

Influence of *Allium sativum* and *Citrus limon* Oil Extracts and *Bacillus thuringiensis israelensis* on Some Biological Aspects of *Culex pipiens* Larvae (Diptera: Culicidae)

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Abstract: The extensive use of chemical pesticides inducing resistance in insects beside residues contamination of human food and environmental pollution. So the present study was designed to evaluate biological control of 4th instar *Culex pipiens* (L.) (Diptera: Culicidae) by using plant oil extracts (*Allium sativum* and *Citrus limon*) and entomopathogenic bacteria (*Bacillus thuringiensis israelensis*). Results showed that the fourth instar *C. pipiens* (L.) was highly susceptible to *Bacillus thuringiensis israelensis* followed by *Citrus limon* and *Allium sativum*. In all case, a positive relationship was found between the time of exposure and mortality percentage of larvae. Histopathological studies revealed that there was a clear damage in the midgut epithelial cells. The tissues of gut, muscle, fat body and cuticle were the most severely damaged by the treatment. Also, the destructive damages described in the investigation depend mainly up on the time of exposure and the dosage. The ultrastructural findings of the 4th instar *C. pipiens* (L.) showed the separation of midgut epithelial cells from the basement membrane. Moreover, separation of columnar and goblet cells together with vacuolezed appearance was demonstrated. The nuclei of columnar cells were clumped into large irregular appearance and destruction in microvilli. It was concluded that the plant oil extract (*A. sativum*, *C. limon*) and Bti proved to be a promising controlling agent to fourth instar *C. pipiens* larvae.

Key words: *C. pipiens* (L.) (Diptera: Culicidae) • Plant oil extracts (*Citrus limon* and *Allium sativum*) • Entomopathogenic bacteria (*Bacillus thuringiensis israelensis*) • Histopathology

INTRODUCTION

Female mosquitoes are one of the most world-wide important insect pests that affect the health of human being and domestic animals. They require a blood meal for egg production and produce a painful bite as they feed. While feeding, they can transmit a number of diseases-causing organisms to human and animals. These diseases includes: encephalitis, dengue fever, filariasis, yellow fever and malaria [1]. Resistance of mosquitoes to insecticides, including organochlorines, organophosphate, carbamates and pyrethroids was considered to be a recent evolutionary adaptation to environmental changes. Response of mosquitoes to sequential application of chemical insecticides for their control extended to less than a century. Therefore, use of biological control is recommended to avoid this resistance

[2,3]. In this respect, several biological controls have been tested in many parts of the world to evaluate their potential in control of mosquito as a vector [4,5]. Plant may be an alternative source of mosquito control agents because they constitute a rich source of bioactive chemicals [6-8]. Also, toxins from certain strains of bacteria, like *B. thuringiensis* var. *israelensis* (Bti) and *B. sphaericus* (Bs) were shown to be highly effective against mosquito larvae at very low dosage and safe to non-target organisms [9-12]. *B. thuringiensis* (Berliner) is a rod-shaped, aerobic, spore-forming bacterium which is characterized by its production of two major classes of insecticidal toxins, beta-exotoxin reviewed by Sebesta *et al.*, [13] and other major toxins mainly endotoxins or crystal protein [14-18]. These authors concluded that exotoxins have negligible role or no role in the larvicidal activity on the bacterium while, Lahkim *et al.*

[19] observed that the toxic effect of *B. thuringiensis* on *A. aegypti* was most probably due to the endotoxins. Toxic action on the treated *C. pipiens* larvae with laboratory strain of *B. thuringiensis* H-14 revealed drastic effect on larval mid-gut epithelium and resulted in extended pathological activity in adults [20]. Moreover, *B. sphaericus* strain Faiyoun and Bti caused ultrastructural changes in the midgut of *C. pipiens*, *An. pharoensis* and *A. caspius* which differed according to the mosquitoes species. Also, the *B. sphaericus* and *B. thuringiensis israelensis* have different cytotoxic effect on the midgut mucosa of the three mosquito species [21, 22].

The current work was carried out to evaluate some biological aspects and the histopathological alterations in 4th instar *Culex pipiens* (L.) following exposure to in plant oil extracts (*A. sativum* and *C. limon*) and Bti on.

MATERIAL AND METHODS

Insect: Fourth instar *C. pipiens* (L.) (Culicidae) were selected as the target insect for the present study (Fig. 1a). The fourth instar larvae were collected from nearby untreated sites in and around Cairo. These field larvae were maintained for many generations, according to the method of Hafez [20].

Plant Oil Extracts: Two essential oils were used in the present study, *A. sativum* (garlic oil extract) and *C. limon* (lemon oil extract). They were obtained from El-Gomhouria Company, Cairo, Egypt.

Tested Bacteria: The entomopathogenic bacteria *B. thuringiensis israelensis* (Bti) in the form of liquid concentration was obtained from Chema industries. Chema @ Chema. Com. eg.

Exposure to Plant Oil Extracts: Fourth instar *C. pipiens* (L.) were exposed to three different concentrations (3, 5 and 10 µl/25 L.) of two types of plant oil extracts (*A. sativum* and *C. limon*). The extracts were diluted in 10 ml distilled water (1 ml/ 10 ml H₂O) and mixed with two drops of tween just prior to use and each concentration was replicated four times. Twenty five 4th instar *C. pipiens* (L.) per replicate were placed into plastic petri dishes (5 cm in diameter). Petri dishes were stored at room temperature in the dark, according to the method of Thomas and Callaghan [23].

Exposure to Entomopathogenic Bacteria (Bti): Fourth instars of *C. pipiens* (L.) were exposed to three different concentrations (1, 3 and 5 µl/25 L.) of the Bti bacteria. Dilutions were made in distilled water as bacterial suspensions. Four replicates each of twenty five of 4th instar of *C. pipiens* (L.) was tried in each concentration [24, 25]. As a control, a similar numbers of larvae were used. The mortality was determined after the indicated period of time post-treatment by counting the number of a live larva in each dish and the number of the dead ones was then deduced for each replicate. Larvae were considered dead (Fig. 1 b) when they did not respond to needle stimuli. The mortality of larvae was daily recorded. The LD₅₀, (the concentration of plant oil extracts and Bti which kill 50% of larvae group of the samples), was computed based on the data obtained from the mortality percentage.

Histopathological Techniques: Light microscopical analysis: Fourth instars of *C. pipiens* (L.) were exposed to lethal dose (10 µl/25 larvae) of plant oil extracts (*A. sativum* and *C. limon*) and (5 µl/25 larvae) of entomopathogenic bacteria (Bti). Control and treated larvae were fixed after 8, 12, 24 and 48 hrs. of exposure to plant oil extracts. In case of Bti, control and treated larvae



Fig. 1-a: A Healthy 4th instar *C. pipiens* (L.). [b] Fourth instar *C. pipiens* (L.) after exposure to entomopathogenic bacteria (Bti). The larvae turn uniformly black after death

were fixed after (30, 90, 120 and 150 min. Serially dehydrated in an ethanol series and embedded in wax. Transverse section were dewaxed and stained with haematoxylin and eosin. The staining method was carried out according to Volman and Peters[26].

Transitional Electron Microscopic (TEM): At least 10 larvae were fixed for transmission electron microscopy at the time of larval removal from contact with plant oil extract (10 $\mu\text{l}/25$ larvae) *C. limon* oil at 48 hrs and entomopathogenic bacteria (5 $\mu\text{l}/25$ larvae) at 150 min. At the time of fixation, heads and siphons of larvae were removed and bodies were nicked with a razor blade. Larvae were fixed in ice cold 5 % glutaraldehyde in sodium codylate buffer, pH 7.4, for 12-24 hrs washed in buffer and post fixed overnight nice cold 1% buffered osmium tetroxide. After rinsing, larvae were refrigerated overnight in 0.5 % aqueous uranylacetate solution, then dehydrated and in filtrated and in bedded in spurr resin [27]. Thin sections were stained with uranylacetate and lead citrate and were viewed using aphilips EM300 electron microscope.

RESULTS

Influence of *A. sativum* (garlic oil extract) on the mortality of 4th instar *C. pipiens* (L.): Fourth instar *C. pipiens* (L.) exposed to three different doses (3, 5 and 10 $\mu\text{l}/25$ L.) of garlic oil extracts showed variable mortality rates during different exposure times. The results are

illustrated in Table 1, Fig. 2. Full mortality rate was reached after 108 hrs at dose of 5 $\mu\text{l}/25$ larvae. Also, 100 % mortality was achieved after a shorter exposure time reached 96 hrs at dose of 10 $\mu\text{l}/25$ L. Results showed lethal or toxic effect following exposure of 4th instar *C. pipiens* (L.) to different concentration (3, 5 and 10 $\mu\text{l}/25$ L.) to *A. sativum* (garlic oil extract). The mortalities of 4th instars *C. pipiens* (L.) were recorded as 100% after variable exposure times. It was found that this exposure times were decreased with increasing the concentration of the garlic oil. It reached 108 and 96 hrs at concentration of 5 and 10 $\mu\text{l}/25$ L, respectively. Generally, plant oil extracts of *A. sativum* induce LD_{50} 121.4, at the three inoculum doses.

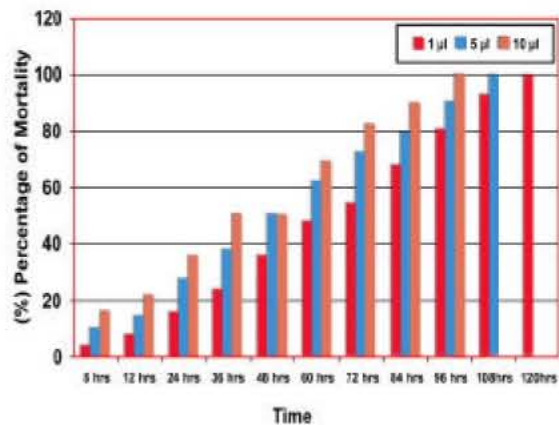
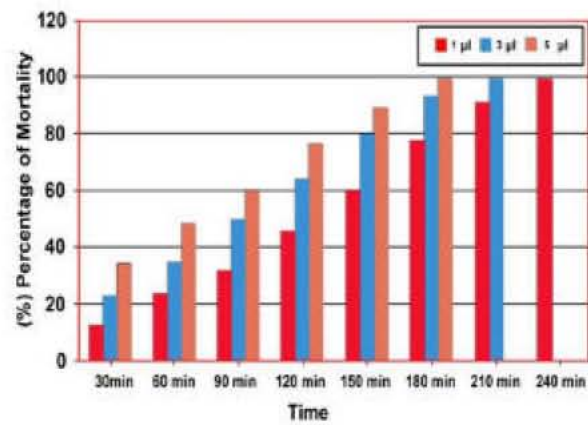
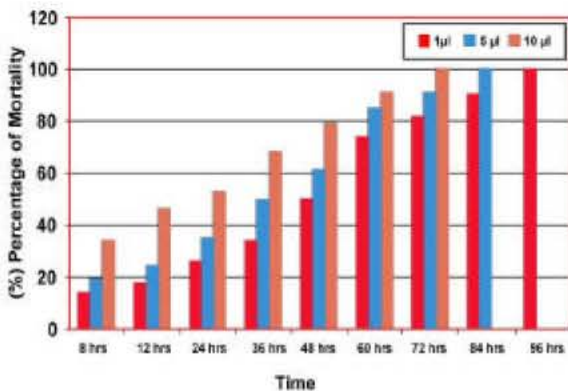
Influence of *C. limon* (lemon oil extract) on the mortality of 4th instar *C. pipiens* (L.): The mortality rates were also affected by both the dose of inoculum and the exposure time (Table 2, Fig. 3). The highest mortality rates (100%) were observed after exposure time of 72 hrs and inoculum dose of 10 $\mu\text{l}/25$ L. The results indicated that 4th instar larvae exposed to variable exposure time gave variable mortality rates at different inoculum doses of 3, 5 and 10 $\mu\text{l}/25$ L. It was recorded that the mortality rates reached 14-100% at 96 hrs. post-exposure. Collectively, results showed a positive relationship between the time exposure of 4th instar larvae to lemon oil extract and the mortality rate of this larva. The control 4th instar larvae survived for 168 hrs. or turned to pupae. Generally, lemon oil extract recorded LD_{50} 73.14, at the three inoculum doses.

Table 1: Effect of *A. sativum* (garlic oil extract) on the fourth larval instar of *C. pipiens*

		Inoculums Dose $\mu\text{l}/25$ larvae					
		3		5		10	
Replicates	Exposure Time in hours	Dead No. (M \pm SE)	M.M. Rate%	Dead No. (M \pm SE)	M.M. Rate%	Dead No. (M \pm SE)	M.M. Rate%
1	4	0	0	0	0	0	0
2	8	2.3 \pm 0.60	4	1 \pm 0.40	10	4.6 \pm .40	16
3	12	5.3 \pm 0.70	8	1.8 \pm 0.40	15	6 \pm 0.40	22
4	24	6.2 \pm 0.10	16	3.0 \pm 0.20	28	7.9 \pm 0.3	36
5	36	7.5 \pm 0.50	24	4 \pm 0.57	38	9 \pm 0.65	50
6	48	9.5 \pm 0.60	36	6.5 \pm 0.70	50	12.5 \pm 1.0	69
7	60	12.8 \pm 0.60	48	8.6 \pm 0.40	62	16 \pm 1.30	71
8	72	14.8 \pm 1.00	54	12 \pm 1.00	73	19 \pm 1.30	82
9	84	19 \pm 0.80	72	13 \pm 0.90	80	23 \pm 0.90	90
10	96	20 \pm 0.30	81	17.8 \pm 0.6	91	24 \pm 0.40	100
11	108	22 \pm 0.70	93	23.9 \pm 0.6	100		
12	120	24 \pm 0.5	100				
LD_{50}				121.4			
p. value				0.005			

Table 2: Effect of *C. limon* oil (lemon oil extract)) on the fourth larval instar of *C. pipiens*

Replicates	Exposure Time in hours	Inoculum Dose $\mu\text{l}/25$ larvae					
		3		5		10	
		Dead No. (M \pm SE)	M.M. Rate %	Dead No. (M \pm SE)	M.M. Rate %	Dead No. (M \pm SE)	M.M. Rate %
1	4	0	0	0	0	0	0
2	8	3 \pm 0.60	14	4.6 \pm 0.80	20	10 \pm 0.70	34
3	12	4 \pm 0.30	18	6.8 \pm 0.70	25	11 \pm 0.40	46
4	24	7 \pm 0.40	26	9 \pm 0.50	36	14 \pm 0.70	60
5	36	9 \pm 0.49	34	11.6 \pm 0.60	52	17 \pm 1.00	72
6	48	13 \pm 1.00	50	14.8 \pm 0.80	69	20 \pm 0.80	85
7	60	17 \pm 1.00	73	18.8 \pm 0.30	80	24 \pm 0.60	90
8	72	21 \pm 0.70	82	22.5 \pm 0.80	92	24.5 \pm 0.16	100
9	84	24 \pm 0.10	90	24.8 \pm 0.70	100		
10	96	24 \pm 0.60	100				
LD ₅₀				73.14			
p. value				0.003			

Fig. 2: Mortality rate of 4th instar *C. pipiens* (L.) after exposure to *A. sativum* (garlic oil extract).Fig. 4: Mortality rate of 4th instar *C. pipiens* (L.) after exposure to *B. thuringiensis israelensis* (Bti).Fig. 3: Mortality rate of 4th instar *C. pipiens* (L.) after exposure to *C. limon* oil (lemon oil extract)

Influence of entomopathogenic bacteria (*B. thuringiensis israelensis* Bti) on the mortality of 4th instar *C. pipiens* (L.): Effect of *B. thuringiensis israelensis* on 4th instar *C. pipiens* (L.) (Table 3, Fig. 4) showed that the mortality rates were also affected with both exposure time and inoculum dose. It ranged from 12-100, 23-100 and 34-100% at inoculum level 1, 3 and 5 $\mu\text{l}/25$ L, respectively. Results also demonstrated that the mortality rates increased as either the exposure time or inoculum dose increased. The highest mortality rate was reached 100% at inoculum doses of 1, 3 and 5 $\mu\text{l}/25$ L. after exposure time reached 240, 210 and 180 minutes, respectively. The most rapid mortality rates were recorded after exposure to lemon oil and Bti bacteria. *C. pipiens* mortality

Table3: Effect of *B. thuringiensis israelensis* (Bti) on the fourth larval instar of *C. pipiens*

		Inoculums Dose μ l/25 larvae					
		1		3		5	
Replicates	Exposure Time in minutes	Dead No. (M \pm SE)	M.M. Rate %	Dead No. (M \pm SE)	M.M. Rate %	Dead No. (M \pm SE)	M.M. Rate %
1	25	0	0	0	0	0	0
2	30	1 \pm 0.49	12	5.3 \pm 0.30	23	9 \pm 0.50	34
3	60	4 \pm 0.47	24	8.3 \pm 0.60	35	13 \pm 0.61	49
4	90	6.8 \pm 0.60	32	11 \pm 0.70	50	16 \pm 1.00	60
5	120	10 \pm 0.74	46	15 \pm 0.73	69	18 \pm 0.80	79
6	150	13 \pm 0.88	60	19.6 \pm 0.60	80	22 \pm 1.00	90
7	180	17 \pm 0.84	78	23 \pm 0.68	92	24 \pm 0.90	100
8	210	20 \pm 0.54	91	25 \pm 0.0	100		
9	240	24 \pm 0.40	100				
LD ₅₀				69.2			
p. value				0.000			

Each replicate contains 25 larvae.

M.: Mean.

M.M.: Mean mortality.

There was a significant influence of time on mortality rate ($P < 0.05$).

increased for all biological controls with an increase in exposure time to 120 hrs. Substantial differences were observed in the effect of exposure time upon *C. pipiens* larvae mortality between plant oil extracts and entomopathogenic bacteria (Bti).

Histopathological findings of control non-treated 4th instar *C. pipiens* (L.): The histological change of midgut, fat body, cuticle and muscular tissues of non-treated controlled 4th instar *C. pipiens* (L.) were illustrated in Fig. 5 a, b, c. The midgut of the larvae is considered the principle regions of absorption and digestion. Its tissue appeared typically columnar cells with dense granulated cytoplasm and obvious nuclei (Fig. 5a). The midgut was also lined with a characteristic infolded peritrophic membrane in which the mitochondria were appeared. The midguts are associated longitudinal and the circular muscle layers are usually associated with the alimentary canal. These layers cause rhythmical peristaltic movement by which the food moves through the alimentary canal. The fat tissues appeared as compact masses with normal sheath, distinct nuclei and filled with fat droplets (Fig. 5b). The muscular tissue is composed of striated fiber. These stations appeared to be difficult to detect. Each muscle fiber is consists of a number of parallel fiberillae sarcostyles. The nuclei are arranged immediately beneath the sarcolemma. The circular muscles fibers constitute principle layer of muscles, which it closed to the basement membrane of the epithelia cells (Fig. 5c). The longitudinal fibers together with the circular ones are appeared few and widely spread.

Histopathological Findings of Treated 4th Instar *C. pipiens*

(L.): Effect of *A. sativum* (garlic oil extract): It was shown that the midgut of the larvae was the most damaged region. Obvious and diverse effect was noticed at 8 hrs post-treatment. The cells of midgut began to detach from each other together with hardly detaching chromatin material with the nucleus and no definite cells shape at 12-24 hrs. (Fig. 6a). Lyses of cuticle layer with large vacuole fragmentation were found (Fig. 6b). After 48 hrs exposure to *A. sativum*, maximal damaged effect was clear with disappearance of gut epithelial layer (Fig. 6d). Moreover, fat body and muscle fibers loose their impact form and the cells appeared scattered without oily droplets or cell contents (Fig. 6c).

Effect of *C. limon* (Lemon Oil Extract): The greatest histopathological effect of *C. limon* oil extract was found in midgut region, fat tissue and muscles of the larvae. At 8 hrs post-treatment, the gut region appeared damaged. This damage found in the apical portion of the gut, whereas the columnar cell appeared swollen with sometimes distinct protrusion into gut lumen (Fig. 7a). After 24 hrs post-treatment, the epithelial gut cells started to elongate and separate. However, at 48 hrs post-treatment, the muscle fibers and fat bodies were actually completely damaged (Fig. 7d). The cytoplasm was appeared vacuolated with enlarged nuclei of gut cells (Fig. 7a). The cells were found to dislodge, slough and detach from each other with separation of cuticle layers (Fig. 7d). Fused cell mass of undifferentiated epithelial

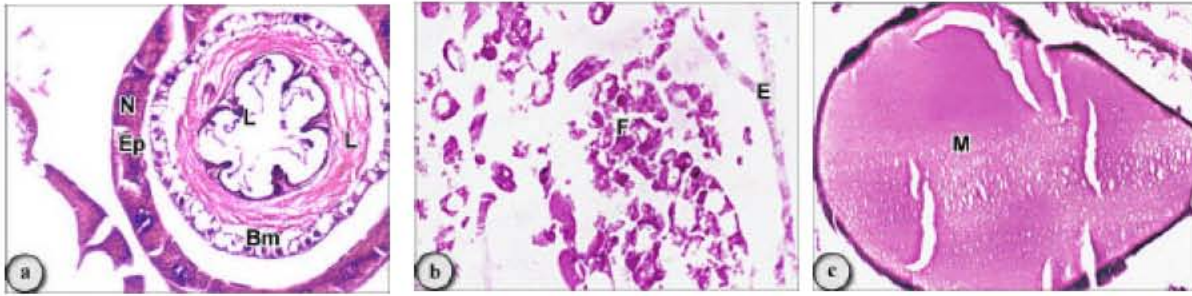


Fig. 5a-c: Histopathological findings of midgut region of control un-treated 4th instar *C. pipiens* (L.) (H and E, 400X) showing: basement membrane (Bm), epithelial cells (Ep), lumen (L), cuticle (C), fat body (F), muscle fibre (m).

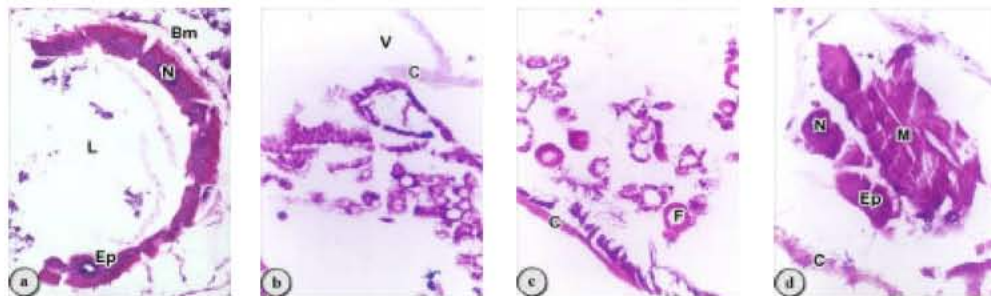


Fig. 6a-d: Histopathological findings of 4th instar *C. pipiens* (L.) midgut region treated with 10 µl *A. sativum* oil (H and E, 400X) showing: [a] At 8 hrs. post-exposure; [b] At 12 hrs. post-exposure; [c] At 24 hrs. post-exposure and [d] At 48 hrs. post-exposure.

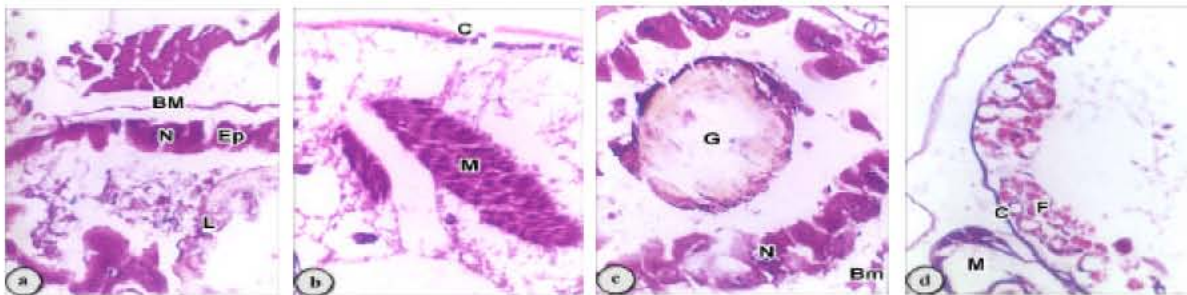


Fig. 7a-d: Histopathological findings of 4th instar *C. pipiens* (L.) midgut region treated with 10 µl *C. limon* oil (H and E, 400x) showing: [a] At 8 hrs. post-exposure; [b] At 12 hrs. post-exposure; [c] At 24 hrs. post-exposure and [d] At 48 hrs. post-exposure.

cells was found with appearance of undigested food particles in gut lumen (Fig. 7c). At 12 hrs post-treatment, the muscle fibers were damaged and some extend (Fig. 7b).

Effect of Entomopathogenic Bacteria *B. thuringiensis israelensis* (Bti): It was found that the first site of infection was observed in the midgut at 30 min. Post-treatment (Fig. 8a). The epithelial showed separated and undifferentiated masses. At 90 min. post-treatment, the basement membrane of the epithelial cells was

slightly detached (Fig. 8b). Moreover, at 120 min. post-treatment, the basement membranes were become highly vacuolated and almost disintegrated (Fig. 8c). The effect of *B. thuringiensis israelensis* was clearly obvious at 150 min. post-treatment. The infected larvae showed clear fissures and partial degeneration of muscles. These muscles showed to loose their typical striation and became shorter (Fig. 8d). The fat tissues showed clear shrinkage and the wall of cytoplasm became irregular and completely separated from each other (Fig. 8d).

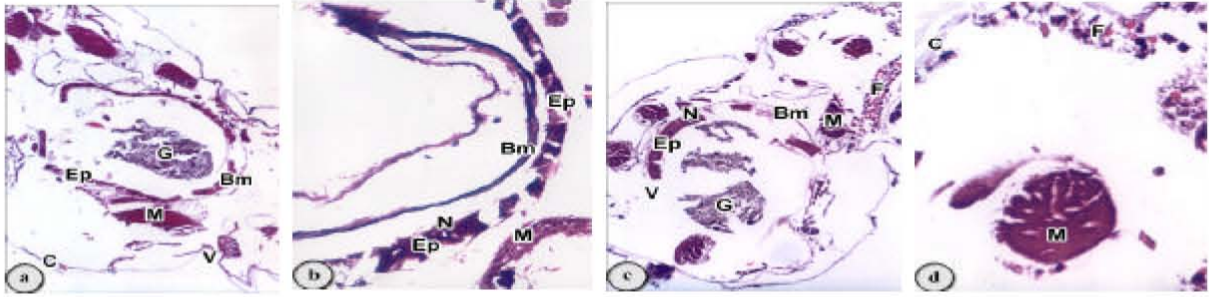


Fig. 8a-d: Histopathological findings of midgut region of 4th instar *C. pipiens* (L.) treated with 5 µl *B. thuringiensis israelensis* (Bti) (H and E) showing: [a] At 30 min. post-exposure (100 x); [b] At 90 min. post-exposure (400x); [c] At 120 min. post-exposure (100 x) and [d] At 150 min. post-exposure (400x).

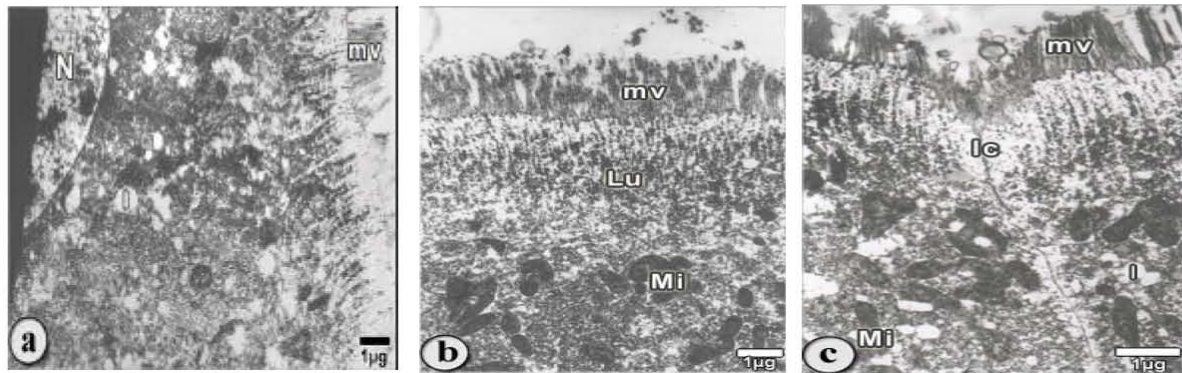


Fig. 9: Ultrastructure findings (TEM) of control un-treated larvae midgut of 4th instar *C. pipiens* (L.) showing: [a] micro-villi (mv), nucleus (N), lipid like granules (I); [b] electron dense vesicles and mitochondria (mi), micro-villi (mv) and [c] intercellular junction (Ic), micro-villi (mv) and mitochondria (mi).

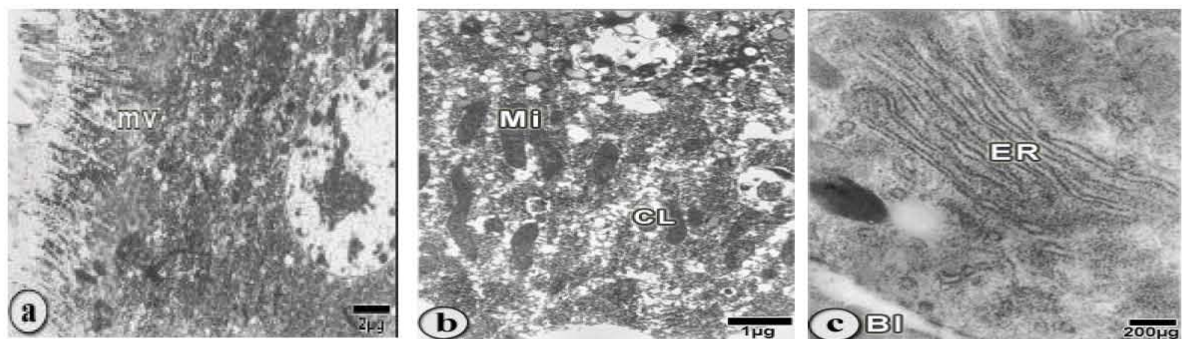


Fig. 10: Ultrastructure findings (TEM) of control-un treated larvae midgut of 4th instar *C. pipiens* (L.) showing: [a] Basal part of cell with nucleus (N), Lipid like droplets (I), cytolysosomes (CL), [b] Numerous mitochondria (mi), cytolysosomes (CL) and [c] portion of a midgut cell with staked rough endoplasmic reticulum (ER) and basal labyrinth (Bl).

Ultrastructure Findings (TEM) of the Midgut of 4th Instar *C. pipiens* (L.)

TEM of Control Non-treated Larvae Midgut: TEM of normal healthy insect gut showed a great diversity from an insect to another. However, basic similarities for digestion and pathogen transmission were almost found

in the midgut. It was also found that the midgut was lined with peritrophic membrane through which the ingested particles pass. The anterior midgut (Fig. 9 a, b) was found to be provided with numerous microvilli with complex infolding of basal plasma membrane or basal labyrinth. This labyrinth was deeply extended into the cell

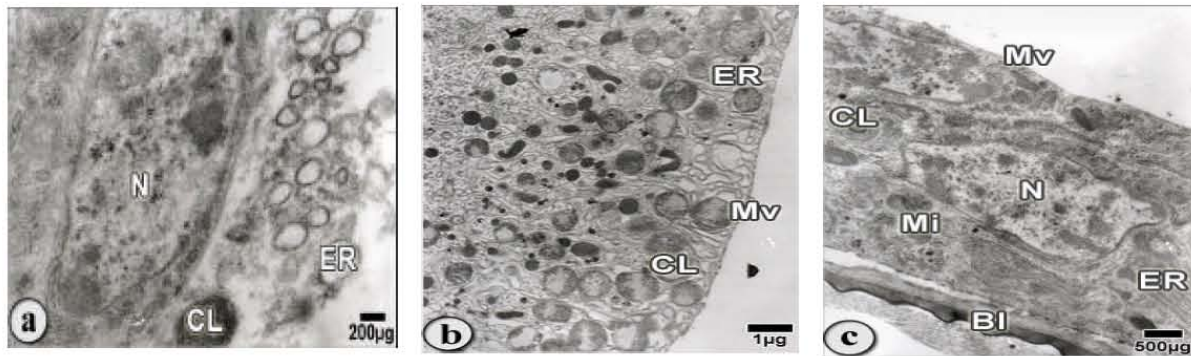


Fig. 11: TEM of larvae midgut of 4th instar *C. pipiens* (L.) at 48 hrs. post-exposure to *C. limon* oil showing: [a] Nucleus (N) dilation of endoplasmic reticulum (ER) and cytolysosomes (CL); [b] Irrigular degenerating microvilli (mv), endoplasmic reticulum (ER) and cytolysosomes (CL) and [c] Increasing in number and size of cytolysosomes (CL) basal labyrinth (Bl), mitochondria (mi), microvilli (mv) and nucleus (N).

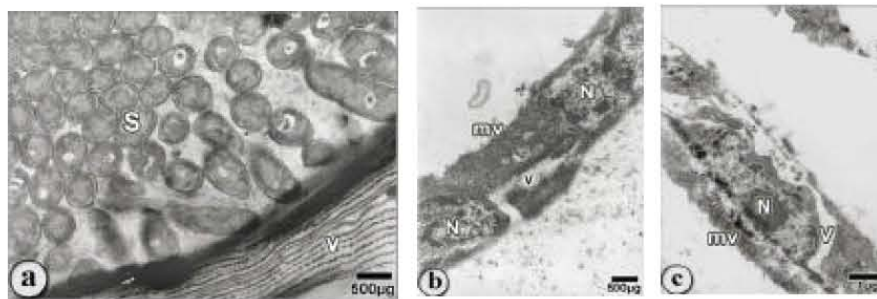


Fig. 12: TEM of larvae midgut of 4th instar *C. pipiens* (L.) at 150 min. post-exposure to *B. thuringiensis israelensis* (Bti) showing: [a] spore core (s) and stomodeal valve of midgut (v); [b] nucleus (N), microvilli (mv) and vacuole (V) and [c] lysis of microvilli (mv) and degenerated nucleus (N) together with vacuolization (V).

(Fig. 9a). Moreover, it was found that there were intercellular junction closely jointed throughout the all midgut length (Fig. 9c). In addition, small bounded bodies were seen containing whorls of lamellae and dense granules as cytolysosomes (Fig. 9a and 10a). The cytoplasmic organelles were seen with larger rough endoplasmic reticulum (Fig. 10c) in which the ribosome may be localized. These ribosomes were appeared in sections as stacks or sheets (Fig. 10c). Mitochondria were observed numerous in gut cells with special features in both its structures and distributions. The Golgi apparatus, if present, was unimpressive and poorly developed. Large lipid-like granule accumulations were observed deposited around the nucleus (Fig. 10a).

TEM of Larvae Midgut of *C. pipiens* Treated with Plant Oil Extract (Lemon Oil): TEM of midgut treated with *C. limon* oil showed a higher sequential degenerative pattern at 48 hrs post-exposure (Fig. 11) with appearance clear cells lysis of midgut. Moreover, severe pathological lesions including apical swelling and degeneration of the

nucleus were recorded. It was found also there were degeneration of cytoplasmic organelles together with dilatation of mitochondria and endoplasmic reticulum (Fig. 11a). The mitochondria were showed with strong electron-dense matrices and the cell cytoplasm had patches of endoplasmic reticulum. However, less advanced cytoplasm degeneration was observed in the basal labyrinth, if compared with the rest of the cell (Fig. 11c). In addition, the cytoplasm in the midgut showed larger and more numerous cytolysosomes (Fig. 11b).

TEM of Larvae Midgut of *C. pipiens* Treated with *B. thuringiensis israelensis* (Bti): TEM showed that the midgut was the most damaged region. It recorded obvious and diverse responses to entomopathogenic bacteria *B. thuringiensis israelensis* at 150 min. post-exposure. Numerous entomopathogenic bacteria were seen located in stomodeal valve and anterior midgut (Fig. 12a). These bacteria were found to induce bores in general cell wall profile forming a thick outer layer, unstained intermediate layer and a thin inner layer. Most *B. thuringiensis* showed

to have dense cytoplasmic ground substance with a few prominent nuclear region (Fig. 12a). No corresponding bacteria were seen in the control non-treated larvae. Also, TEM showed that the midgut contents were shown to throw into zigzag folds (Fig. 12a-c). It was found also there were lysis of microvillus and degeneration of nucleus together with vacuolization. The midgut cells were seen separated from itself and loss of microvilli (Fig. 12c).

DISCUSSION

C. pipiens complex mosquitoes are important vectors of animal and human diseases including filariasis, encephalitis and various other viruses. Their control over the past fifty years has been through the use of chemical insecticides. However, the development of resistance to all the synthetic compounds used against them has reduced the efficiency of insecticide treatments. Several studies have looked at the possibility of using plant extract as larvicides to control *C. pipiens* [28-31]. This study found that the two used plant oil extracts *A. sativum* and *C. limon* as well as entomopathogenic bacteria (Bti) had larvicidal effect on the 4th instar of *C. pipiens* (L.). The more highly effect were obtained by entomopathogenic bacteria Bti ($LD_{50}=69.2$) followed by *C. limon* ($LD_{50}=73.17$) and lastly *A. sativum* ($LD_{50}=121.4$). The obtained results demonstrated that the mortality rates increased as the concentration of the plant extract increased. Similar results were previously obtained [20, 32, 33]. These authors reported that *Citrus* oils and *B. thuringiensis* H-14 caused serious latent effect to adults and larval stages of *C. pipiens* and *M. domestica*. Moreover, this deleterious effect was particularly obvious in lemon oil treatments. Aromatic plants and their essential oils are very important sources of many compounds that are used in different respects. The oil of 41 plants were evaluated for their effects against 3 instar larvae of *A. aegypti*, *An. stephenis* and *C. quinquefasciatus* [34]. Thirteen oils from 41 plants (camphor, thyme, amyris, lemon, cedar wood, Frankincense, dill, myrtle, juniper, black pepper, verbena, helichrys and sandalwood) induced 100% mortality after 24 hrs, or even after shorter periods [34].

Govindarajan *et al.* [40] found that methanol leaf extract of *Cassia fistula* was lethal to the larvae of *C. quinquefasciatus* and *An. stephenis* with LD_{50} values of 17.97 and 20.57 mg /L, respectively. Otherwise, our results revealed that *C. limon* extract was more toxic than *A. sativum* extract. In this work, it was found that the

mortality rates did not increase over time and the lower doses of garlic oil did not kill all larvae. In this respect, Thomas and Callaghan [23] used lemon peel and garlic extracts as a larvicidal activity against *C. pipiens* (L.). They reported that both garlic and lemon were toxic to mosquito. Garlic was more persistent than lemon with no significant difference in killing between fresh and ~4.5-day-old treatments. Habeeb *et al.* [41,42] found that the toxicity of the *C. Limon* was attributed to the main compounds of the essential oil dL-Limonene (46.99%) and Myrcenol (21.85%) against the ectoparasite camel tick *Hyalomma dromedarii*. On the other hand, Chungsamarnayrt and Jansawan [43] stated that the peel oils of *Citrus reticulata* and *C. maxima* showed higher larvicide activities two times than of limonene. In addition, Rajan *et al.* [44] described a trial designed to determine if a short-term ingestion of garlic would make individuals less attractive as targets for mosquitoes (*A. aegypti*). They found that chronic ingestion of garlic powder rather than raw garlic might have a different effect on mosquito feeding. In the recent times, bioprospecting for plants that show bioactive properties has yielded many chemicals that can be used in controlling mosquitoes. Crude extracts of 4 terrestrial and 3 mangrove plants were assayed against 2nd-3th larval instar of *A. aegypti* [45] among the plants tested, *Cordia curassavica* showed the highest level of activity for all the extract tested and the *Azadirachta indica* showed the least activity. Mosquitoes in the larval stage are attractive targets for pesticides because mosquitoes breed in water and thus, it is easy to deal with them in this habitat. The use of conventional pesticides in the water source, however, introduces many risks to people and the environment. Natural pesticides, especially those derived from plants, are more promising in this aspect. Aromatic plants and their essential oils are very important sources of many compounds that are used in different respects. The oils of 41 plants were evaluated for their effects against 3 instar larvae of *A. aegypti*, *An. stephenis* and *C. quinquefasciatus* [34]. Thirteen oils from 41 plants (camphor, thyme, amyris, lemon, cedar wood, Frankincense, dill, myrtle, juniper, black pepper, verbena, helichrys and sandalwood) induced 100% mortality after 24 hr or even after shorter periods [34].

The idea of the possibility of including toxins other than α -endotoxins in the toxicity of *B. thuringiensis* H-14 to mosquito larvae was derived from guessing experiments which showed that adults developed from larvae survived after treatment with this bacterium were influenced particularly in their egg fecundity which is a phenomenon known to be due to the α -exotoxins

[13, 20, 35-38]. In the present study, high larvae mortality at comparatively low concentration of Bti bacteria indicates high susceptibility of *C. pipiens* larvae to a particular bacteria strain. Our findings further imply that the high mortality rate may be related not only to the concentration of Bti bacteria but also to other factors. The mode of action of *B. thuringiensis* on lepidopterous larvae has been under study, following, the appearance of pioneering works carried out by Heimpel and Angus [39]. These authors gave a through account of the general symptoms of intoxication as well as changes in blood pH and midgut histopathology that follow ingestion of the bacteria by a wide range of lepidopterous larvae. Since the discovery of *B. thuringiensis israelensis* de Barjac (Bti) and efficacious isolates of *B. sphaericus* Neide, formulation of these bacteria have become the predominant non-chemical means employed for control of mosquitoes larvae at several locations [17]. The efficacy of Bti formulations has been demonstrated in a variety of habitats against a multitude of species of mosquitoes. Moreover, *B. sphaericus* formulations have been utilized predominantly in organically enriched habitats against *Culex* species, but they are also active in a variety of habitats having low organic enrichment against numerous species and across several genera. Due to their efficacy and relative specificity, both Bti and *B. sphaericus* can be ideal control agents in integrated biological control agent, environmental, personal protection and the judicious uses of insecticides are combined.

Histopathological studies on infected 4th instar of *C. pipiens* (L.) demonstrated various and progressive destructions to different target tissue of infected larvae. The tissues mostly affected are the midgut, muscles, fat bodies and the cuticle. The intensity of the damage caused to each of this tissue was depended in the first place upon the nature of the tissue and the period after infection. Based on previous histopathological studies carried out by several investigators, the midgut region of the infected larvae is the first site where cellular response is observed [46]. The present study demonstrated that the 4th instar *C. pipiens* (L.) treated with plant oil extracts (*A. sativum*, *C. limon*) and Bti showed a highly damaged midgut, fat body, muscles. The lesions were increased by increasing the time of exposure. A significant differences were obviously noticed between oil extracts and Bti. Moreover, there was a significant difference between effect of *A. sativum* and *C. limon* oil extracts. These results are in agreement with previous studies [46-48] on this insect. The authors found that exposure of *C. pipiens* (L.) to oil extracts reflected great damage in the midgut

and the epithelial layer which appeared vacuolated, swollen cells, appearance of mass of cellular material in the midgut and finally the epithelium cells lost their normal appearance. Similarly, Massoud and Labib [49] mentioned that the oleo-resin oil of myrrh (*Commiphora molmo*) induced histopathological deformities with the different tissues of *C. pipiens*. The gut apical portion of columnar cells was swollen and sometimes, distinct elongation protruded into its lumen as a bulbous aversion. Sometimes, the apical part of columnar cells appeared empty. In completely paralyzed larvae, the tissues showed vacuolated cytoplasm with enlarged nuclei of gut cells. The cells were dislodged, sloughed and detached from each other, the fat tissue lost its compact form, cells appeared scattered, sometimes without the oil droplets or cell contents and complete disintegration of that tissue in most treated sections. The present work contributes farther to the histopathological changes produced by *B. thuringiensis* in the midgut tissues of the 4th instar of *C. pipiens* (L.). Histopathological changes were seen in the midgut including separation of the epithelial cells from the basement membrane. Columnar and goblet cells of the midgut were also observed in the epithelial cells. Mitochondria were more condensed and endoplasmic reticulum appeared swollen with dilated cisterns. The most obvious ultracellular changes noted in the midgut epithelium of *C. pipiens* (L.) were reduction of microvilli. This giving the midgut a vacuolar appearance. This statement coincides with the observations of Labib and Dawaud [21] and Charless *et al.* [50]. In this aspect, Clark *et al.* [22] described the midgut of *A. aegypti* (L.) by using scanning electron microscopy. The anterior ventriculus (stomach) region is found to have much lower mitochondrial densities than other midgut region. The transitional region is distinguished by apical surface architecture and by region-specific effects of cry Bti IVB in which, toxin causes holes ranging from 1.0 to 7.0 μ in diameters. On the other hand, Davidson [27] found that no striking histopathological changes were observed in the larvae midgut cells of *C. pipiens* (L.) after several hours feeding on *B. sphaericus* strain ssII-I (belonging to the h2a, 2b serotype, toxic at the vegetative stage). In the same way, *C. pipiens* larvae fed on *B. sphaericus* spores of strain 1593 (belonging to the H5a, 5b serotype) showed only slight separation of the midgut cells at the intercellular junctions, during the first hours of the intoxication [51]. However, Charless and de Barjac [52] found that a very early cytological alteration occurred in *C. pipiens* larvae fed on *B. sphaericus* and *B. faiyoun*, but without significant dilatation of latero-basal intercellular spaces.

It was concluded that the plant oil extract (*A. sativum*, *C. limon*) and Bti proved to be a promising controlling agent *C. pipiens* larvae. Attention needs to focus on the potential of these tools under field conditions.

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