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Scanning Electron Microscopy of Cuticular Deformations in *Culex pipiens* Linnaeus, 1758 (Diptera: Culicidae) Larvae Associated with Larvicidal Effect of Nanoemulsion of *Pimpinella anisum* L. Essential Oil

Eman Abdel-Nasser[1], Jehan A. Hafez[1], Rawda M.Badawy[1], Sameh A. Rizk[2], Hassan H. Hefni[3], Radwa M. Azmy[1]*

[1]Department of Entomology, Faculty of Science, Ain Shams University
[2]Department of Chemistry, Faculty of Science, Ain Shams University
[3]Department of Petrochemicals, Egyptian Petroleum Research Institute
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***Corresponding author: radwa.azmy@sci.asu.edu.eg**

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ABSTRACT

Culex pipiens Linnaeus, 1758 is the chief vector of dangerous diseases such as lymphatic filariasis in tropic and subtropic regions. The current study aimed to investigate the larvicidal effect of nanoemulsion of *Pimpinella anisum* L. essential oil by studying the associated cuticular deformations in *Culex pipiens* larvae via scanning electron microscopy. The nanoemulsion was prepared using ultrasonication method. The mean droplet size of the nanoemulsion was 116nm, with poly dispersity index equal to 0.21. The LC_{50} of the nanoemulsion was 40.1ppm. The third instar larvae were treated with the LC_{50} of the prepared nanoemulsion. The cuticular deformations possibly inhibited the ability of the larvae to continue developing leading at the end to the death of these treated larvae. This is the first report to demonstrate the association between the nanoemulsion of *Pimpinella anisum* and the cuticular changes of *Culex pipiens* larvae. The results of the present study proved that nanoemulsion of *Pimpinella anisum* EO can be considered as a promising candidate in the integrated management programs to control *Culex pipiens*.

INTRODUCTION

Culex pipiens Linnaeus, 1758 (Diptera: Culicidae) is a vector of high concern throughout the world since it is the chief vector of many serious diseases to humans and several domesticated animals **(Ibrahim *et al.*, 2023)**. An important consequence of mosquito bites is the transmission of viral diseases such as Zika, dengue virus, and thw west Nile fever, which cause encephalitis, meningitis, and microcephaly. The vector control which is directed toward the immature larval stage can be applied at the aquatic breeding sites. Unfortunately, control using chemicals is the most used method, despite the effectiveness of chemical control, there are several problems such as the toxic impact of the insecticides on the ecosystem and the development of resistance in mosquitoes.

Hence, there is a rising focus on new safe bio-insecticides with novel modes of action to avoid the side effects of synthetic insecticides **(El-Sadawy *et al*., 2018)**.

Nanotechnology is a branch of science focusing on new approaches in different fields including insect pest management **(Azmy, 2021; Farahat *et al.*, 2024)**. In recent years, nano-insecticides gained an increasing interest because of their great ratio of surface area to volume which leads to a better chemical reactivity and catalytic action at lower doses than those of the bulk material **(Azmy *et al.*, 2019)**. Some forms of nanoinsecticides raised the mortality rates of several insect pests **(Thabet, 2022)**. The effects of nano-emulsions based on essential oils on the larvae of *Culex pipiens* were recognized and exhibited dysfunctions in the larval physiology, histology and development after treatment **(Azmy *et al.*, 2021a)**. Fortunately, the target stage of the insect grows in water bodies such as swimming pools and accumulated rain. Such water bodies are good targets for nanoparticle spray. Nanoemulsions of many essential oils were stated to induce insecticidal impact against different mosquito genera **(Azmy *et al.*, 2021b)**. Anise, *Pimpinella anisum* L., is an important ancient medicinal herb in the Mediterranean region including Egypt. It is a flowering herbaceous plant belonging to the family Apiceae or Umbelliferae, with white flowers and small green to yellow seeds **(Ali *et al.*, 2017)**.

Hydrodistillation is the method used for ages to produce essential oils; several studies have revealed that *Pimpinella anisum* is rich in bioactive compounds, such as tannins, phenols, carotenoids, and fatty acids. The essential oil of *Pimpinella anisum* constitutes mainly trans-anethole **(Özcan & Chalchat, 2006)**. There are insufficient studies using scanning microscopy technique to study the effects of larvicides from plant sources on mosquito larvae. To our awareness, no work has been conducted to examine the morphological effects of nanoemulsion of *Pimpinella anisum* essential oil on the integument of *Culex pipiens* larvae. Therefore, this work aimed to evaluate the larvicidal effect of nanoemulsion from anise essential oil on the third larval instars of *Culex pepiens* larvae by monitoring the morphological malformations. The cuticle of the treated larvae was observed for any changes using scanning electron microscope to detect the probable interruption in penetration.

MATERIALS AND METHODS

Mosquito larvae

The third instar larvae of *Culex pipiens* were obtained from the Egyptian Research Institute of Medical Entomology. The larvae were kept at 80% pH, 25± 2°C and a photoperiod of 12L: 12D. One hundred larvae were subjected to the LC_{50} concentration of the nanoemulsion of *Pimpinella anisum* L. essential oil for 24 hours. The LC_{50} value was calculated through Probit analysis of the data acquired from the larvicidal bioassay;

the value was 40.1pm **(Abdel-Nasser *et al.,* 2023)**. Three survived larvae and three untreated larvae were examined using the scanning electron microscope.

Nanoemulsion

The nanoemulsion was prepared using Tween 20 and distilled water according to **Azmy *et al.* (2019)** using a Sonicator at Electrom Microscope unit, faculty of Science at Ain Shams University.

Behavioral and morphological observations

The behavioral and morphological changes in the treated larvae were observed using a stereo microscope to visualize the changes in body parts after 24h of exposure, the changes in treated larvae were screened based on three replicates. Additionally, optical images of treated and control larvae were documented.

Scanning electron microscopy (SEM)

SEM study was conducted at the Egyptian Petroleum Research Institute; specimens were dehydrated in an ascending series of alcohol (10% -100%) for five minutes in each, then in xylene. Afterward, they were fixed on stubs made of aluminum with a sticky tape. Subsequently, they were coated with 200 A° gold using a sputter coater. Finally, scanning electron micrographs of the cuticle of normal larvae and the cuticular changes in the treated larvae were taken. Observations were mainly focused on the head capsule, thorax, abdominal segments, and siphon.

RESULTS

Stereomicroscopic study

Stereomicroscopic images of control larvae revealed normal morphological features of the different body parts including the head, thorax and segments of the abdomen. With a large number of bristles on the surface of the whole body, the normal respiratory organ looked short and tubular (Fig. 1A).

On the other side, the treated larvae with LC_{50} of *Pimpinella anisum* nanoemulsion revealed several morphological changes when compared to the control larvae. The malformations were in different body parts with major changes involving rupture, constriction, shrinking, extension or distortion. The color of body parts also changed whether they became blackish, whitish or pale with spots of pigmentation. The deformation of the head region included flattening and blackening of the cephalic capsule, neck elongation and reduction and shortening of head bristles (Fig. 1B, C). The thorax region showed general deformation, darkening and significant swelling of some treated larvae (Fig. 1C).

The deformities in the abdomen included demolition of the pigmentation, abdomen curving and thinning and whitish coloration in the digestive tract. It was observed that the anal segment was malformed and swollen (Fig. 1B,C).

Stereomicroscopic images of the pupa did not show significant change before and after treatment (Fig. 2).

Scanning electron microscopy

In the case of the control larvae, scanning electron microscopy images revealed that the body of the larvae showed an intact surface of cuticle and normal coverage of the body with hairs; the control larvae had normal rounded head with hardened capsule and mouth brushes. The thorax of the control larvae appeared with normal features (Fig. 3A, B). The terminal region carried a typical anal segment, siphon, anal papilla and pectin teeth (Fig. 3C, D).

Behavioral observation

The control group of larvae displayed normal behavior, while the treated group showed some behavioral changes such as irregular movement compared to the control group. After 30 minutes of treatment, the treated larvae became more irritated with wriggling movements and paralytic symptoms. This behavior continued for more time, and then at the end the affected larvae displayed slow movement with more time away from the water surface. Some larvae developed a distinct c-shape as the head got near the end of the body (Fig. 7).

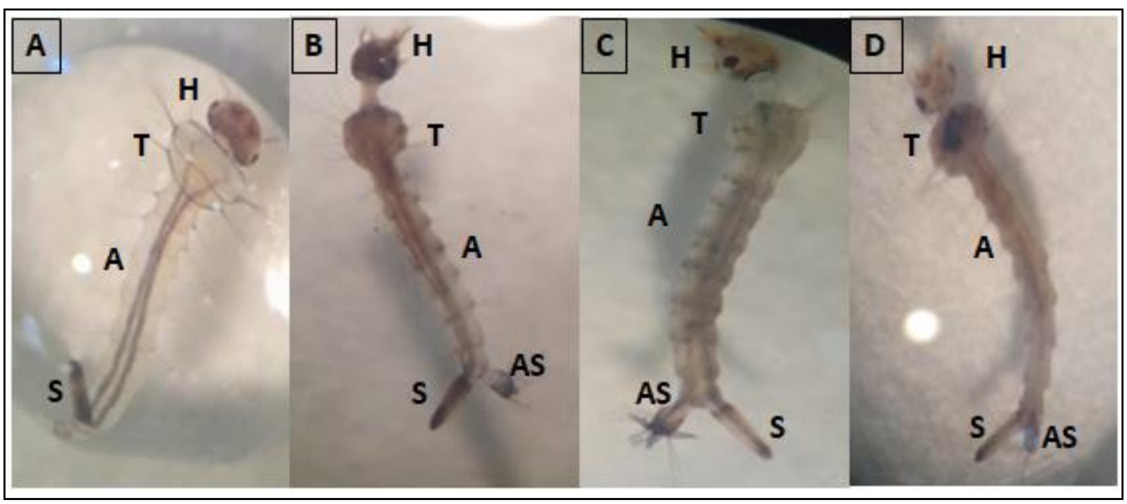

Fig. 1. Stereomicroscopic images of third instar larvae of *Culex pipiens*. **A:** Control larvae with normal appearnce; **B:** Treated larvae with *Pimpinella anisum*. H: Head, T: Thorax, A: Abdomen, S: Siphon, AS: Anal segment. The images show changed color of body parts, deformation of the head region included flattening and blackening of the cephalic capsule, neck elongation and reduction. The thorax region showed darkening and significant swelling

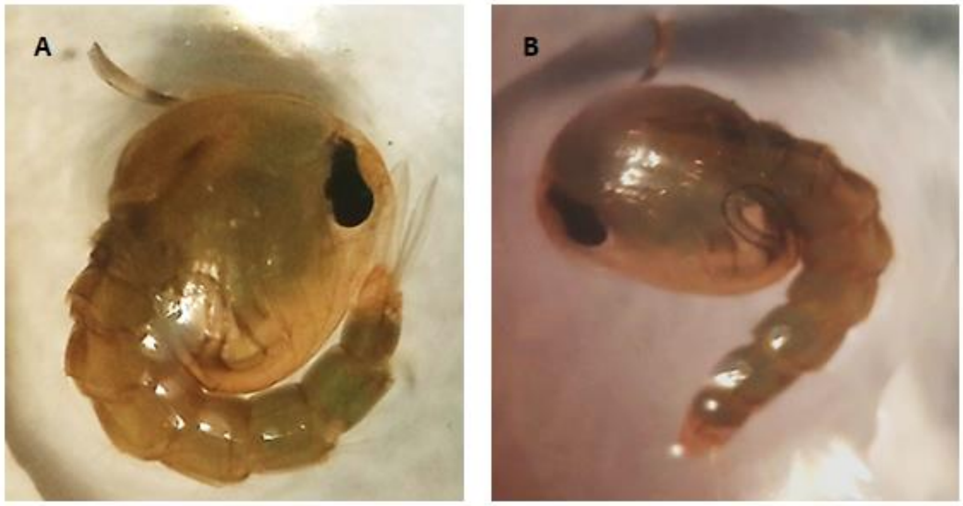

Fig. 2. Stereomicroscopic images of pupa of *Culex pipiens*. **A:** Control; **B:** Treated pupa with nanoemulsion of *Pimpinella anisum* showed no significant changes by stereomicroscope

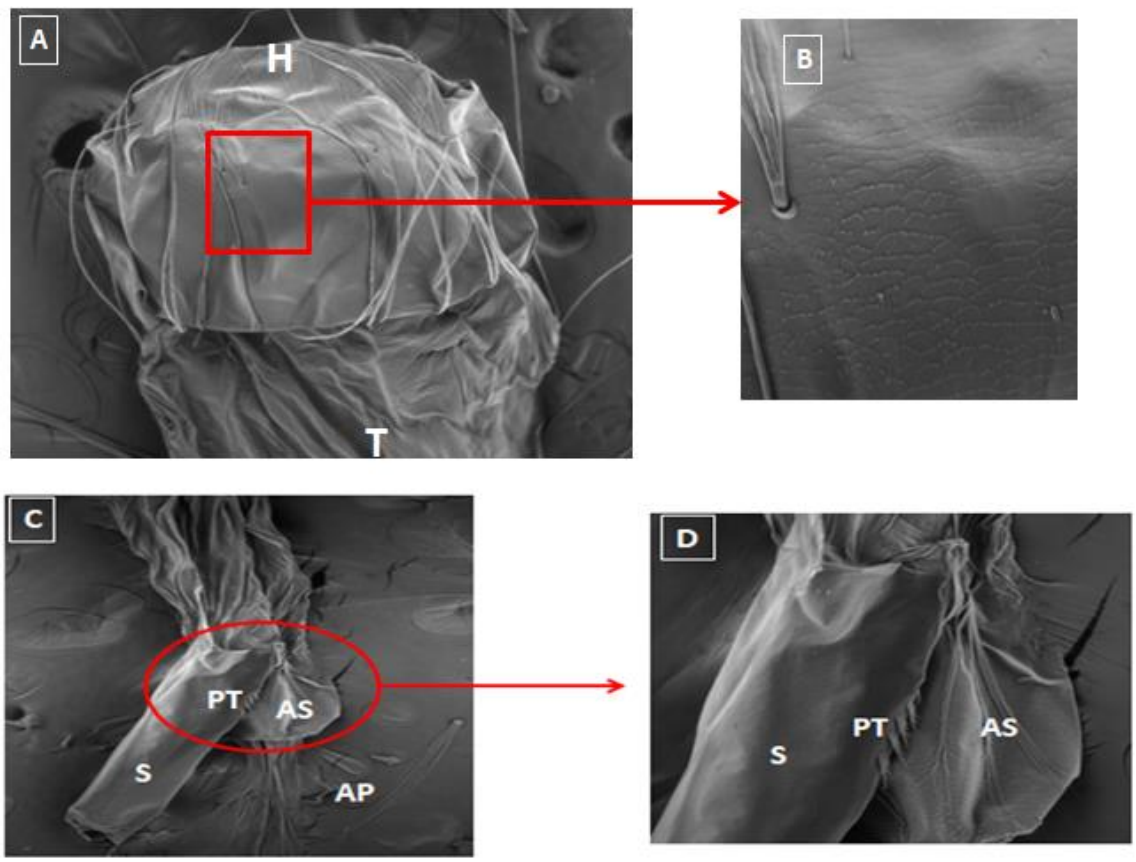

Fig. 3. Scanning electron images of normal third instar larvae of *Culex pipiens* showing healthy intact integument. **A:** Dorsal view of head and thorax regions (100 X); **B:** More magnification (500 X) showing the intact cuticle; **C:** Dorsal view of terminal region

(100 X); **D:** More magnification (250 X) to show the intact cuticle. H: Head; T: Thorax; S: Siphon; PT: Pectin teeth; AS: Anal segment; AP: Anal papillae

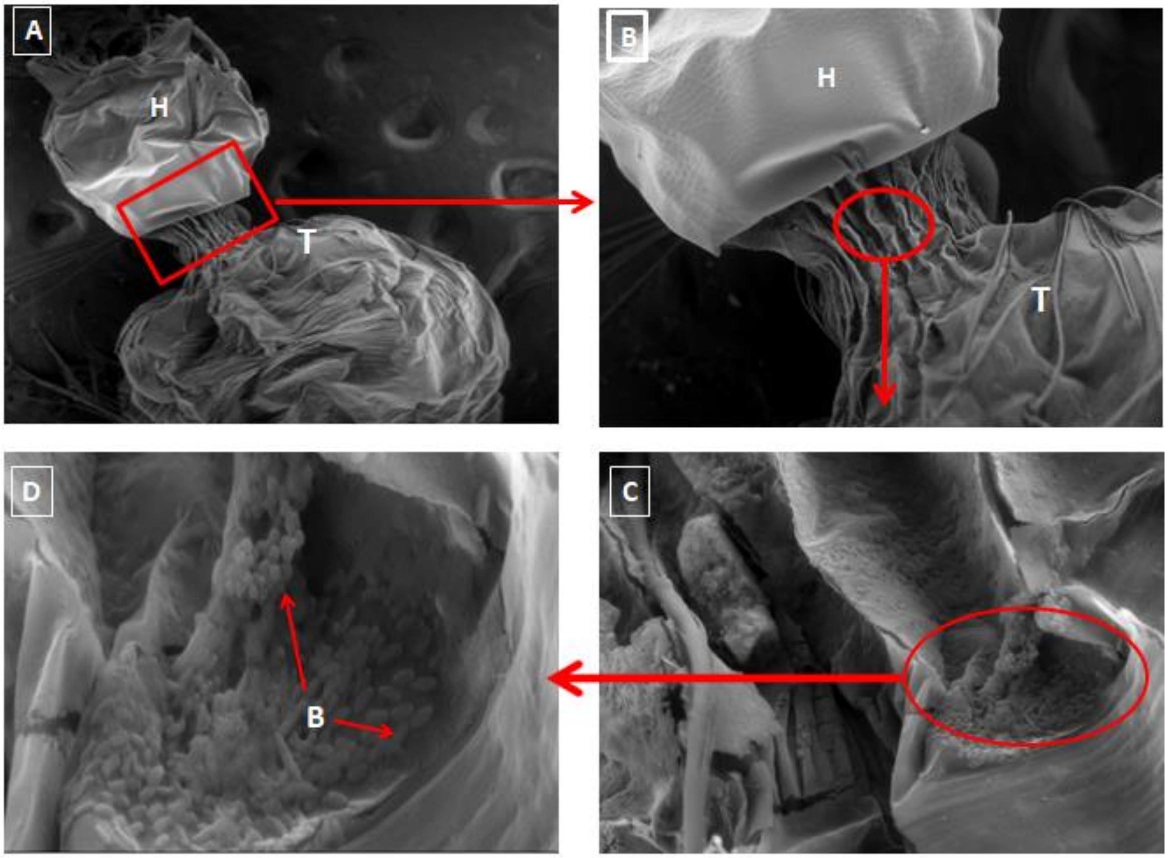

Fig. 4. Scanning electron images of head and thorax regions of treated third instar larvae of *Culex pipiens* with nanoemulsion of *Pimpinella anisum*. **A:** Dorsal view (74 X); **B:** More magnification (250 X) showing cracks in cuticle; **C:** Clusters of bacterial cells (2.50 K X); **D:** Different types of bacterial cells including cocci and bacilli bacteria (3.50 K X). H: Head; T: Thorax; B: Bacterial cells. The scanning images show shrinkage and cracks in the integument of the treated larvae and infection with bacteria

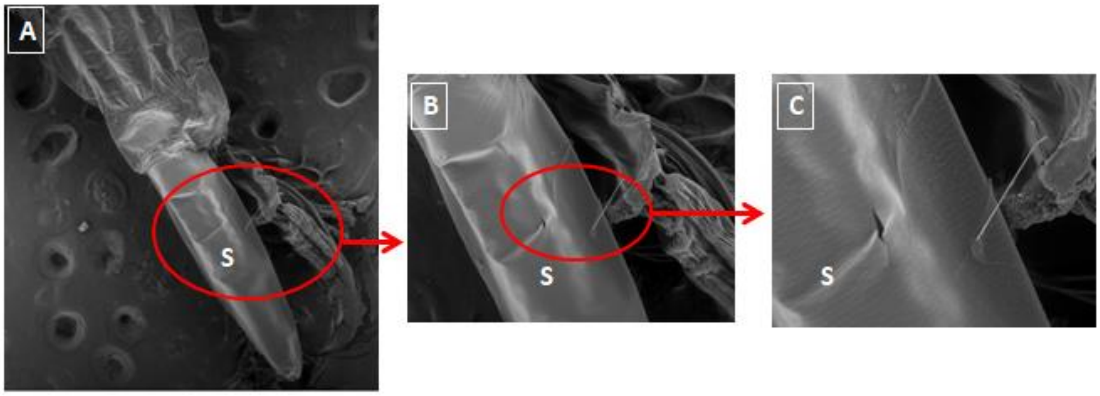

Fig. 5. Scanning electron images of terminal region of treated third instar larvae of *Culex pipiens* with nanoemulsion of *Pimpinella anisum*. **A:** Dorsal view of (74 X); **B:** More magnification (250 X) showing cracks in cuticle; **C:** More magnification (500 X). **S:** Siphon. The scanning images show cracking in the siphon

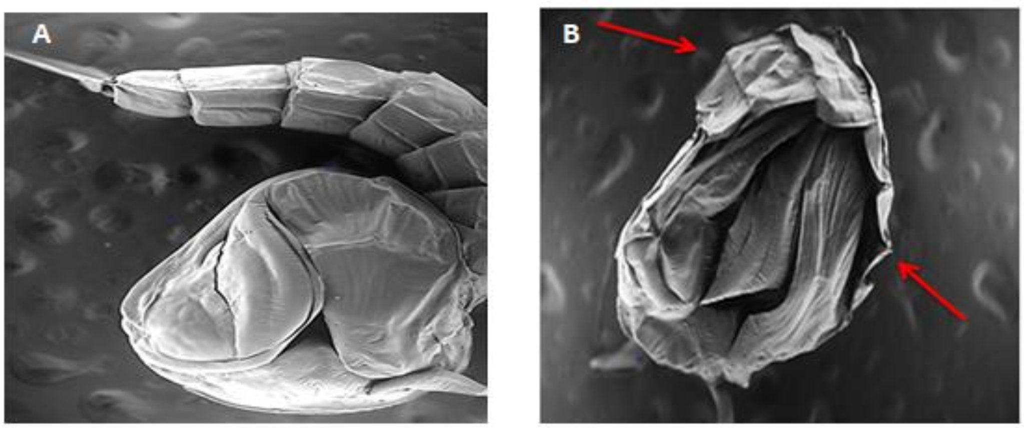

Fig. 6. Scanning electron images of pupa of *Culex pipiens*. **A:** Control (100 X); **B:** Treated pupa with nanoemulsion of *Pimpinella anisum* (73 X). The scanning images show shrinkage and malformation in the treated pupa

Fig. 7. Stereomicroscopic images of third instar larvae of *Culex pipiens* treated larvae with *Pimpinella anisum* showing the behavioral changes including a c-shape form

DISCUSSION

The current study revealed that the cuticular layer was a site of abnormalities in the larvae of *Culex pipiens* induced by the treatment with nano-emulsion of *Pimpinella anisum* essential oil. Integument plays a significant role in resistance to penetration of insecticides; penetration of the cuticle is one of the insecticides modes of action **(Miresmailli & Isman, 2014)**. The insect cuticle slows down the inoculation rate of insecticide into internal organs to enhance the efficiency of detoxification mechanisms. Decrease in thickness or cracks of cuticle increases the penetration rate through the cuticle which increases the rate of inoculation of larvicide into the insect organs. The hydrophobic epicuticle is considered as a final barrier between the outside environment and the insect. The small size of insects results in a high ratio of surface area to volume which leads to short paths to the internal nervous system. As a result, insects are susceptible to contact poisons that enter through the outer integument. The first step in the mechanism of action of contact insecticides is the superficial contact of the insecticide with the insect body, this process is related to the insect behavior and the physical characteristics of the insecticide, specially its droplet or particle size **(Azmy, 2024)**. The nano-size of the droplets of the nanoemulsion enhances fast and easy penetration through the larval cuticle. Due to the smaller size and larger surface area of nanodroplets, they can penetrate the body of larvae easily and interrupt the normal life cycle by disturbing breathing, feeding and cell division. The second step is the penetration of the toxic substance through the integument. The thickness of the cuticle affects the penetration of oils; the thin setae membranes are susceptible areas. The big extension of the interface between insects and toxicants may be the principal cause of the high toxicity of oily solutions. The results of the current research validate the morphological study on *Aedes Aegypti*, *Anopheles stephensi*, and *Culex tritaeniorhynchus* larvae treated with the nanoemulsion of *Thymus vulgaris*. This study reported the induced morphological changes in the larval cuticle including damage of the bristles, head, disintegration of abdominal segments and decrease in body length. In addition, cuticular changes were detected in the larvae of *Culex quinquefasciatus* in larvae treated with chitosan nanoparticles and lavender nanoemulsion using a scanning microscopy including cuticle stiffening **(Taktak *et al.*, 2022)**. Malformations in the anal papillae were recorded in larvae of *Aedes aegypti* treated with ethanolic extract of three Piper species; the morphological malformations included cuticle dysfunction and anal papillae shrinkage. The chemical of anise essential oil is dominated by (E)-anethole **(Adham *et al.*, 2013)** as the major active compound. A probable explanation for the cuticular alterations could be that anise essential oil inhibited the synthesis and deposition of chitin. Chitin is crucial for the polymerization of the cuticle and its deficiency indicates the delay of the cuticular growth which affects the process ecdysis, and causes incorrect deposition and disorganization of chitin. Some studies have attributed these induced ultrastructural and morphological alterations to the lipophilic monoterpene constituents of essential oils **(Adham *et al.*, 2013)**.

The behavioral change represented by the crippling activity of larvae is done by brushing the mouth with the tail to remove the larvicidal solution touching the body. The darkening of body may be due to the damage of changes in the peritrophic matrix or epithelial cells of the larvae **(Ravi *et al.*, 2018)**. Alterations in the integument may lead to damage in the internal organs of larvae. Consequently, the enhanced toxicity could be due to the faster penetration through the damaged parts of the cuticle indicated by cracks and corrosion of the cuticle **(Mostafa *et al.*, 2021)**. Moreover, the deformation of anal segment detected in the present work may cause destruction of the anal segment hydrophobic surface **(Yu *et al.*, 2015)**. Furthermore, the injuries in anal papillae disturb the osmotic and ionic regulation leading to the entry of water medium into the tracheal trunk, which causes troubles in the larval respiration system, and finally suffocation of the larvae **(Kassem *et al.*, 2018)**, and this may explain the presence of bacterial cells as a sign of degeneration of the body of the larva.

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