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Original article

# Larvicidal, ovicidal activities and histopathological alterations induced by *Carum copticum* (Apiaceae) extract against *Culex pipiens* (Diptera: Culicidae)

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ABSTRACT

An experiment was carried out, firstly, to determine the possible toxicity of *Carum copticum* (Apiaceae) extract against *Culex pipiens* (Diptera: Culicidae), and, secondly, to study the histopathological alterations in the midgut of *Cx. pipiens* as a result of treatment with *C. copticum* extract. Larvicidal and ovicidal activities of *C. copticum* extract against the larvae of *Cx. pipiens* was determined according to World health organization (WHO). The inhibition effect of *C. copticum* was assessed by determining the mortality of the treated larvae and eggs. The histopathological effect of the *C. copticum* extracts on midgut epithelium of the larvae was examined under both light and transmission electron microscopy. The crude extract of *C. copticum* exerted 100% mortality for *Cx. pipiens* after 24 h at 200 µm/ml, and zero hatchability (100% mortality) at 150 µm/ml for *Cx. pipiens*. The histopathological study showed that larvae treated with *C. copticum* extract had cytopathological alterations of the midgut epithelium. The study provided information on various effects of *C. copticum* extract against *Cx. pipiens*.


## 1. Introduction

Mosquitoes are the most dangerous insect pests affecting humans and animals worldwide, transmitting a number of epidemic and fatal diseases (WHO, 2010; Aziz et al., 2014). In Saudi Arabia, different local mosquito vectors are spread all over the country (Al-Khuriji et al., 2007; Al-Ghamdi et al., 2008; Ahmed et al., 2011; Al-Ahmed, 2012). The use of pesticides to control mosquito vectors is widespread but, in recent years, there has been increasing public concern about the potential consequences of the excessive use of synthetic pesticides. These concerns centre on the potential health and environmental hazards associated with conventional synthetic pesticides. Risks include the non-specificity of pesticides (i.e. the fact that non-harmful and even beneficial insects are killed indiscriminately along with target species, such as mosquitos); the build-up of toxins in the water courses and the water supply due to run-off, and the potential environmental and public health consequences of this; and, not least the fact that repeated use of a single synthetic pesticidal ingredient can result in resistance amongst the target populations. For these reasons, researchers are increasingly focusing their attention on the development of biodegradable phytopesticides. Biodegradable pesticides of plant origin mitigate the long term environmental effects of pesticide use, and, furthermore, pests rarely develop resistance against pesticides of plant origin (Maurya et al., 2012). Al-Khreji (2005) reported that, *Culex pipiens* is the most common species of mosquitoes in Saudi Arabia. Also, Omar (1996) reported that in Saudi Arabia, the bancroftian filariasis could be introduced by the local mosquitoes *Cx. pipiens*. This type of mosquito is important as the main vector of several viral diseases (Darwish and Hoogstraal, 1981) and filariasis (Harb et al., 1993).

Essential oils derived from plants are an important source of potential insecticides (Adebayo et al., 1999; Gbolade et al., 2000), exhibiting inhibitory activity against a range of pests including bacteria, fungi and termites. Essential oils have also been shown to play an important role in controlling several mosquito species and as having larvicidal activities (Cheng et al., 2004). The Family *Apiaceae* mainly comprises annual, biennial or perennial herbs, often with culinary uses. It includes the genus *Carum*, various species of which are known to have bioactivity. The exact extent of

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bioactivity varies significantly between the individual species within the genus, however; probably due to geographical and ecological factors which affect the production of carbon-based bioactive secondary metabolites, although different compositions of chemical constituents in the medicinal seeds may result in various degrees of bioactivity (Yu et al., 2015). Among these species is Ajwain, *Carum copticum*, which is an aromatic, grassy, annual plant with white flowers and small brownish fruits, growing in Iran, Pakistan and Egypt (Zargari, 1991; Sahaf and Moharramipour, 2008). *Carum copticum* is known to have medicinal properties, and its oil has been used as a pharmaceutical and in flavouring.

The midgut of insects plays an important role in the secretion of digestive enzymes and absorption of nutrients (Christophers, 1960). Allelochemicals have been proven to exert a detrimental effect on the digestive epithelial cells and further decrease the survivability of the insect. For instance, mosquito larvae treated with plant extracts, namely *Melia azedarach*, *Derris urucu* and *Capparis cartilaginea* have been reported to experience extensive damage on the midgut epithelium and peritrophic matrix (Gusmão et al., 2002; Al-Mehmadi and Al-Khalaf, 2010; Abutaha and Al-Mekhlafi, 2014). Extracts of *Copaifera reticulata* cause partial or complete destruction of midgut epithelial cells via cytoplasmic vacuolization, enlargement of intercellular spaces, and alteration of microvilli, while also affecting the nuclei and nucleoli (Abed et al., 2007). Extracts of *Magonia pubescens* and *Sapindus saponaria* were also reported to cause serious damage to the midgut epithelial cells via processes including cytoplasmic vacuolization (Arruda et al., 2003). Number of essential oils have a toxicity against stored-product insect pests (Isman, 2000; Sahaf et al., 2007; Sahaf and Moharramipour, 2008). There are no reports on the insecticidal activity of *C. copticum* against *Cx. pipiens*. This paper, therefore, reports research conducted to determine the possible toxicity of the extract of *C. copticum* against *Cx. pipiens* larva. In addition, the midgut of *Cx. pipiens* treated with *C. copticum* extract were examined in order to determine whether there were any histopathological alterations as a result of the treatment.

## 2. Materials and methods

### 2.1. Plant material and preparation of C. copticum methanol extract

Fruits of *C. copticum* were purchased from a local crude herbal drugs store in Salman Dir'iya in Riyadh, Kingdom of Saudi Arabia. Identification of the plant and deposition of voucher specimen was done in the Department of Botany and Microbiology, College of Science, King Saud University. Crude extracts were obtained by maceration of 70 g of seeds in 700 ml of different solvents that is 95% methanol, ethyl acetate and distilled water. The extracts were left overnight at 150 rpm and 30 °C (centrifuge, Sigma, Germany). After 48 h, the extracts were filtered using Whatman filter paper No. 1 and the solvents evaporated using a rotary evaporator (Heidolph, Germany) at 45 °C. Dried extracts were stored in a dark amber-coloured bottle. All the concentrations of the extracts were based on the dry weight of the extracts.

### 2.2. Experimental mosquitoes

*Cx. pipiens* larvae were obtained from a colony maintained within the Department of Zoology, College of Science, King Saud University. The larvae were reared in a plastic tray (24 × 35 × 5 cm) and were fed on 'Liquifry' (Interpet Ltd, Dorking, U.K.) until pupation. The pupae were then transferred to a cup containing tap water and allowed to develop further in our insectary. Adults were held at 28 ± 1 °C, 70–85% relative humidity and a photoperiod of 12 h light 12 h dark. They were provided with 10% glucose solution as well as a 1 week old chick to serve as a source for blood meals.

#### 2.2.1. Larvicidal bioassay

Based on the preliminary tests, five concentrations (25, 50, 100, 150, 200 μg/ml) of the crude extract. Ten 4th instars *Cx. pipiens* larvae were placed in each well of sterilized standard 12-well tissue culture test plates (Nunclone Delta Surface, Thermo Fischer Scientific, Denmark) with 2 ml of tap water and each of the five concentrations of methanol crude extract. The number of dead larvae was counted 24 and 48 h after exposure and the percentage of mortality was expressed as an average of three experiments. Methanol was used as a negative control.

#### 2.2.2. Ovicidal activity

Ovicidal activity was assessed using the method of Su and Mulla (1998), slightly modified. *Cx. pipiens* mosquito eggs were lifted from the newly established colony reared in the main breeding cage and then exposed to concentrations (25, 50, 100, 150 μm/ml) of the *C. copticum* extract in 100 ml plastic containers. Each experiment was replicated three times along, with the solvent was used as a control. The hatch rates after treatment were expressed using the following formula:

$$\%\text{of egg mortality} = \frac{\text{No. of hatched egg}}{\text{Total No. of eggs}} \times 100 \quad (1)$$

### 2.3. Histopathological studies

#### 2.3.1. Light microscopy

A histological evaluation of the digestive system was performed using fourth instar larvae (treated and control). The larvae were trimmed by removing the head, thorax and tail segments. The remaining parts were fixed in 10% neutral buffered formalin for 72 h. An automatic tissue processor (Sakura, Japan) was used to dehydrate and clear the tissue samples. The specimens were then embedded in paraffin blocks using an embedding station (Sakura, Japan) and a rotary microtome was used to cut sections 4 μm thick (Leica-RM2245, Germany) and stained with H&E stain. The stained sections were observed under light microscopy, with images being taken using a digital microscopic mounted camera (OMX1200C, Nikon, Japan).

#### 2.3.2. Electron microscopy

The ultrastructure of the midgut epithelia of treated Cx. *pipiens* and control larvae were examined using a transmission electron microscope (TEM) (Jeol Ltd., model JEM-100CX II) at 80 kV 24 h post-treatment. The midgut was fixed in glutaraldehyde (2.5%) in a cacodylate buffer (0.2 M), pH 7.2, the sample was further processed in a cacodylate buffer containing sucrose (7.2%), post-fixed in osmium tetroxide (1% for 1:45 h), dehydrated in graded acetone and embedded in Epon. Later, Sections were stained with lead citrate and uranyl acetate (Reynolds, 1963).

### 2.4. Statistical analysis

$LC_{50}$, $LC_{95}$, slopes, and standard error values were estimated according to Finney (1971). Two isolates were considered as not being significantly different in their toxicity if their $LC_{50}$ 95% confidence limits overlapped (Litchfield and Wilcoxin, 1949).

## 3. Results

Methanol extract was the only solvent gave a toxicity to larvae of *Cx. pipiens*. The methanol extract of *C. copticum* seeds showed a

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**Table 1**
Mosquito larvicidal activity of extracts of *C. copticum* against 4th instar larvae *of Cx. pipiens*.

| Species mosquito | Time | (%) Mortality Concentration (µg/ml) | | | | | $LD_{50}$ (µg/ml) | $LD_{90}$ (µg/ml) |
|---|---|---|---|---|---|---|---|---|
| | | 25 | 50 | 100 | 150 | 200 | | |
| *Cx. pipiens* | 24 | 0 ± 00 | 6.67 ± 4.71 | 20 ± 8.16 | 70 ± 8.16 | 100 ± 00 | 122.26 | 190.26 |
| | 48 | 6.67 ± 4.71 | 30 ± 4.71 | 60.33 ± 9.42 | 83.33 ± 8.16 | 100 ± 00 | 92.29 | 168.56 |

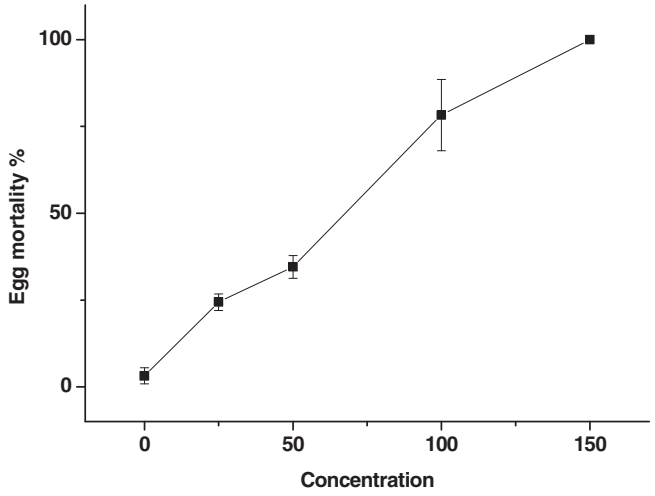


**Fig. 1.** Ovicidal activity of methanol extract of *C. copticum* against *Cx. pipiens*.

larvicidal activity against *Cx. pipiens*, with 200 µm/ml causing 100% mortality after 24 h. Furthermore, a concentration of 150 µm/ml resulted in no *Cx. pipiens* eggs being hatched (100% mortality) (Table 1 and Fig. 1).

The midgut epithelium of control larvae exhibited flattened regular cells with a pale clear cytoplasm and regular microvilli lining the apical surface under light microscopy (Fig. 2A). In contrast, treated larvae exhibited destruction in the midgut epithelial cells and cytopathological alterations, such as the existence of vesicles of various sizes, destruction of microvilli and swollen cells (Fig. 2B).

Under TEM, the structure of the epithelial cells and their components in sections of the control larvae appeared normal and kept their integrities (Fig. 3A–C). Treated larvae, when viewed under TEM, however, revealed disrupted microvilli in the midgut (Fig. 3D) along with cell disintegration, degradation of chromatin and nucleoli (Fig. 3E). The mitochrondria appeared with degraded cristae and almost free of internal contents (Fig. 3F).

## 4. Discussion

Essential oils possess a wide spectrum of biological activities including anti-microbial, fungicidal, insecticidal, insect repellant, herbicidal, acaricidal, and nematicidal (Noutcha et al., 2016). Seo et al. (2012) stated that *C. copticum* can be used as a botanical insecticide. Although other members of the *Carum* genus have been reported to be toxic to mosquito larvae. For example, *C. ptroselinum* has been reported to exhibit larvicidal action against the larvae of *Cx. pipiens*, with $LC_{50}$ values of 152.94 ppm (Khater and Shalaby, 2008). In our report, the $LC_{50}$ value of the crude extract of *C. copticum* was 92.29 µg/ml); this is within the effective range according to the classification of Thangam and Kathiresan (1996), classification of an $LC_{50}$ of less than 100 mg/L, in addition, *C. copticum* oil, 0.1 mg/ml caused 100% larval mortality against *A. aegypti* mosquito larvae.

In the current study, *C. copticum* extract showed a promising ovicidal activity, this might be due to the volatile compounds present in the oils. These results are in accordance with Su and Mulla (1998), who tested the neem products against *Culex tarsalis* and *Culex quinquefasciatus*, Saghal and Pillai (1993), used permethrin and deltamethrin against *Aedes aegypti*, *C. quinquefasciatus* and *Anopheles stephensi*, Oudo et al. (1998) used the seed extract of *Atriplex canescens* against *C. quinquefasciatus*, and Grosscurt (1977) used *Solanum trilobatum*, against *Culex* mosquitoes. In addition, Warikoo et al. (2011) reported that some of essential oils from *Mentha piperita*, *Ocimum basilicum*, *Rosmarinus officinalis*, *Cymbopogon nardus* and *Apium graveolens* exhibited oviposition deterrent activity against *Ae. aegypti*.

The histopathological changes in treated insects with alternative insect control as a toxic action were previously investigated (Charles, 1987; Davidson and Titus, 1987; Singh and Gil, 1988; Silva-Filha and Peixoto, 2003), and with botanical insecticides were also studied (Nasiruddin and Mordue, 1993). Bakkali et al. (2008) stated that, the cytotoxic effects on living cells depending on the type and concentration of essential oils. These findings suggest that at least in part, the encountered beneficial effects of essential oils

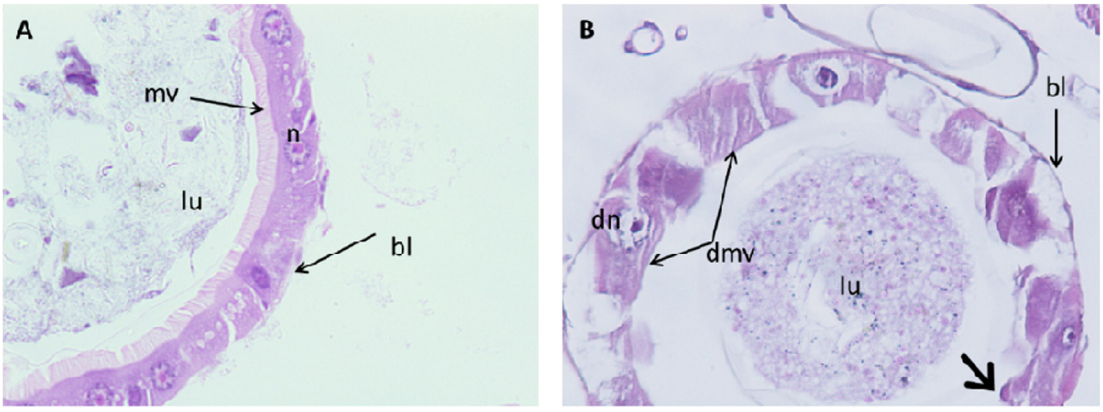


**Fig. 2.** Longitudinal section of midgut of *Cx. pipiens* larvae (40×). (A) The midgut epithelial cells of a control larva. (B) A larva under treatment with *C. copticum* extract showing the effect after 24 h of exposure. Midgut epithelium with cell vacuolization and apical protrusion (arrow), extract, degraded microvilli (DMV) degenerating epithelial cells (DEC), degenerating peritrophic membrane (PM) and degenerating nuclei (DN).

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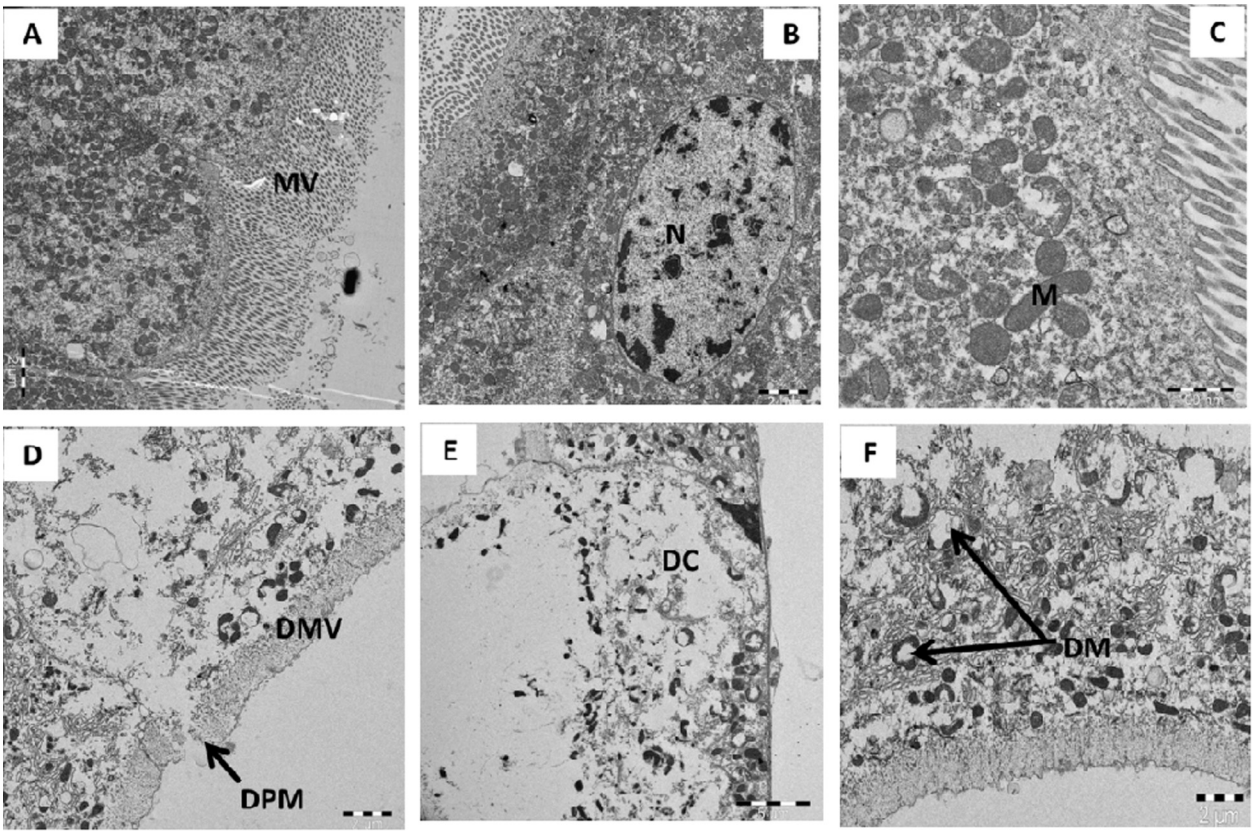


**Fig. 3.** Transmission electron microscopic micrographs showing the cytological effects of treatment with *C. copticum* extract on the ultrastructure of the midgut epithelial tissue of *Cx. pipiens* larvae, 24 h after treatment. (A)–(C) indicate normal nucleus (N), microvilli (MV), and chromatin contents in epithelial cells of control larvae (Scale bar = 2, 2 µm and 100 nm respectively). (D)–(F) indicate contents, degenerated microvilli (DMV) and its degenerating cell (DC) with bubbling and stretching appearance in treated larvae and represent degenerating mitochondria (DM) (Scale bar = 2, 5, and 5 µm respectively) in treated midgut cells. Ultrathin 4 µm sections were analysed with transmission electron microscope model JEOL JEM-100CX II at 80 kV.

are due to pro-oxidant effects at the cellular level. In this study, histomorphological alterations in larvae treated with *C. copticum*, could be observed in the midgut, with cellular destruction, and vacuolization of epithelial cells. These observations are in agreement with the findings of by Abutaha et al. (2015) which showed destruction and detachment of cells within the midgut epithelium of the treated larvae of *Ae. caspius* and *Cx. pipiens* when treated with fungal extract of *Cochliobolus spicifer*.

## 5. Conclusion

The data obtained in this study has provided information on the toxicity of *C. copticum* extract against larvae and eggs of the mosquito *Cx. pipiens*. The methanol extract of *C. copticum* showed the strongest larvicidal and ovicidal activity. The *C. copticum* extract cause a damage in the midgut of *Cx. pipiens* larvae. It is evident that *C. copticum* extract possesses potential as a mosquito insecticide.

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