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Histopathological Alterations in the Larvae of *Culex pipiens* L. (Diptera: Culicidae) Induced by Nanoemulsion of *Pimpinella anisum* L. Essential Oil

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ABSTRACT

Culicid mosquitoes pose significant threats to both animal and human health due to their ability to transmit various parasitic and viral diseases. In light of the problems arising from the overuse of synthetic insecticides, there is an urgent need to explore alternative solutions. Nanotechnology offers promising advancements, enhancing the efficacy of products across various fields, including the food industry, pharmacy, and agriculture. One notable application is the development of nanoinsecticides, which exhibit superior penetration capabilities into target insect pests due to the small size of nanoparticles and their high efficiency resulting from a larger surface area. This research evaluated the effects of a nanoemulsion of *Pimpinella anisum* essential oil on the third instar larval stage of *Culex pipiens*. The lethal concentration (LC50) used for treatment was 40ppm. The studied nanoinsecticide induced significant histopathological and biochemical alterations in the larvae post-treatment. Biochemical assessments revealed a notable decrease in alkaline phosphatase enzyme activity in the treated larvae. Histopathological changes were analyzed using transmission electron microscopy, which showed significant alterations in muscle and midgut tissues after 24 hours of exposure to the nanoemulsion. Observed changes included cytoplasmic vacuolization, elongation of epithelial cells, damage and disorganization of microvilli, and degeneration of the muscle layer.

INTRODUCTION

Vectors of disease present significant challenges to public health, particularly in developing countries due to their economic and medical impacts. Members of the Culicidae family such as *Culex* transmit numerous pathogens affecting both animals and humans, including filarial worms, avian malaria, Saint Louis encephalitis, and the West Nile virus **(Akono *et al.*, 2012)**. Resistance among mosquitoes to chemical insecticides has increased significantly due to their extensive use **(Bigoga *et al.*, 2013)**. Furthermore, these chemical insecticides contribute to water pollution, pose toxicity risks to non-target organisms, and decrease soil fertility **(Azmy, 2024)**.

These drawbacks have prompted control programs to seek biodegradable and eco-friendly insecticides derived from natural plants or plant products with mosquitocidal effects. Natural insecticides may induce various impacts on target insects, including behavioral changes, developmental delays, or disruptions in the neuro-endocrine system that affect metamorphosis and growth. Essential oils have shown strong potential against mosquito larvae, but they must be formulated in a way that prevents the vaporization of volatile components to maintain their biological activity **(Osanloo *et al.*, 2017)**.

Nanoemulsions, developed through nanotechnology, are particularly suitable for application in aquatic breeding sites of mosquito larvae. These formulations allow various active compounds to be encapsulated within the nanoemulsion droplets, enabling immiscible materials such as essential oils to disperse in water, where mosquito larvae thrive.

Several essential oil nanoemulsions, such as those derived from orange essential oil, have been reported as effective larvicides **(Azmy *et al.*, 2019)**. However, there remains a gap in understanding the mechanisms by which key components in essential oils cause larval mortality. Therefore, there is a need to investigate the effects of these botanical insecticides on various biological features, including biochemical and histopathological levels.

This study aimed to evaluate the effects of the nanoemulsion of *Pimpinella anisum* on inducing biochemical and histopathological alterations in the third instar larval stage of *Culex pipiens*.

MATERIALS AND METHODS

Insect rearing

Culex pipiens larvae were reared under optimum conditions at a temperature of 24 ± 3°C, humidity of 75 %, and a photoperiod of 12:12 light/dark hours.

Extraction of essential oil and preparation of nanoemulsion

Essential oil was extracted from the seeds of *Pimpinella anisum* by the hydrodistillation method according to the method of **Meyer (1984)**. Nanoemulsion was formulated using polyoxyethylene 20 sorbitan monolaurate and distilled water, and then subjected to a sonicator with 30kHz frequency for 20min according to the method of **Anjali *et al.* (1984)**.

Biochemical study

The larvae of *Culex pipiens* were subjected to a lethal concentration LC_{50} (40mg/ mL) of the nanoemulsion of *Pimpinella anisum* essential oil according to the method of **Abdel-Nasser *et al.* (2023)**.

After 24 hours of treatment, tissue samples from the larvae were homogenized in a treatment buffer (1g of larval body per ml) for 3 minutes using a grinder. The

homogenates were then centrifuged for 15 minutes at -2°C and 14,000rpm. The supernatant was stored at -5°C until biochemical analysis.

Alkaline phosphatase activity was measured in both control and treated larvae using a buffer with a pH of 10.5. The enzyme activity was assessed using a spectrophotometer at 400nm, following the method outlined by **Laufer and Schin (1971)**. Each test was conducted in triplicate.

Histopathological and ultrastructural study

The histopathological and ultrastructural study was carried out by transmission electron microscope at Mycology and Biotechnology Center, Al-Azhar University. Treated and control specimens were fixed in glutaraldehyde (5%) and were then washed in 70% ethanol according to the method of **Disbrey and Rack (1970)**. The samples were prepared in 0.1 M buffer of sodium cacodylate for three hours at pH 7.2 and were then washed for 20min in cacodylate buffer (0.1M).

Samples were immersed for 100 minutes in osmium tetroxide (1%) prepared in sodium cacodylate buffer (0.1M), and then washed in cacodylate buffer (0.1M). Finally, samples were dehydrated in a gradual series of ethanol. Next, samples were embedded in epoxy resin. Sections with 1μm thickness were cut by a glass knife. Afterward, sections were stained using toluidine blue. Ultrathin sections (50–80nm thick) were cut by a glass knife. Finally, sections were stained by uranyl acetate and lead citrate stains as described by **Reynolds (1963)**.

RESULTS

Biochemical alterations

After treatment of larvae with LC_{50} of the nanoemulsion, results showed a significant difference in the quantity of alkaline phosphatase level in treated larvae from control ones. The results obviously verified that activity of alkaline phosphatase decreased to be in an average equals to 1212± 99 mU/ mg protein compared to 2187± 131 mU / mg protein, in untreated larvae with percentage change equals to 80%, as shown in Table (1).

Table 1. Effect of lethal dose of the nanoemulsion of *Pimpinella anisum* essential oil on alkaline phosphatase in *Culex pipiens* larvae

	Alkaline phosphatase (mU / mg protein)			
	R_1	R_2	R_3	Average
Control	2200	2050	2311	2187±131 a
Treated	1100	1250	1288	1212±99 b

Histopathological study

After treatment of larvae with LC_{50} of the nanoemulsion, results displayed malformations in the skeletal and gut tissues of the treated larvae compared to the healthy tissues in the control larvae. The standard skeletal muscles composed of the parallel striated fibers; each fiber contains many fibrils parallel in sarcoplasm. The fibers are

enclosed by a connective tissue (Fig. 1A). The treated larvae with the nanoemulsion (Fig. 1B) showed disorganization of fibrils and presence of vacuoles between muscle fibers. On the other hand, malformations in the midgut tissues were also noted. The midgut epithelium of control larvae (Fig. 2A) consists of ciliated columnar cells on a basement membrane and microvilli on the cells surface. While, the midgut of the treated larvae showed many alterations including destructed epithelial cells. Moreover, the most observed change noted in the epithelium was the disruption of the microvilli, swollen cells, and abnormal appearance of the epithelium (Fig. 2B).

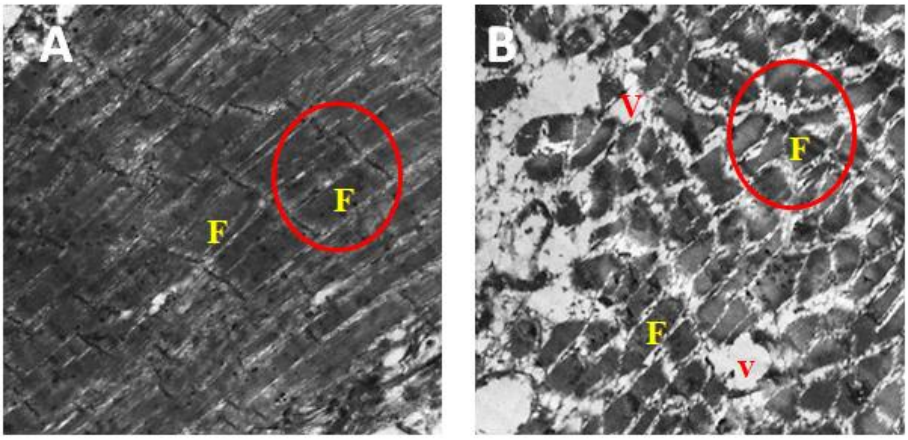

Fig. 1. Ultrastructure micrograph of muscles of *Culex pipiens* larvae (15000X). **A:** Control; **B:** Treated. F: fibril, v: vacule

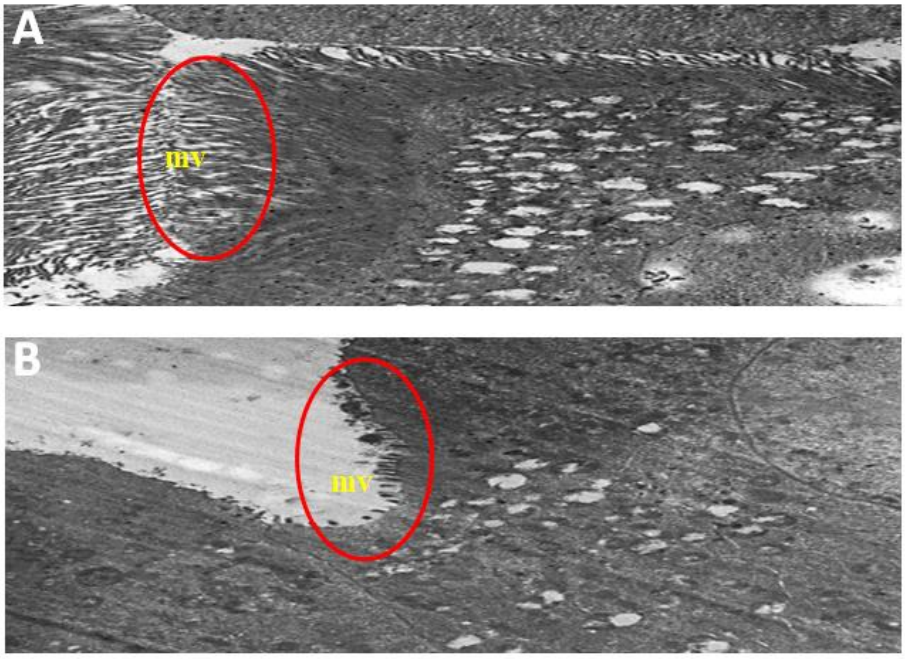

Fig. 2. Ultrastructure micrograph of midgut of *Culex pipiens* larvae (15000X). **A:** Control; **B:** Treated. mv: microvilli

DISCUSSION

Plant-based insecticides offer safe, cheap, and ecofriendly alternatives to the toxic synthetic chemical insecticides **(Azmy, 2021)**. Moreover, resistance of insects to plant-based insecticides is considered slow. In addition, botanical insecticides have residues in the surrounding environment and quickly degrade. Furthermore, nanotechnology enhances the efficiency of the botanical insecticides through formulations including nanoemulsions **(Azmy *et al.*, 2021a, b)**. This study shed light on the impact of nanoemulsion of *Pimpinella anisum* essential oil on the larvae of *Culex pipiens* in an attempt to understand the mechanism of action of this nanoinsecticide in the body of the treated larvae.

The midgut is the functional core of the digestive tract as it undertakes digestion of food and absorption of nutrients; the normal larvae midgut consists of a layer of epithelial cells lied on a basal lamina bounded by muscles; the border of midgut has a striated shape due to the microvilli that line inner side of epithelial cells **(DeRobertis *et al.*, 1965)**. In this study, the obvious alteration observed in the epithelium of the treated larvae midgut was microvilli disruption and the general vacuolization. The results are similar to those showed in insect response to several toxic materials such as the effect of myrrh on *Culex quinquefasciatus* larvae **(Al-Mehmadi & Al-Khalaf, 2010)**. In addition, the results reported by **Zayed *et al.* (2009)** revealed that midgut epithelium is incapacitated to perform its normal digestive and absorptive functions due to the treatment of *Culex pipiens* larvae with *Allium sativum* and *Citrus limon* oil extracts.

Assar and El-Sobky (2003) noted that *Eichhornia crassipes* extract caused an extreme effect on midgut of *Culex pipiens* larvae as some epithelial cells and brush border and were apically degenerated after 72h of exposure, while most of the epithelial cells entirely vacuolated and degenerated. Likely, the ethanol leaf extract of *Dracaena arborea* caused basement membrane detachment, epithelial cells vacuolization, and microvilli disorder in the midgut of *Culex pipiens* larvae **(Mo'men *et al.*, 2022)**.

From another side, the treated larvae showed muscle disorganization and vacuolization of fibrils. Likewise, **Bakr *et al.* (2010)** reported similar observations in treatment of *Schistocerca gregaria* with an extract of rice bran. It is suggested that the malformation and disorganization of muscle fibers hinder the larvae's ability to move properly, preventing them from reaching the water surface to breathe and seek food. This ultimately leads to suffocation and starvation. The histopathological injuries observed in the larvae after treatment are associated with both the ingestion of the nanoemulsion components and their contact with the external tissues of the integument.

The mode of action in the gastrointestinal tract can be understood through the ingestion and subsequent absorption of the active compounds in the essential oil, which are encapsulated in the nanoemulsion droplets. These droplets can cross the larval

peritrophic membrane and penetrate the intestinal epithelial cells. The small size of these droplets facilitates a high rate of penetration into the larvae's tissues. Furthermore, the effect of the active compounds is enhanced due to the large surface area of the nanoemulsion droplets.

In general, detoxification enzymes of insects are considered as the enzymatic defense mechanism against insecticides, and they play important roles to maintain regular physiological functions **(Li & Liu, 2007)** since they include phosphatases **(Zibaee *et al.*, 2011)**. Phosphatases are known to play an important role in physiological processes and considered as marker enzymes to measure the toxic effects of several toxicants on insect pests, they are located in membrane vesicles of brush border of larvae gut. Therefore, phosphatase enzymes are used as biomarkers to evaluate toxicity of botanical and synthetic insecticides. Alkaline phosphatase enzyme is considered as a brush border membrane enzyme which is active in midgut epithelium **(Qari *et al.*, 2017)**. Hence, it may be used as an indicator to determine antifeeding activity in the larvae **(Abdel-Aziz, 2000)**. The results of the current study obviously showed a significant decrease in alkaline phosphatase level after treatment with the *Pimpinella anisum* nanoemulsion. Imbalance in the level of this phosphatase enzyme and the consequent disturbance of different biochemical processes such as tissue differentiation and growth in the larvae. The decreasing of alkaline phosphatase may indicate the loss of detoxification ability of the larvae against the studied nanoinsecticide.

This study contributes in the reduction of the chemical insecticides use, which in return increases the chance for natural control methods of various important medical pests by using botanical insecticides. To our awareness, this research is the first to report histopathological effecs of the nanoemulsion of *Pimpella anisum* essential oil as a nanoinsecticide on midgut of *Culex pipiens* larvae, this research may contribute to an improved knowlege of the mode of action of this nanoinsecticide against *Culex pipiens* larvae.

CONCLUSION

Among the tools to control mosquito vector, bioactive compounds of plant essential oil are strongly recommended. The current laboratory study of larvicidal activity of *Pimpinella anisum* essential oil encapsulated in droplets of nanoemulsion revealed a severe toxicity to the larval stage represented by the third instar of *Culex pipiens*. The promptness of its action, low LC_{50} value, and its induced biochemical and histopathological malformations ensure its biological effectiveness on the target larvae.

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