Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 28(5): 1555 – 1572 (2024)
www.ejabf.journals.ekb.eg

Enzymatic and Ultrastructural Effects of Peels Nanoemulsion *Citrus aurantifolia* Essential Oil on Larvae of *Culex pipiens* L. (Diptera: Culicidae)

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ARTICLE INFO
Article History:
Received: July 2, 2024
Accepted: Sept. 17, 2024
Online: Oct. 8, 2024

Keywords:
Citrus aurantifolia,
Larvicide,
Essential oil,
Nanoemulsion,
Enzymes,
Histopathological effect

ABSTRACT

The effectiveness of lime essential oil (EO) from *Citrus aurantifolia* as a larvicide was investigated on third-instar larvae of *Culex pipiens* mosquitoes, with an LC50 value of 35.4ppm. The larvae exhibited morphological aberrations, including hardened, darkened, and shrunken anal papillae, as well as darkening of the siphon. After treatment, there was a reduction in total body protein (15.3 ± 0.21), along with an increase in the levels of acid phosphatase and alkaline phosphatase (4.67 ± 0.12 and 36.03 ± 0.79, respectively). Conversely, the activity of acetylcholinesterase and glutathione S-transferase decreased (13.07 ± 0.27 and 312.67 ± 7.22, respectively). The treated larvae also showed significant histopathological changes in the cuticle and midgut tissues. These findings indicate that a nanoemulsion of lime EO from *Citrus aurantifolia* is a promising natural larvicide that is biodegradable, environmentally friendly, and effective for controlling *Culex pipiens*, a vector of disease.

INTRODUCTION

Culex mosquitoes are vectors of numerous arboviruses including the encephalitis, West Nile, and Rift Valley fever viruses **(Murugan K. *et al.*, 2012)**. In Egypt, *Cx. Pipiens* Linnaeus, 1758 has been investigated as a vector of several diseases, like Filariasis, which was recorded from all governorates of Egypt **(Ammar *et al.*, 2012; El-Zayat *et al.*, 2018)**. The control of mosquitoes using synthetic insecticides has led to the development of varying levels of resistance, making it increasingly difficult to manage mosquito populations due to the rise of resistant strains **(Bigoga *et al.* 2013)**. Moreover, such chemicals are non-biodegradable and cause a decline in soil fertility, water pollution, and toxicity to non-target organisms and humans. The disadvantages of using synthetic larvicides created a need to develop new efficient, biodegradable, and target-specific larvicides. The effectiveness of plant extracts was investigated against mosquito larvae to face this obstacle of resistance **(Bigoga *et al.*, 2013; Gonza´lez *et al.*, 2015; ; Azmy *et al.*, 2021; Khan *et al.*, 2021)**. Essential oils are gaining attention because of

their safety, being easily extractable, eco-friendly, biodegradable, with low or no toxicity against vertebrates and mammals **(Khater *et al.* 2015)**, and their most chemical components are more effective against countless insect species **(Wagan *et al*., 2018)**. Moreover, EOs reduce the ecologically damaging effects of synthetic insecticides and are regarded as a new class of ecological products for insect pest management **(Regnault-Roger, 1997; Sendi & Ebadollahi, 2013)**. Larvicides based on EOs are presented as alternatives at breeding sites **(Ghosh *et al.* 2012; Keyal *et al.*, 2016)**. Penetration through the body cuticle of larvae is crucial for insecticidal activity and one of the mechanisms of insecticides **(Kasai *et al*. 2014)**. Essential oils should be formulated to enhance poor water solubility oils, prevent the vaporization of volatile compounds, and preserve biological activity **(Bakkali *et al*., 2008; Goshen & Magdassi, 2009; Osanlo *et al*., 2017)**. Nanoemulsions of EOs are submicron emulsions with droplet sizes lower than 100nm **(McClements, 2011)**; such formulation enhances the solubility of poorly water-soluble oils **(Magdassi *et al,* 2013)**. Several nanoemulsions of EOs have been reported as larvicides such as the nanoemulsions of eucalyptus, rosemary, orange, and clover **(Sugumar *et al*., 2014; Duarte *et al*., 2015; Azmy *et al.*, 2019; El Gohary *et al*., 2021)**. The present study aimed to assess the larvicidal efficiency of a nanoemulsion of *C. aurantifolia* EO via the ultrasonic emulsification method against third-instar larvae of *Cx. pipiens*, comparing it with a conventional formulation method.

MATERIALS AND METHODS

1. Maintenance of insect colony

Cx. pipiens larvae were collected from Abu Rwash region, Giza Governorate. The larvae were reared in the Research and Training Center on Vectors of Diseases (RTC), Ain Shams University, under optimum conditions, including humidity of 75 ± 5%, temperature of 25 ± 2°C, and a photoperiod of 16:8 hours light/dark; the bioassay was performed using the third generation. The adult was given a 10% sucrose solution, and the females were fed a blood meal from a pigeon host **(Kasap & Demirhan, 1992)**.

2. Extraction of oil and nanoemulsion preparation

Essential oil extraction from peels of *Citrus aurantifolia* was subjected to hydro-distillation for 3 hours **(Azmy *et al*., 2019)**. The bulk emulsion EO was prepared using surfactant (Tween 20) and distilled H_2O according to the method of **Duarte *et al.* (2015)**. The bulk emulsion was subjected to an ultrasonicator in Central Laboratory, Faculty of Science, Ain Shams University (Ultrasonics, USA/ digital ultrasonic cleaner cd 4830), frequency 30 kHz for 30 minutes according to the method of **Anjali *et al.* (2010)** and **Azmy *et al.* (2019)**. Droplet size and polydispersity index were measured by using a particle size analyzer (Malvern-UK, 4700) according to **Sugumar *et al.* (2014)** at the Egyptian Petroleum Research Institute. The viscosity of the nanoemulsion was measured

by Ostwald viscometer at a temperature of 25± 0.5ºC. Moroevoer, experiments were performed in triplicate according to **Abbas *et al.* (2010)**. The stability of the nano-emulsion was checked by storing at 25 and 4ºC for a month. Additionally, the nano-emulsion was subjected to centrifugation at 10,000rpm for 30min and then was observed for any creaming or cracking according to the guidelines of **Ghosh *et al.* (2013)**.

3. Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis was conducted for the EOs of *Citrus aurantifolia* in the Central Lab, Faculty of Science, Ain Shams University using a Shimadzu GC-MS-QP 2015 plus (Kyoto, Japan), by injecting 0.5µl of the EO in Hewlett Packard chromatograph model 597, provided with flame ionization detector (FID) and 50cm HP capillary column. The carrier gas was helium, and the gas flow rate was 1ml/ minute. Diluted samples (1v/ v) were injected with a split ratio of 15:1, and the injected volume was 10µl. The quantities of the components were detected by comparing the area of the resulting peak with the data from WILEY/ NIST and Tutor Libraries **(Beckley *et al*. 2014)**.

4. Biochemical activity

Tissue samples of the treated third instar larvae with LC50 of *C. aurantifolia* EOs (nanoemulsions, 35.4ppm and EOs dissolved in the absolute ethanol 60.4ppm (1/100 v/v)) were recorded according to **Azmy *et al.* (2019)**. Treated and untreated samples were homogenized (gram/ml) after 48 hours of treatment using saline solution of 0.9% in a chilled grinder for 3 minutes. Homogenates were centrifuged at 14000rpm for 15 minutes at -2°C in a refrigerated centrifuge. The supernatant was stored at -5°C until use for investigations. Three replicates were carried out for each test. The total protein content was investigated by Folin- Cicocalteu method **(Lowry *et al.*, 1951)**. Acid phosphatase activity was measured in untreated and treated tissue samples according to **Laufer and Schin (1971)**. The same method of acid phosphatase activity was applied for the alkaline phosphatase activity, but instead of the acid buffer, an alkaline buffer was used. The activity of both enzymes was measured by a spectrophotometer at 400nm. The activity of acetylcholinesterase (AChE) was measured at 515nm according to the method described by **Simpson *et al*. (1964)**. Acetylcholine bromide (AChBr) was used as a substrate at a level of 6×10^{-3} M. Glutathione S-transferase activity (GST) was determined in tissue samples according to the method described by **Habig *et al*. (1974)**. Additionally, the increment in absorbance at 340nm was recorded.

5. Morphological investigations

After 24 hours, the morphological changes of larvae treated with the EO nanoemulsion were reported and compared to untreated larvae. A Leica EZ4HD with an integrated three mega-pixel CMOS camera was used to make morphological observations **(Perumalsamy *et al.*, 2013)**.

6. Histological and ultrastructural studies

The investigation was carried out at the Regional Center of Mycology and Biotechnology (RCMB), Al-Azhar University. Transmission electron microscope (TEM) JEM1011 was used. Control and treated samples with LC25 of the third instar larvae after 48 hours were subjected to ultrastructural studies according to **Disbrey and Rack (1970)**. The samples were fixed in 5% glutaraldehyde and were then washed in 70% alcohol **(Disbrey & Rack, 1970)**. Ultra-thin sections (50-80nm thick) were cut by using glass knives. Sections were stained with uranyl acetate stain followed by lead citrate **(Reynolds, 1963)**. The sections were stained for 15 minutes in the dark. Furthermore, the stained sections were then washed with water and left on filter paper. The solution of lead citrate was centrifuged at 5000rpm for 15 minutes; drops of the supernatant were transferred to the wax. A single grid was floated on a drop of the stain, allowing the sections to be exposed to the stain for 15 minutes. The stained sections were then washed with 0.02N NaOH, followed by water, and left on filter paper before examination.

7. Statistical analysis

The biological data were reported as mean ± SE. Student t-test was used to compare the data of control and treated groups. Data between treated groups were analyzed using SPSS 22 software, and the level of significance was tested using the one-way analysis of variance (ANOVA).

RESULTS

1. Extraction of the EOs, and gas chromatography-mass spectrometry (GC-MS)

The essential oil (EO) extracted from *C. aurantifolia* was transparent with a slight greenish tint and had the characteristic aromatic odor of lime. Qualitative analysis of the constituents of the extracted EO was conducted using GC-MS. The main components are summarized in Table (1). Limonene was the major constituent, comprising an average of 98.8%, while the minor components included α-pinene (average 0.21%) and β-pinene (average 0.96%).

Table 1. The major components identified in *Citrus aurantifolia* EO by using GC-MS

Peak	RT	Area % (Average rate)	Compound	Molecular formula	M.wt (gm/mol)
1	5.317	0.21	7Chlorobicyclo[4.1.0]hept-3-ene (α-Pinene)	C_7H_9Cl	128.599
2	5.844	0.96	Spiro[2.2]pentane-1-carboxylic acid, 2-cyclopropyl-2-methyl- (β-Pinene)	$C_{10}H_{14}O_2$	166.22
3	6.497	94.31	Bicyclo[6.1.0]non-1-ene Limonen	C_9H_{14}	122.211
4	6.955	4.52	1,4-Pentadiene Limonene	C_5H_8	68.119

2. Biochemical activities

The biochemical changes in the total body proteins of *Cx. pipiens* larvae after 48 hours of treatment with LC_{50} were investigated. The LC_{50} values of newly formulated nano-emulsions of the extracted EOs and the conventional method showed a significant reduction of proteins (Table 2). After 48 hours of treatment with LC_{50} of the formulated nano-emulsions of the extracted EOs and the EOs dissolved in absolute ethanol, the activities of the enzymes of *Cx. pipiens* larvae were measured (Table 3). The activity of the acid phosphatase significantly increased after the treatment by *C. aurantifolia* EO dissolved in absolute ethanol. In contrast, the treatment with the nano-emulsion of EO did not induce a significant difference. In contrast, the treatment with the nano-emulsion of EO significantly increased the activity of the alkaline phosphatase, while EO dissolved in absolute ethanol did not induce a significant difference. The obtained findings, on the other hand, indicated that the activity of acetylcholinesterase was reduced by 11% only after treatment with *C. aurantifolia* nano-emulsion compared to the activity of the enzyme in untreated larvae. With a percentage change of 6.9 and 13.4, the activity of the GST was substantially reduced after treatment with the nano-emulsion EO and EO dissolved in absolute ethanol, respectively.

Table 2. Effect of LC_{50} of *Citrus aurantifolia* on the total protein of *Cx. pipiens* larvae

Treatment	Total protein (mg/g.b.wt.) Mean ±SE
Untreated	22.7 ± 0.36 a
Nano -emulsion of *C. aurantifolia*	15.3 ± 0.21 b
C. aurantifolia EO dissolved in ethanol	16.4 ± 0.43 b

Means with the same letters are not significantly different.
Each value represents a mean of three replicates.

Table 3. Effect of LC_{50} values of different treatments of *C. aurantifolia* on the activity of acid phosphatase, alkaline phosphatase, acetylcholinesterase, and glutathione S-transferase of *Cx. pipiens* larvae

	Treatment				
Enzyme	Untreated	EO-Nano-emulsion	% of change	EO Dissolved of ethanol	% change
Acid phosphatase (mU phenol/mg protein) (Mean ±)	4.24±0.11 a	4.67±0.12 a	+ 10	5.50±0.15 b	+ 29.7
Alkaline phosphatase (mU phenol/mg protein) (Mean ±)	32.37±0.68 a	36.03±0.79 b	+ 11.3	35.40±0.91 ab	+ 9.4
Acetylcholinesterase (ugAchBr/min/mg protein) (Mean ±)	14.77±0.28 a	13.07±0.27 b	-11	14.03±0.55 ab	-5
GST(umolesub conjugated/min/mg protein) (Mean ±)	335.00±8.66 a	312.67±7.22 b	-6.9	290.00±5.77 c	-13.4

Means with the same letters are not significantly different. Each value represents a mean of three replicates.

3. Morphological observations

Compared with the control larvae, the larvae treated with the EO nanoemulsion showed morphological abnormalities. Fig. (1) shows darkened syphons and shrank anal papillae, as well as darkened and hardened anal papillae.

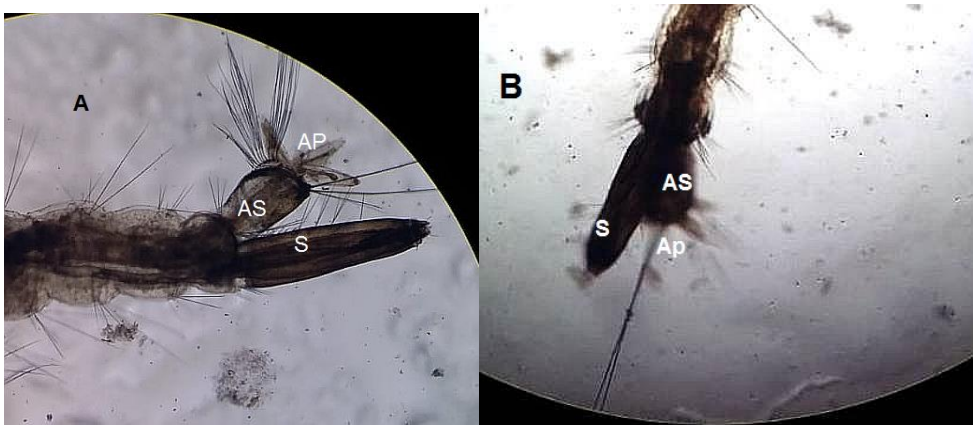

Fig. 1. Photomicrographs showed the terminal segment of the third instar larvae of *Cx. pipiens* Linnaeus, 1758 (10×): **(A)** the control larvae; **(B)** larvae treated with LC_{50} of the EO nanoemulsion of *C. aurantifolia* (L.) Osbeck. AS: anal segment; S: siphon; AP: anal papillae

4. Histological studies

Various histological changes in the normal structure of the peritrophic membrane occurred in larvae treated with *C. aurantifolia* EO nano-emulsion (Fig. 2B) compared to

the untreated larvae (Fig. 2A). It became unequal in thickness; either very thick as indicated by the solid black arrows or very thinner as indicated by the dashed black arrows. The epidermal layer also has enlarged nuclei as shown by the red arrows. The peritrophic membrane was separated in the larva treated with EO dissolved in absolute ethanol (Fig. 2C), as indicated by the black arrows, as well as many enlarged nuclei of the epidermal layer, as indicated by the red arrows. In addition, as shown by the black circles, the food callus and vacuoles were slightly smaller.

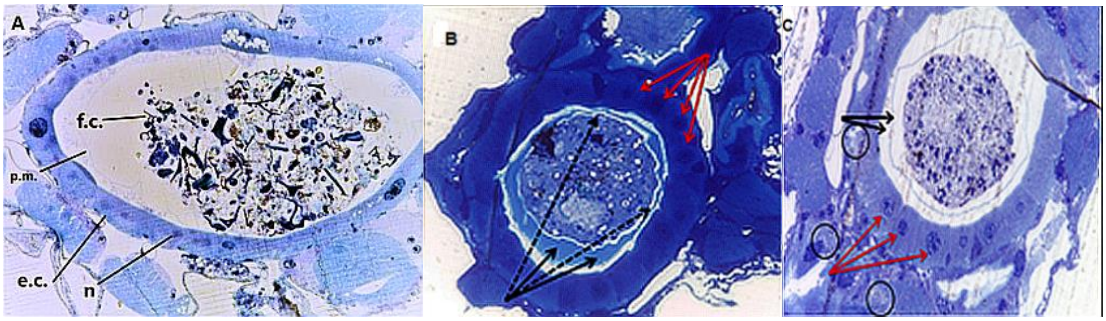

Fig. 2. Semi-thin cross-section of the third instar larva of *Cx. pipiens* Linnaeus, 1758 (x=40); **A:** Untreated, **B:** Treated with *C. aurantifolia* EO nano-emulsion, **C:** Treated with *C. aurantifolia* EO dissolved in absolute ethanol

5. Ultra-structural studies

5.1. Integument

The presence of projections or papillae bounded by the epicuticle in the cuticle of untreated third-stage larvae was observed (Fig. 3A, B). The cuticle of the larvae treated with *C. aurantifolia* EO nano-emulsion was disorganized and undifferentiated into epicuticle and endocuticle layers (Fig. 3C). The epicuticle layer became discontinuous and lost its projections or papillae. The dashed black arrow showed a separation between the epicuticle and the endocuticle; the endocuticle appeared devoid of lamellae, and some vacuoles appeared in the epidermal layer, as shown by the red circles. The epicuticle layer became discontinuous and lost its projections or papillae bounded in the larva treated with *C. aurantifolia* EO dissolved in absolute ethanol (Fig. 3E), as shown by the solid black arrows. As shown by the dashed black arrows, the endocuticle layer appeared disorganized and lost its striated structure.

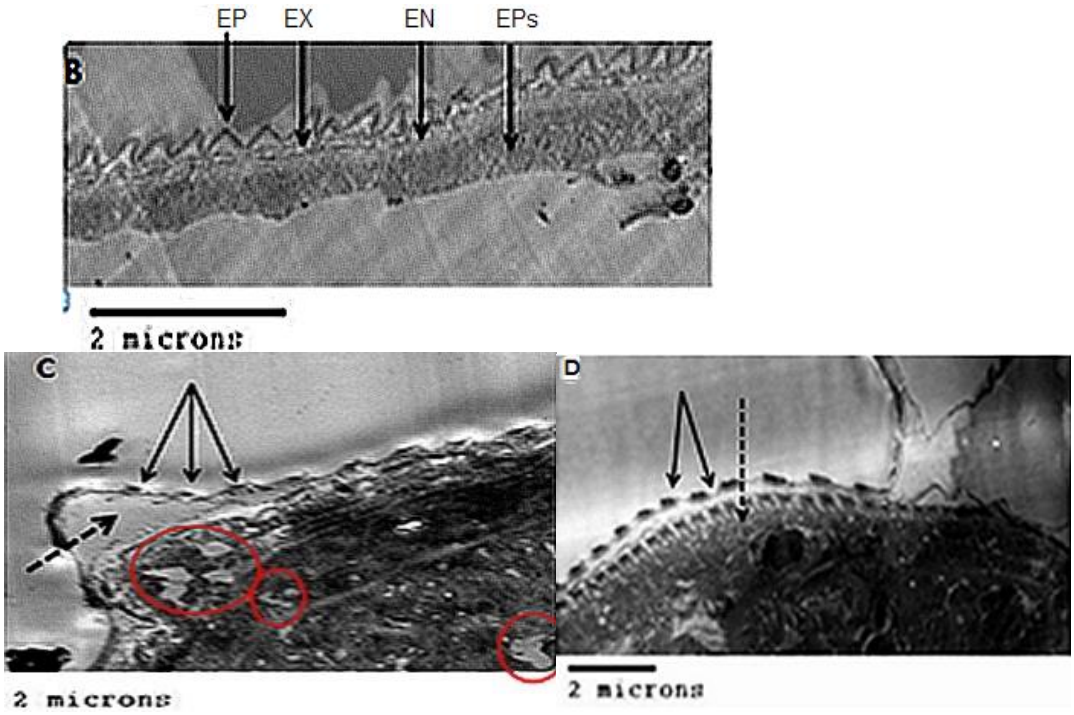

Fig. 3. TEM microphotograph of the cuticle layers of third instar larva of *Culex pipiens* Linnaeus, 1758; **B:** Untreated (x=10000), **C:** Treated larva with *C. aurantifolia* EO nano-emulsion (x=10000), **D:** Treated larva with *C. aurantifolia* EO dissolved in absolute ethanol (x=5000) and organized lamellae

5.2. Midgut

The TEM microphotograph of the epithelium of the untreated larva's cross-sectioned midgut (Fig. 4A, B) consists of a single layer of ciliated columnar cells resting on a basement membrane and surrounded by circular and longitudinal muscle fibers, as well as several microvilli (mv) on the surface of epithelial cells. The epidermal layer is protected from the food callus (fc) by the peritrophic membrane (pm). The TEM microphotograph of the treated larva's cross-sectioned midgut revealed various changes, including destructed epithelial cells with vacuolated cytoplasm caused by the degeneration of cell organelles, which caused cavities in the cytoplasm. The disruption of the microvilli in the midgut epithelium was the most recognizable ultrastructural change, giving the midgut a vacuolization appearance. The epithelial layer was vacuolated, swollen cells, masses of cellular material appeared in the lumen, and the epithelium eventually lost its normal appearance. Microvilli degeneration was observed in larvae treated with *C. aurantifolia* EO nano-emulsion and EO dissolved in absolute ethanol (Fig. 4C).

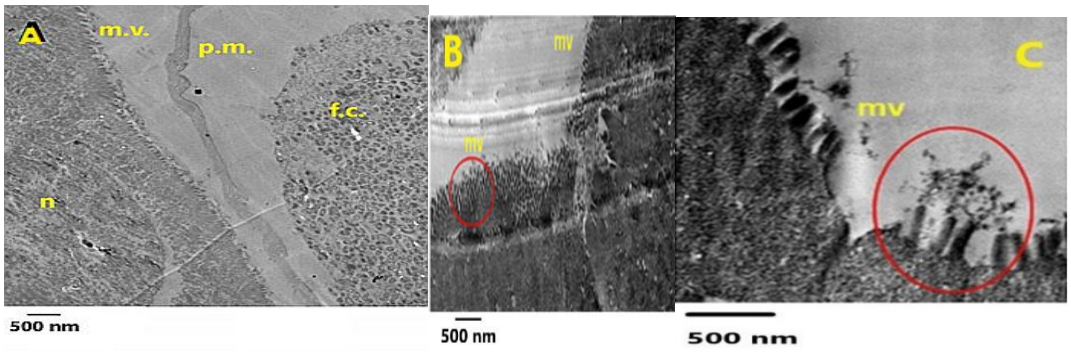

Fig. 4. TEM microphotograph of the cross-sectioned midgut of the larva of *Cx. pipiens* Linnaeus, 1758nano-emulsion; **A:** Untreated (x=5000), (fc) food callus, (mv) microvilli, (pm) peritrophic membrane, (v) vacuole, **B:** Treated with *C. aurantifolia* EO nano-emulsion (x=80000), **C:** Treated with *C. aurantifolia* EO dissolved in absolute ethanol(x=20000)

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DISCUSSION

The global preference for phytochemicals in malaria vector control may arise from their unique characteristics **(Duke, 1992)**. The need for safe and effective alternatives has emerged because of environmental pollution and the development of mosquito resistance to chemical insecticides **(Pavela, 2015)**. In comparison to synthetic insecticides, herbs with insecticidal properties have a significant advantage **(Turan & Mammadov, 2020)**. The most significant advantages of these insecticides are that resistance develops slowly leaving no residues in the environment since they are derived from renewable resources and degrade easily **(Roel, 2001)**. Additionally, they are non-toxic to humans and domestic animals **(Duke, 1992)**.

Citrus aurantifolia (lime) belongs to the Rutaceae family of fruits, which includes lime and orange. Essential oils are non-toxic and are listed as GRAS (Generally Recognized as Safe) by the FDA (Food and Drug Authority of USA). They are used as flavoring agents because many volatile compounds can be found in lime oils. EOs can be used in a variety of ways, such as medications, flavoring agents, cosmetics, antifungals/bacteria, and oils from the citrus peel of fruits that are effective against mosquitoes **(Lee *et al*., 2006)**. Additionally, citrus peel is a primary waste, as a result using EOs to control mosquitoes is a more environmentally friendly alternative than using synthetic pesticides. Furthermore, EOs are volatile, have a strong odor, and have a lower density than water **(Bakkali *et al*., 2008)**. **Fernandes *et al.* (2014)** used the EOs nano-emulsion to increase its water solubility with insecticidal activity. According to **Anjali *et al.* (2011)**, the higher efficiency of the nano-formulation compared to the bulk emulsion may be due to the nano-emulsion droplets' smaller size, which increases surface area, facilitates the penetration of nano-emulsion, and increases the rate of accumulation into the larvae. Several studies of the nano-emulsion EOs as eucalyptus **(Sugumar *et al*., 2014)**, castor **(Sogan *et al*., 2018)**, and sweet orange **(Azmy *et al*., 2021)** comply with our

results for the investigation of *C. aurantifolia* EOs which were found to be more effective than its bulk emulsion with ethanol. Treatments with different essential oils (EOs) or their constituents have a neurotoxic effect **(Kostyukovsky *et al.*, 2002)**. **Saad (2013)** reported that EOs nano-emulsion of *C. aurantifolia* has larvicidal activity due to the major component limonene. **Obembe and Opeyemi (2022)** demonstrated that among the various species of citrus, higher concentrations of the extract resulted in increased mortality rates of *Culex* larvae.

Overall, the treated larvae showed a clear and notable decrease in their protein levels. Protein binding with foreign substances, such as the tested EOs, may be the cause of the treated samples' decreased protein levels **(Ghosh *et al*., 2012)**. According to **Sharma *et al.* (2011)**, anopheline larvae treated with *Artemesia annua* extract showed a substantial decrease in total protein. The decrease in protein levels may be the result of harmful effects on some cells that secrete the decreased proteins in the *Cx. pipiens* larvae that are being treated. According to **Hazarika *et al.* (2018)**, the plant EO may have interfered with hormones that control the amounts of protein synthesis in the treated samples, which may have contributed to the decrease in protein content.

In insects, detoxification enzymes are typically shown to be the enzymatic defense against foreign substances **(Li & Liu, 2007)**. The chemicals that have insecticidal properties are reacted against by the detoxification enzymes. According to **Zibaee *et al*. (2011)**, they consist of phosphatases, glutathione S-transferase, and general esterases. One lysosomal marker enzyme that is active in the gut is called acid phosphatase. As a brush boundary membrane marker enzyme, alkaline phosphatase is particularly active in membrane-transfer-related tissues such as intestinal epithelial cells and Malpighian tubules **(Qari *et al.*, 2017)**. Accordingly, it may be employed as a criterion to assess antifeedant efficacy **(Abdel-Aziz, 2000)**. The present study's results unmistakably show a significant increase in the ACP and ALP following treatment with the tested larvicides.

As noted by **Shekari *et al*. (2008)**, an increase in ALP in the current investigation may suggest that this enzyme is involved in the detoxification process against the tested larvicides. Acetylcholinesterase is an esterase that degrades the neurotransmitter acetylcholine to stop nerve impulses. The current study's results showed a significant decrease in AChE enzyme activity when compared to the control group; similar findings were published by **Liu *et al.* (1990)** and **Nasr *et al.* (2017)**. This inhibiting action is somewhat consistent with previously reported findings for different insect species by other plant extracts, like *M. domestica* by azadirachtin **(Saeed *et al*., 1987)**. The tested larvicides led to a decrease in the enzyme activity of Glutathione S-transferase (GST) according to the results. This outcome aligns with the findings of **Nasr *et al.* (2017)**, who observed a notable reduction in GST activity in *Plutella xylostella* larvae when treated with a sub-lethal concentration of *Oregano vulgare* extract.

The current study's morphological findings showed that aberrations were most commonly found in the anal papillae. The treated larvae displayed darkening and deformation of the anal papillae due to the newly formulated nano-emulsions. Likewise, the mosquito larvae treated with seaweed extract showed similar aberrations in the anal papillae, consistent with the findings reported by **Yu *et al*. (2015)**.

The negative impact on the anal papillae disrupts the ion regulation in larvae and leads to an imbalance in homeostasis. Additionally, the distortion of the larval anal segment as seen in the current study is believed to result in damage to the hydrophobic surface of the anal segment, allowing the entry of water medium into the tracheal trunk, which adversely affects the larvae's respiratory system **(Kumar *et al*., 2010; Bianco *et al.*, 2013)**.

The structure of the cuticle and midgut was found to have many histological and ultrastructural alterations. The histological changes were found after treatment with both EOs nanoemulsions and EOs dissolved in absolute ethanol. Both the nano-emulsions and the EOs dissolved in absolute ethanol caused the cuticle disorganization profile of the treated larvae. The projections or papillae bounded to the epicuticle layer became loose as it became discontinuous. The epicuticle and endocuticle were separated, the endocuticle lost lamellae, and some vacuoles existed in the epidermal layer. These observations were noticed when **Younes *et al.* (1999)** treated larvae of *Spodoptera littoralis* with EOs of *Zygophyllum coccineum*. Changing in the normal structure of the peritrophic membrane as it became unequal in thickness, the epidermal layer with enlarged nuclei, vacuolization appearance of the epithelial layer, changes in the food callus, and decrease of the adipose tissues observed after treatment of the third larval instar of *Cx. pipiens* with LC_{25} of both the nano-emulsions of *C. aurantifolia* EO and the EO dissolved in absolute ethanol. In all treatments, the microvilli were malformed and degenerated, and masses of cellular material appeared in the lumen. The histopathological alterations in the midgut of *Cx. pipiens* larvae were consistent with **Assar and El-Sobky (2003)**. **Hamouda *et al.* (1996)** reported that the epithelial layer of the midgut of *Cx. pipiens* were vacuolated, and masses of cellular material appeared in the lumen after being treated with *Artemisia judaica*. **Ndione *et al.* (2007**) investigated the toxicity of neem oil against *Aedes aegypti* larvae and found various damage in the exposed larvae's midgut epithelium. The midgut cell changes are attributed to the fact that this part of the digestive tract, which is responsible for insect digestion, is close to toxic elements, resulting in death **(Seye *et al*., 2006)**. According to **Zerroug *et al.* (2017)**, the interaction of the gut contents with the hemolymph may result in larval death **(Al-Mehmadi & Al-Khalaf, 2010)**. A histological study on the 4th larval instar of *Cx. pipiens* treated with *Eucalyptus globulus* leaves aqueous extract reveal different and progressive damage to the larvae's intestinal tissue, resulting in the mixing of gut cells content with hemolymph, which causes larval death. The ultrastructural analysis in our study showed that treatment with nano-emulsions has similar effects on the cuticle, muscles, and midgut, indicating that nano-formulations of

EOs have the potential to be used as an efficient larvicide against *Cx. pipiens*, with the advantages of environmental protection and low concentrations that do not impact non-target species. According to our findings, the lime oil nanoemulsion containing *C. aurantifolia* EO with a droplet size of 20.7± 2.6nm was found to be effective in controlling mosquito *Cx. pipiens* larvae.

CONCLUSION

The *C. aurantifolia* nanoemulsions demonstrated a potent effect on *Cx. pipiens* larvae. The studied nanoemulsions had an impact on the cuticle, midgut, and muscle ultrastructure as well as histopathology in mosquito larvae. The studied larvae's protein underwent alteration as a result of the tested nanoemulsions. These findings will significantly lessen the need for synthetic pesticides, which will reduce pollution in the environment.

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الملخص العربًى

تم دراسة فعالية مبيد اليرقات لزيت الليمون العطري *Citrus aurantifolia* (EO) على يرقات العمر الثالث لبعوض *Culex pipiens* (LC_{50} 35.4 جزء في المليون). وقد اظهرت النتائج الانحرافات المورفولوجية مثل تصلب وتغميق وانكماش الحليمات الشرجية بجانب تغميق السيفون. كان هناك انخفاض في بروتين الجسم الكلي بعد العلاج (15.3 ± 0.21)، وزيادة في مستويات الفوسفاتيز الحمضي والفوسفاتيز القلوي (4.67 ± 0.12 و 36.03 ± 0.79، على التوالي)، في حين كان هناك انخفاض في نشاط استيريز الأسيتيل كولين والجلوتاثيون S-Transferase (13.07 ± 0.27 و 312.67 ± 7.22 على التوالي). أظهرت اليرقات المعالجة تغيرات نسجية مرضية واضحة في أنسجة الجلد والأمعاء الوسطى لليرقات المعالجة. تعتبر دراسة المستحلب النانوي EO من *Citrus aurantifolia* مبيد يرقات طبيعي واعد، وهو قابل للتحلل البيولوجي وصديق للبيئة، ويمكن استخدامه للسيطرة على البعوض *Cx pipiens* الناقل للمرض.