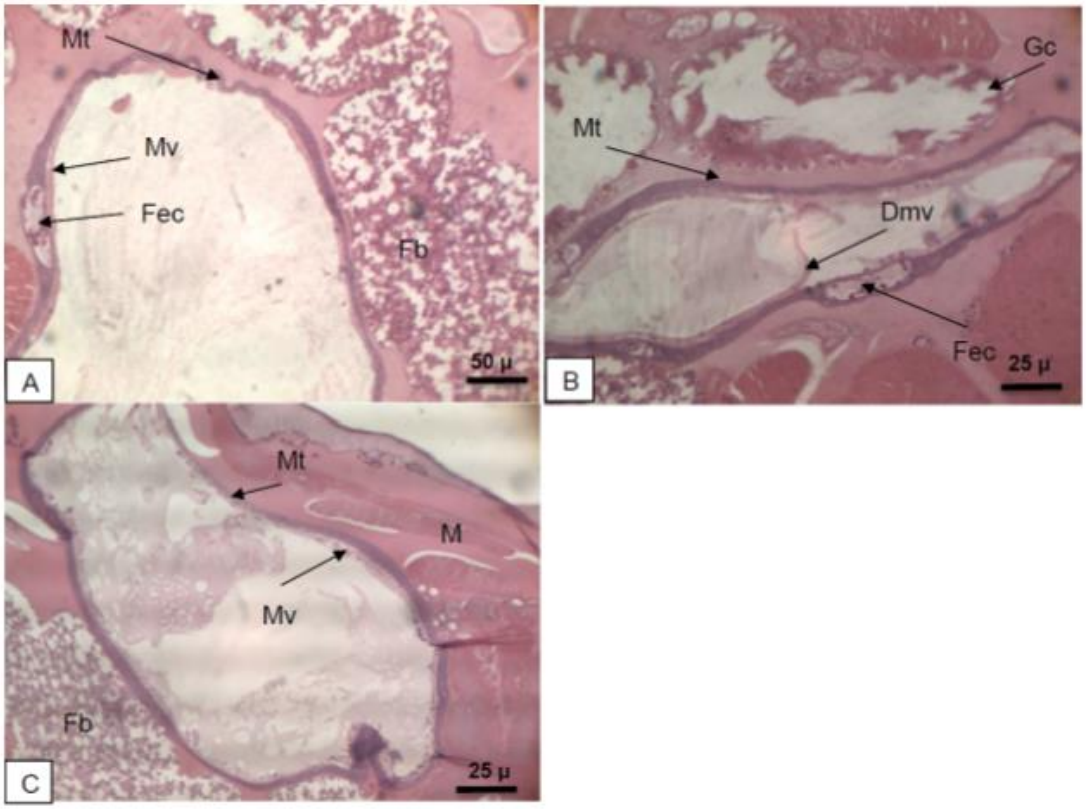

Fig. 5: Photomicrographs of T.S. of *D. ciliates* larvae showing A) normal Malpighian tubules showing single layer of flattened epithelial cell with clear microvilli; B& C) Malpighian tubules treated with Azadirachtin showing detachment and coagulation of microvilli. (Dmv, detached microvilli; Fb, fat bodies; Fec, flattened epithelial cell; Gc, gastric ceacae and Mt, Malpighian tubule).

DISCUSSION

Azadirachtin (15% EC) has an insecticidal effect against the destructive lesser pumpkin fruit fly *D. ciliatus*. Little literature studied the effect of neem extracts and their derivatives on this fruit fly. In India antiovipositional effects of neem extracts against *Dacus spp*. on vegetables were observed. While, aqueous extracts of neem seeds and neem oil when sprayed against *D. ciliatus* in Togo could not reduce the pest attack and the degree of damage to the plants (Jacobson, 1988).

Other scientists studied the effect of neem extracts or their active ingredients on other fruit flies. Singh and Singh (1998) declared that extracts of neem seed kernel have an anti-oviposition effect on both *B. cucurbitae* and *B. dorsalis*. Previous investigation of Di Ílio *et al*. (1999) demonstrated significant reductions in *Ceratitis capitata* fecundity when the flies were exposed for 24 h to diets containing aqueous neem seed extract Deterring of egg-laying of *D. cucurbitae* and *D. dorsalis* recorded by 0. 25 % of aqueous extract of neem seed kernel (Singh, 2003). Akhtar *et al.* (2004) recorded a lower number of the settling of *B. zonata* females flies on fruit treated with petroleum ether extract of the sweet flag followed by flies on neem acetone extract. Neem oil and neem seed water extract reduced the percent of infestation in the melon fruit fly, *B. cucurbitae* at 1%, 2%, and 3% (Khattak *et al*., 2009). The acetone extract of six indigenous plants was effective against cucurbit fruit fly, *B. cucurbitae* (Siddiqi *et al.*, 2018). Ali *et al*. (2011) stated that minimum melon fruit fly adult numbers were found after treatment with Methomyl and Neem, respectively. Aqueous extract of neem

seed kernel has repellent activity against *B. zonata* (Ilyas *et al*., 2017). They also found significant oviposition inhibition in guava treated with petroleum ether extract of *Azadirachta indica* compared with control. Commercial formulations of neem greatly reduce the damage caused by fruit fly species. Additionally, they could block the development of female ovaries (Khan *et al.,* 2007).

Many scientists studied the toxic effect of other plant extracts against other fruit flies. *Acorus calamus* extracts had chemosterilant action on *B. cucurbitae* adults (Shankutala and Thomas, 2001a). Hasyim *et al*., 2007 stated that camphor isolated from *Elshltozia pubescens* show strong attractant properties to the fruit fly, *B. tau* males is useful in trapping experiments. Rehman *et al*. (2009) studied the effects of ethanol extracts of *Peganum harmala* L. seeds on the olive fruit fly, *B. oleae*. They recorded adult repellency, delaying larval growth, and reduction in oviposition rate. Acetone extract of *Curcuma longa* induced higher mortality percent in newly emerged adults of *B. zonata* (Siddiqi *et al.,* 2011). Ethanolic leaf extract of *Ruta graveolens* has insecticidal properties toward *C. capitata* adults (Ghabbari *et al.,* 2018).

The adult of *D. ciliatus* that emerged from pupae treated with a median lethal concentration of Azadirachtin show various degrees of morphological abnormalities. Insects with crumpled, distorted wings had observed so dispersion of adults is prohibited also female insects show swollen abdomen with deformed ovipositor thus egg deposition is completely ceased.

The morphological aberration of insects recorded after treatments of many insects by neem extracts and derivatives. Saxena *et al.* (1981) observed some *Cnaphlocrosis medinalis* emerging from apparently normal pupae often had deformed wings and occasionally failed to emerge from the pupal cases when treated with neem oil. Ivbijaro (1986), noticed few pupae that survive metamorphosed into malformed adults when *Tribolium castaneum* treated with neem ethanolic extract. Similar results were described by many authers by natural plant extracts on many other insects. Shalaby *et al*. (1998) reported the presence of an adult with crumpled wings after treatment of *Culex pipiens* and *Musca domestica* with citrus oils. Hussein (2002) on *C. pipiens* larvae after treatment with *Thevetia peruvine* active compound, stigmasts-en-3-ol came with similar results. Khater (2015) recorded complete adult eclosion but adults show crumpled wings following treatment of *C. pipiens* larvae with *Ruta graveolens* and *Raphanus sativus* petroleum ether extracts.

The morphological abnormalities of Azadirachtin insecticide may be a result of inhibition of the release of prothoracicotropic hormones and allatotropins (Mordue and Blackwell, 1993, and Williams and Mansingh, 1996). Sharma (1992) investigated the growth-inhibiting properties of Azadirachtin. By topical application of Azadirachtin, the development of *Corcyra cephalonica* was inhibited and at higher doses lead to disturbance of both larval-pupal and pupal-adult moulting was interpreted as interference with the morphogenetic hormone.

The treatment of third larval instar of *D. ciliates* with a median lethal concentration of Azadirachtin induced histological alterations in the cuticle and digestive system. The complete disintegration in the epicuticle in some regions. The appearance of vacuoles between cuticle and epidermis has been observed. The destruction of muscular cells and fragmentation of muscular layers observed. The gastric caecal cells show serious damage, some cells

appear hypertrophied. The treatment induced severe effect on the mid-gut, where shrinkage in some epithelial cells and swelling of other cells were noticed. The boundaries between epithelial cells were damaged. The brush border of Malpighian tubules showed coagulation and detachment from epithelial cells.

Many researchers mentioned the histological changes in cuticle and epidermis of insects following treatment with plant extracts and active compounds of plants. Degeneration in the hypodermal cells in addition to some cuticular abnormalities was reported with Azadirachtin treatment against the larvae of *Epilachna varviests* (Schluter and Schulz, 1983 and Schluter, 1986). Khalaf *et al*. (2009) found, detachment of cuticle from hypodermis, disintegration in the hypodermis, and destruction of the basement membrane when *Synthesomyia nudesita* larvae treated with *Cupressus macrocarpa and Alpinia officinarum* volatile oils. Blockage of cuticle secretion in fifth instar larvae of greater wax moth, *Galleria mellonella* after treatments with Azadirachtin (Ünsal and Güner, 2016). The histopathological effect of Azadirachtin on muscles was slight degeneration by the occurrence of fissures. The appearance of the fissure and the fragmentation attributed to the destruction of the sarcolemma.

The treatment with a median lethal concentration of Azadirachtin induced severe damages in the midgut. The midgut of insects is responsible for the production of enzymes and absorption of the digestive products (Chapman 1998; Cruz-Landim 1999 and Vatanparast *et al.,* 2012). Vacuolation in the midgut cells appeared in the cytoplasm of *B. oleae* after treatment with *Bacillus thuringensis* (Dimitriadis and Domouhtsidou 1996 and Ruiu *et al*., 2007). Neem oil affects the new pupal epithelium of the midgut epithelium in the lacewing *Ceraeochrysa claveri*, loss of microvilli of midgut cells from treated pupae observed. Cellular injuries such as the appearance of vacuoles in the cytoplasm (Scudeler *et al*., 2014). Al-Mekhlafi (2018) recorded the destruction of microvilli, swallowing, and appearance of vacuoles in the midgut of *C. pipiens* larvae after treatment with methanol extract of *Carum copticum*.

This study reveals the efficacy of neem compound, Azadirachtin in the control of both larvae and pupae of pumpkin fly, *Dacus ciliates.* It is possible to minimize the economic damage result from this fly to cucurbits with safe and environmentally friendly compounds.

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ARABIC SUMMARY

التأثيرات الهستوباثولوجية للازاديركتين على ذبابة القرع داكس سيلياتس (رتبة ذات الجناحين: تيفيرتيدي)

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تمت دراسة النشاط الإبادي للازاديركتين (١٥٪ مركب مستحلب) ضد العمر اليرقي الثالث و العذارى لذبابة القرع داكس سيلياتس. تم تسجيل نسب الموت بعد ٢٤ساعة من المعاملة وتبين ان العمر اليرقي الثالث أكثر حساسية لهذا المركب وكان التركيز نصف المميت (٧٤و٢٣ جزء في المليون) من الاعمار العذرية حيث التركيز النصف المميت (٩٧و٤٩ جزء في المليون).

أدت معاملة العذارى بالتركيز النصف المميت الي ظهور حشرات بأجنحة مجعدة و اناث ببطن منتفخة والة وضع البيض مشوهة كما أدت معاملة العمر اليرقي الثالث بالتركيز النصف المميت الي ظهور تغيرات هستوباثولوجية في طبقة الجليد(كيوتيكل) والجهاز الهضمي. تبين تحلل طبقة الابيكيوتيكل و خلايا طبقة تحت الجليد(الابيديرمز) نفسها وظهور فجوات بين الجليد وطبقة تحت الجليد. وقد احتوت العينات المعالجة على ألياف عضلية متحللة وغير منتظمة من حيث التركيب العضلي المعروف. واظهرت المعاملة تأثير شديد علي الردوب المعدية حيث اتساع التجويف واتساع التجويف بين الخلايا وضمورفي الخلايا. وقد أوضحت العينات الهستولوجية في خلايا المعي المتوسط نتيجة المعاملة وجود ضعف ملحوظ في طبقة العضلات وانفصال الغشاء القاعدي وظهور فجوات في الخلايا الطلائية واختفاء الزغيبات. واظهرت ايضا تكتل وانفصال زغيبات الخلايا الطلائية لأنابيب ملبيجي.