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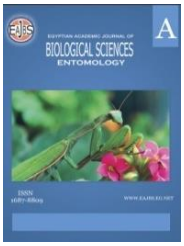

Larvicidal Activities and Histopathological Alterations Induced by Margosa Oil on Human Filarial Vector, *Culex pipiens* Mosquito

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ABSTRACT

Margosa oil (*Azadirachta indica*) extract as biopesticides were examined against *Culex pipiens* mosquito larvae under laboratory conditions to detect the toxicity of margosa oil extract and study the histopathological alterations in the midgut of treated larvae. The inhibition influence of plant extract was investigated by detecting the mortality rates of larvae post-treatment with LC_{50} value11.59 ppm. The crude extract of margosa oil recorded 90% mortality for *Cx. pipiens* at 80 ppm. In contrast, the lowest concentration of extract recorded 30% mortality. Histological analyses showed that treated larvae had histopathological changes in the midgut epithelium. The treatment at high concentrations of margosa extract showed more damage in the gizzard and mid-gut region, leading to death. On the other hand, the treated larvae with low concentration exhibited little changes in the gizzard dendrites. The study provided that margosa oil is favorable as a larvicidal agent against *Cx. pipiens*, naturally revolved biopesticides would be an alternative to synthetic insecticides.

INTRODUCTION

Mosquitoes are vectors of many dangerous human diseases including malaria, filariasis, Japanese encephalitis, and yellow fever leading to millions of deaths annually (An *et al.*, 2020 and Hamama *et al.*, 2022). Extensive usage of chemical insecticides for mosquito control caused troubles related to physiological resistance to vectors, and negative implications on the environment and human health (Benelli *et al.*, 2017). Many plant extracts have been registered as insecticides for suppressing all mosquito stages as well as repellents for mosquito biting and are considered a safe method for mosquito control (Prabhu *et al.*, 2011 and Vivekanandhan *et al.*, 2018).

Margosa oil, known as neem oil (*Azadirachta indica*) native to India, belonging to the family Meliaceae is a fast-growing evergreen tree. They are spread in tropical and subtropical areas worldwide (NRC, 1992). Neem seeds have about 99 biologically active compounds involving azadirachtin, nimbin, nimbidin and nimbolides are major molecules (Locantoni *et al.*, 2006). Many of these products have antifeedant, ovicidal activity, fecundity suppression besides molting defect, morphogenetic disorder, and changes in insect behavior (Schmutterer, 2002; Isman, 2006 and Ofusori *et al.*, 2010). Azadirachtin is one of the most the most affective phtophageous insecticides from the neem tree, *Azadirachta indica*, and it has been shown to be the key agent for controlling mosquitoes

(Suman *et al*., 2010). Neem products have low toxicity to birds, fish, and mammals. In addition to this, insect growth regulatory activity of neem weakens the cuticle defense system of the larvae causing easy penetration of pathogenic organisms into the insect system. The eco-friendly neem products with the powerful anti-mosquito property are actually favored in the vector control program worldwide due to their efficacy against a wide range of insects, low environmental impact, and less opportunity for resistance development (Singh *et al.*, 2011; Govindarajan *et al*., 2016 and Ayinde *et al.*, 2020). The current work aimed to investigate the larvicidal activity of the margosa oil formulation against mosquito, *Cx. pipiens*.

MATERIALS AND METHODS

Insect Rearing:

Larvae of *Cx. pipiens* were reared in the Zoology Laboratory, Faculty of Science, Zagazig University, Egypt at 27 °C ± 2 °C temperature, 80 ± 5% relative humidity (R.H.), and 14 h light: 10 h dark. The adults were reared in cages (35 × 40 × 35 cm), and the females laid eggs three days post-feed on blood. Egg rafts were hatched in a glass tray filled with water. Larvae were reared in plastic cups (20 × 25 × 5 cm) and fed on yeast.

Emulsified Margosa Oil Formula (stock solution):

Emulsified margosa oil was prepared by mixing 1 ml of margosa oil with 10 ml of distilled water. The solution was shaken strongly to confirm the completely dissolved oil in the water. This was done up to 1 L with the addition of extra distilled water to get a 1000 ppm stock solution (Oyedele *et al*., 2000).

Larvicidal Bioassay:

Tests were investigated on third-instar larvae of *Cx. pipiens* using five concentrations (5, 10, 20, 40, and 80 ppm) of the margosa oil for 120 hrs. Twenty-five larvae for each concentration were utilized for the examinations in 250 ml plastic beakers have different concentrations of margosa oil. The treatments were replicated four times, and each replicate set has a control group. Cumulative mortalities of larvae were recorded daily.

Histopathology of Midgut:

Treated *Cx. pipiens* third instar larvae with the lowest and highest concentrations of margosa oil and control larvae were fixed in glutaraldehyde (2.5%) in a cacodylate buffer (0.2 M), pH 7.2, the samples were fixed in 2.5% glutardehyde (Sigma) in 0.1 M cacodylate buffer (pH 7.2) for about 10 minutes, and rinsed into fixative for 1-2 hr. after put in 0.1 M cacodylate buffer, samples were post-fixed in 1 % OsO_4 in the same buffer for 1hrs, then rinsed, dehydrated in an ethanol series, and embedded in araldite epoxy resin. Semi-thin sections were cut on a Leica EM KMR2 ultra-microtome to examined under light microscopy. These sections were stained with toluidine blue and photographed using a light microscope (Bancroft & Stevens, 1996) at the Central Laboratory, Zagazig University.

Statistical Analysis:

The mortality rates were analyzed using SPSS version 14; using a one-way analysis of variance (ANOVA) and then pairwise comparisons according to Tukey's HSD tests. The median lethal concentration (LC_{50}) and other statistics at 95 % confidence limits were set by log probit analysis. All differences were considered significant at $P \leq 0.05$. The efficiency of neem oil on the mosquito larvae was determined from the formula Abbott, (1925):

$$Larvicidal\ agent\ (LA\ \%) = \frac{(\text{M Test} - \text{M Control})}{100 - \text{M Control}} \text{ X } 100 \qquad (1)$$

Where (M Test) the percent of the showed lethal rate of larvae in the treatment and (M Control) the control larvae.

RESULTS AND DISCUSSION

Toxic Effects of Margosa Oil on *Cx. Pipiens*:

The toxic effects of the neem product (margosa oil) on mosquito larvae were presented in Table 1. The margosa oil has shown larvicidal activity on *Cx. pipiens* larvae with a mortality rate from 30 to 90% after exposure to different concentrations ranging from 5 to 80 ppm for 120 hrs, the mortality of third instars larvae of *Cx. pipiens* increased significantly according to the concentrations (df= 4; F = 57.3; P = 0.000 < 0.05). The margosa oil killed 50% of *Cx. pipiens* larvae at the concentration of 11.59 ppm (LC_{50}). The mortality probability of the margosa oil on *Cx. pipiens* mosquito larvae were distributed in Figure 1. These findings agreed with Ndione *et al*. (2007) who reported that the neem products show significant bioactivity against *A. aegypti* larvae. Also, it has been reported that the emulsified formulations of neem oil showed significant larvicidal activity against mosquitoes, involving, *Aedes*, *Anopheles* and *Culex* (Gianotti *et al.,* 2008; Dua *et al*., 2009 and Benelli *et al.,* 2015). Moreover, Ayinde *et al.* (2020) reported that neem oil achieved larval toxicity against *Anopheles gambiae* 5 days post-treatment. Neem oil ingestion causes irregular molts, development reduction, and increased death rate. Azadirachtin interacted with the synthesis of an "ecdysteroid" hormone, which is necessary for the insect molting process that allows larvae to be molted and develop. Indirectly, azadirachtin influences the insect's neurosecretory system by blocking the emission of morphogenetic peptide hormones which are responsible for secreting juvenile hormones (Chaudhary *et al*., 2017). Consequently, the larvae were unable to molt, stay in the larval stage, and eventually died. If the larvae succeed to get in the pupal stage, there is a probability that they will be sterile without any capacity for reproduction (Prajapti, 2005).

Table 1: Dose-dependent larvicidal activity of margosa oil against third instar larvae of *C. pipiens*

Conc., ppm	**Mortality% mean ± SE**	**LC_{50} ppm**	**(95% LCL–UCL) ppm**	**Chi-square X^2**	**Regression equation**
5	$30^{a} \pm 0.866$				
10	$48^{b} \pm 0.408$	11.59	9.44 -14.58	9.468	Y = 1.0 X – 2.5
20	$60^{b} \pm 0.478$				
40	$74^{c} \pm 1.190$				
80	$90^{d} \pm 0.645$				

LC_{50}, a lethal concentration that kills 50% of the exposed larvae; UCL, upper confidence limit; LCL, lower confidence limit; Significant at $P \leq 0.05$; SE: stander error. Letters indicate the degree of significance based on Tukey's HSD tests between concentrations.

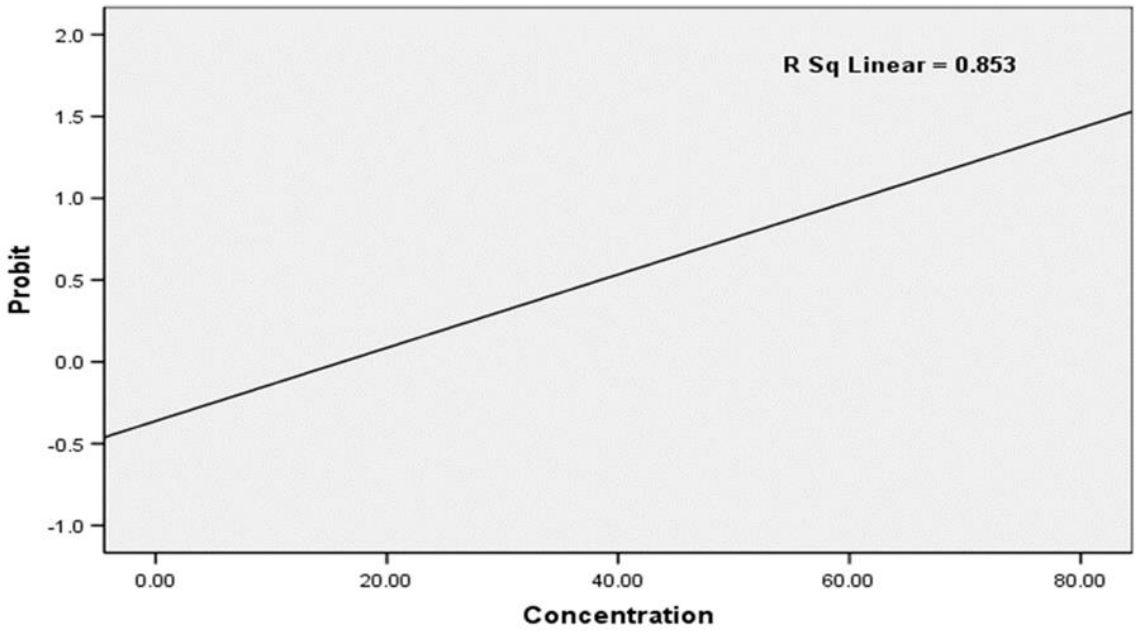

Fig.1. Probability analysis of mortality of *Cx. pipiens* mosquito larvae by margosa oil.

Histopathological Effects:

Histological analysis of the control *Cx. pipiens* larvae showed that in the gizzard part the epithelial cells illustrated with the preserved cytoplasm, and tooth-like denticles made from the intima surrounded by muscular layer and peritoneal membrane (Fig. 2a). Midgut exhibited a well-preserved layer of columnar epithelial cells which line with tiny projections (microvilli) that have a large surface area for absorption of digested food and a normal-shaped nucleus (Figs. 2b, c). This coincided with Abutaha *et al.* (2015) and Al-Mekhlaf (2018) who illustrated the normal structure of the middle area of the *Cx. Pipiens* gut.

Mosquito larvae treated with margosa oil showed changes in their intestinal tract (Figs. 3, 4). These regions are studied because they are directly in contact with a toxic substance (*azadirachtin*) of neem products. Further, the alternation of these regions is associated with the concentration of margosa oil used. Thus, treated larvae at low concentration exhibited little changes in the gizzard denticles which started invaginated inside the lumen and decrease lumen size (Fig. 3 a). Moreover, damage in the midgut region and cytopathological changes were observed, like the presence of vacuoles of many sizes, and the rupture of microvilli and swollen cells. However, some cell nuclei appeared normal (Fig. 3b). On the other side, the treatment at high concentration showed more damage in the gizzard and midgut regions. The malformed gizzard illustrated that the lumen is divided into two parts by deformed denticles that lose their normal shape (Figs. 4a, B) and subsequently leads to gizzard malfunction and larval death. While the treated larval midgut with a high amount of margosa oil exhibited alterations (Figs. 4c, d) with the appearance of cell destruction, epithelial cells vacuolization, disorganization of tissue with spacing among the cells, and some destruction of muscle fibres, and some cells observed absence of cytoplasmic borders. These clarifications were coincident with the finds described by Ndione *et al*. (2007) reported *Azadirachta indica* was toxic to larvae of *Aedes aegypti* and caused serious deterioration to the epithelial columnar cells, a perturbation of alimentary flow, and rupture of some cells in the posterior portion of the gut. Al-Mekhlaf (2018) showed the demolition and degenerating of cells within the midgut epithelium and cytopathological alterations of the treated larvae of *Culex pipiens* when exposed to *Carum*

copticum extract. Also, midgut damages in treated insects with neem oil were investigated by David *et al.* (2002) and Silva-Filha &Peixoto (2003). Previous studies have reported that some plant extracts cause extensive harm to the epithelium layer and peritrophic membrane of filarial vector *Cx. quinquefasciatus* and other mosquito species (Al-Mehmadi & Al-Khalaf, 2010; Pradeepa *et al.*, 2015 and Senthil-Nathan, 2020). Selin-Rani *et al.* (2016) and Abdullah (2009) reported that the phytoextract may destroy the gut epithelium and is the main cause of irregular metabolic rate and decline in the enzyme-action. Midgut cell demolition is related to digestive and detoxifying enzyme dysregulation (Senthil-Nathan *et al*., 2008). Moreover, this was proven by histological studies of the mosquitoes that showed damage to midgut cells, after treatment with different plant compounds (Yu *et al*., 2015). Further, in mosquitoes, treatment with botanical compounds was related to altered protein (Senthilkumar *et al*., 2013, Fallatah, 2014).

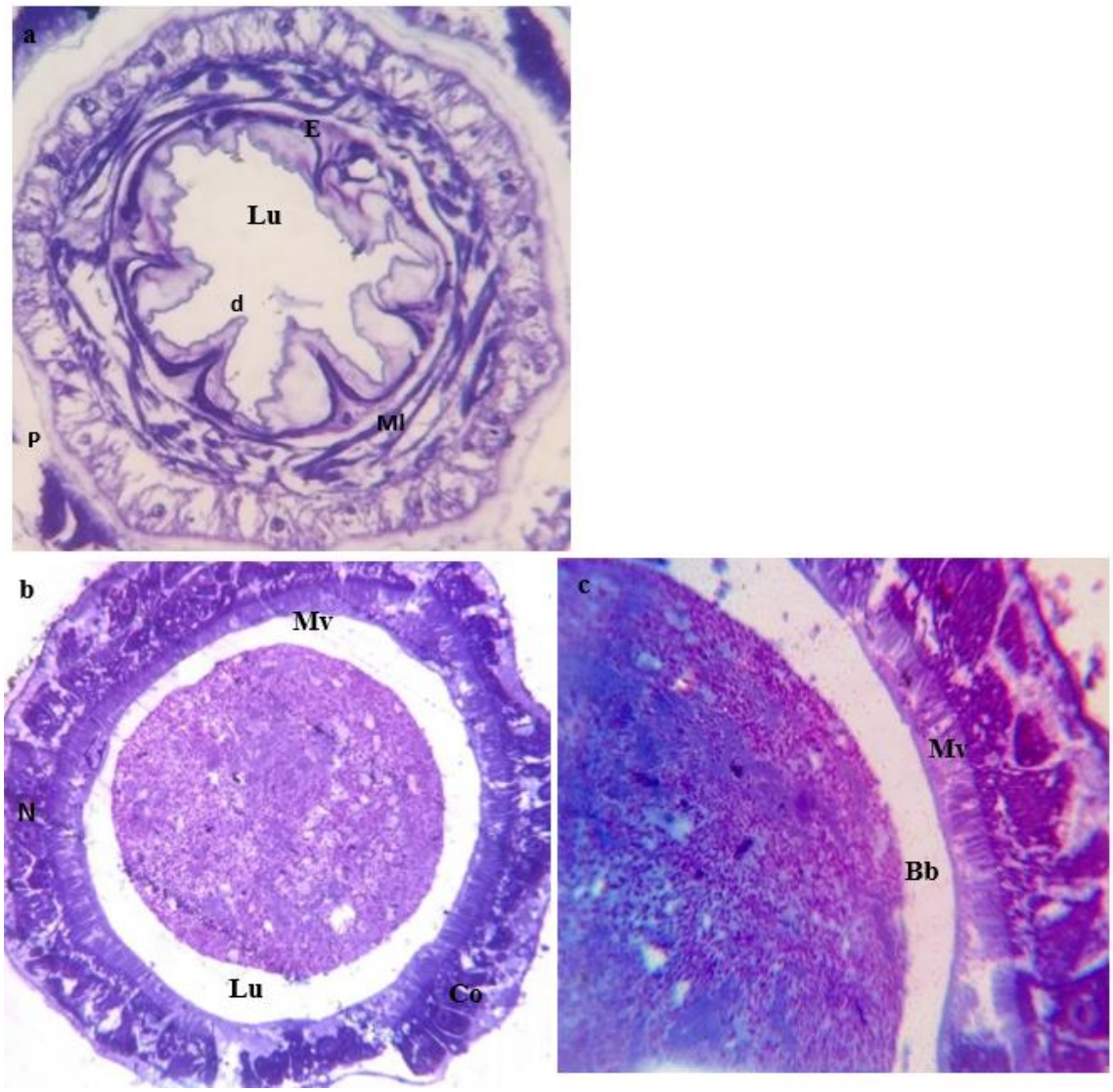

Fig. 2. Transverse section part of control larva of *Culex pipiens*. (a) the gizzard with normal denticles (d) with teeth and epithelium cells (E) surrounded by muscular layer (Ml) and peritoneal membrane (P) and lumen (Lu). (b) observed normal midgut with columnal epithelial cells (Co) had a nucleus (N) and the cells had Microvilli (Mv) of brush border (Bb).

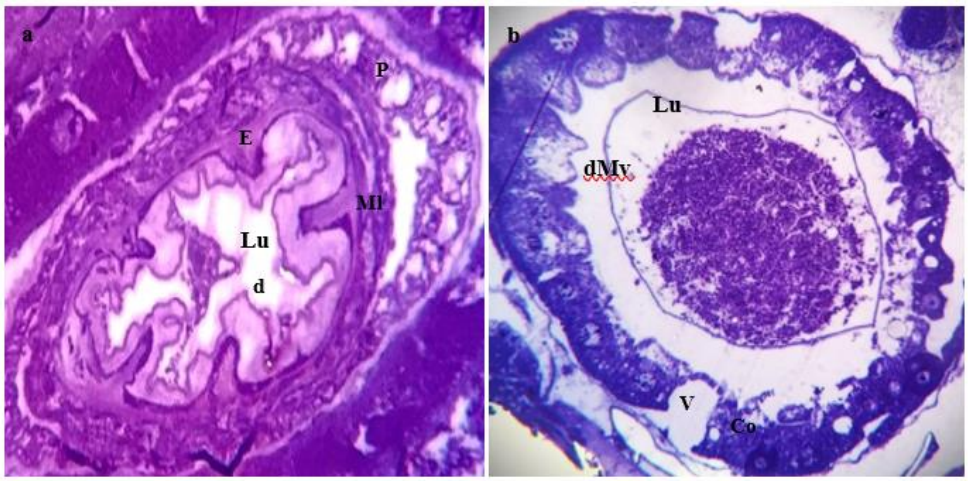

Fig. 3. Transverse section part of control larva of *Culex pipiens* treated with low conc. (5 mg/l) of Margosa oil (a) the treated gizzard showed deformed denticles (d) with broad teeth and epithelium cells (E) surrounded by muscular layer (Ml) and vacuolated peritoneal membrane (P). (b) treated midgut with damaged columnal epithelial cells (Co) had vacuoles (V) and destructive Microvilli (dMv).

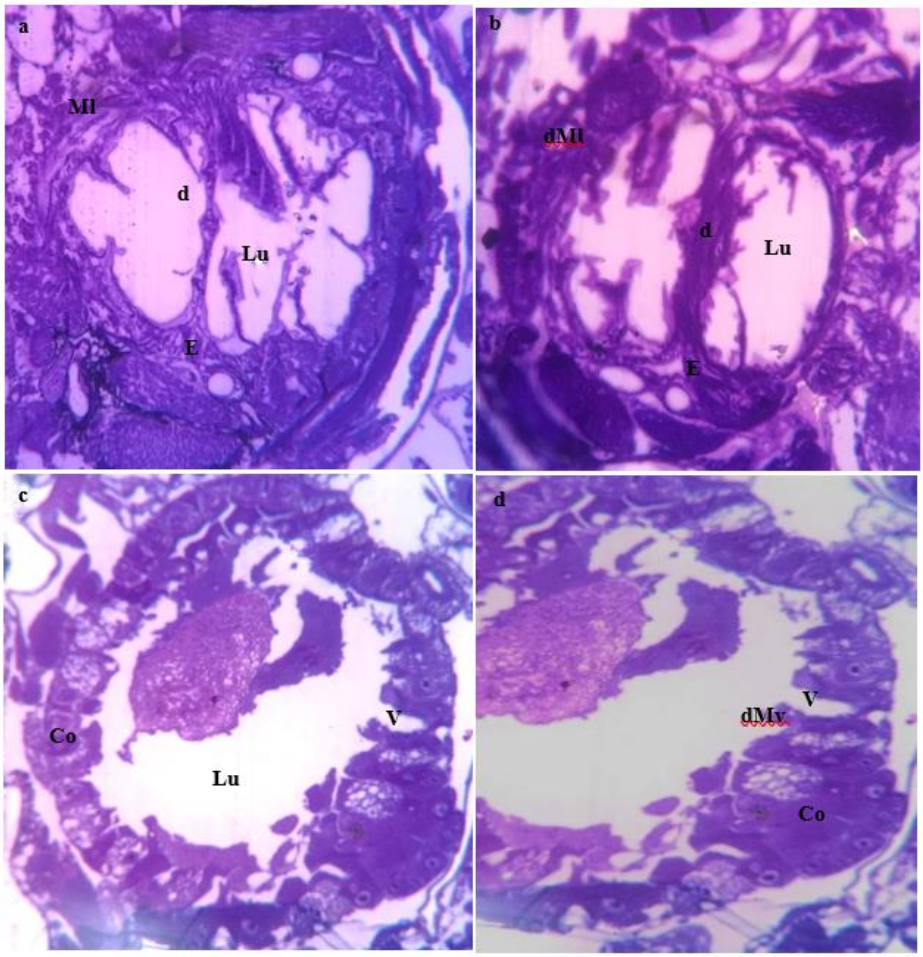

Fig. 4. Transverse section part of control larva of *Culex pipiens* treated with high conc. (80 mg/l) of Margosa oil (a, b) the treated gizzard showed deformed denticles (d) and epithelium cells (E) surrounded by an abnormal muscular layer (dMl) and destructive peritoneal membrane (P). (c, d) treated midgut with damaged columnal epithelial cells (Co) had vacuoles (V) and degenerative Microvilli (dMv).

Conclusion

The neem oil examination in this study was an effective larvicide against *Cx. pipiens* larvae. In addition, micromorphological alterations of treated mosquito larvae with neem oil confirm the reason for larval death could be observed in the middle region of the gut, with cellular destruction and disorganization, spacing between cells, vacuolization of epithelial cells, and gizzard malformation. These results indicated that neem oil is a destructive agent for the control of filariasis vectors *Cx. pipiens.*

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ARABIC SUMMARY

أنشطة مبيدات اليرقات والتغيرات النسيجية المرضية التي يسببها زيت المارجوزا على البعوض الفيلاري البشري الكيولكس بيبينز

وجيهة عبد الله مصطفى وفاطمة محمد هاشم
شعبه الحشرات قسم علم الحيوان كليه العلوم جامعه الزقازيق

تم فحص مستخلص زيت المارجوزا كمبيد حيوي على يرقات البعوض كيلوكس بيبينز تحت ظروف معملية للكشف عن سمية مستخلص زيت المارجوزا ودراسة التغيرات النسيجية المرضية في المعى المتوسط لليرقات المعالجة. تم تقييم تأثير التثبيط للمستخلص النباتي من خلال تحديد تركيز اللازم معدل وفيات 50% من اليرقات (LC_{50}) بقيمة 11.59 جزء في المليون. سجل المستخلص الخام من زيت المارجوزا معدل وفيات بنسبة 90% في كيلوكس بيبينز عند 80 جزء في المليون. بينما سجل أقل تركيز للمستخلص معدل وفيات 30%. أظهرت التحليلات النسيجية أن اليرقات المعالجة لها تغيرات مرضية خلوية في المعي المتوسط. أظهرت المعالجة بالتركيز العالي لمستخلص المارجوزا أن المزيد من الأضرار في الحوصلة ومنطقة الأمعاء الوسطى تؤدي إلى الوفاة. من ناحية أخرى، أظهرت اليرقات المعالجة ذات التركيز المنخفض تغيرات طفيفة في الحوصلة. أوضحت الدراسة أن زيت المارجوزا واعد كعامل مبيد لليرقات ضد كيلوكس بيبينز ، المبيدات الحيوية ستكون بديلاً لمبيدات الحشرات الكيميائية.