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Original Research Article

Evaluation of Toxicity of the Ethanol Extract of *Euphorbia antiquorum* L. in *Drosophila melanogaster*

Tran T. Men[1]*, Nguyen H. Son[2], Huynh H. Phien[1]

[1]*College of Natural Sciences, Can Tho University, Can Tho city 94000, Vietnam*
[2]*Faculty of Basic Sciences, Vinh Long University of Technology Education, Vinh Long city 85000, Vietnam*

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ABSTRACT

Many plant extracts exhibit insecticidal activity and are considered an environmentally friendl for pest management. They act through various effects on the target insects, including repellenc inhibition, and structural and physiological changes. This study was carried out to evaluate the pc *Euphorbia antiquorum* L. to inhibit the growth and development of fruit flies. The plant extract great influence on the growth and development of *Drosophila melanogaster* fruit flies thr different survey criteria, such as the rate of pupation, larval formation, the energy storage comp carbohydrates, proteins, lipids, and levels of enzymes belonging to the esterase group (AchE, *α*- and *β*-carboxyl) and phosphatase (AcP and AkP) of adult flies after 14 days surveying. At a 1 concentration, the extract affected the growth and the development of fruit flies; the number o larvae was 2.25 times lower than that of the control treatment as a standard food; 80.99% of the at the pupal stage and were not able to develop to the adult stage. Total flavonoid and polypheno were determined to be 466±6.35 mg GAE/g extract and 198±2.33 mg QE/g extract, respectively. The present findings show that *Euphorbia antiquorum* L. has a potential for use as an insecticidal

***Keywords*:** *Drosophila melanogaster*, enzyme inhibition, extract, *Euphorbia antiquorum* L., inhibit.

Introduction

Vietnam is a country with developed agriculture, so the current intensive cultivation requires a new effective and environmentally friendly pest management method. Using secondary plant metabolites with insecticidal effects is one of the green methods for pest management. Natural compounds, including terpenes, flavonoids, alkaloids, polyphenols, cyanogenic glucosides, quinones, amides, aldehydes, thiophenes, amino acids, saccharides, and polyketides, are demonstrated to be biologically active against insects. Many compounds belonging to the class; alkaloids, iridoids, terpenes, flavonoids, naphthoquinones, anthraquinones, coumarins, phenylpropanoids, and flavonoids isolated from plants and fungi have effective insecticidal activity.[1] These compounds play important roles in natural ecological activities, such as attractants, biocides, fungicides, repellents, growth regulators, insecticides, pathogens, and allergens, acting as a promising source for new pest control agents or biopesticides.[2] Scientifically introduced as *Drosophila melanogaster*, flies are also known as fruit flies because they love the smell of fermented fruits such as apples, bananas, and grapes. This fly species is not an agricultural pest but has typical features of insects. Some species of the fruit fly genus are also harmful to fruits; therefore fruit fly could be used as an excellent model organism for studying insecticidal activity.[1] *Euphorbia antiquorum* L. (*E. antiquorum*) is a plant distributed mainly in tropical and subtropical regions of Asia.[3] Essa *et al*.[4] reported that the chemical constituents of plants belonging to the family Euphorbiaceae have insecticidal activity. In particular, *Euphorbia antiquorum* L. is known to be able to kill mosquito larvae and other insects.

*Corresponding author. E mail: ttmen@ctu.edu.vn
Tel: +84.907.416.657

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In 2008, De Silv*a et al*.[5] also investigated the insecticidal activity of *E. antiquorum* extract. The survey results showed that the extract could fight six different insect species, including three vegetable pests (*Myzus persicae*, *Aphis gossypii* and *Aphis craccivora*) and three rice pests (*Nilaparvata lugens*, *Leptocorisa oratorius* and *Scotinophara lurida*). Therefore, this study investigated the toxicity of *Euphorbia antiquorum* L. in a fruit fly model.

Materials and Methods

Research materials

Chemicals used in the study included ethanol 96%, distilled water, gallic acid, quercetin, Folin-Ciocalteu, aluminum trichloride, sodium nitrite, sodium hydroxide, propionic acid, sodium benzoate, acetylcholine chloride, fast blue B salt, α-naphthyl acetate 99%, β-naphthyl acetate 95%, p-nitrophenyl phosphate, 4-nitrophenylphosphoric and some other chemicals.

Sample collection and preparation

E. antiquorum was collected in Can Tho city, Vietnam, in March 2021. Its scientific name was identified by Dr. Nguyen Thi Kim Hue (Department of Biology, College of Natural Sciences, Can Tho University, Vietnam). A voucher specimen (No. CTU-EA018) was deposited in the herbarium of the Department of Biology, College of Natural Sciences, Can Tho University, Can Tho City, Vietnam. After removing the damaged parts, the plant sample was washed, chopped and dried. The materials were then ground into powder using a powder pulverizer machine (Yamafuji 2500, China), and thereafter placed in a cloth bag and soaked in ethanol 96%. The process was repeated 5 times for each 24 h soaking time. The extracts from each soaking were collected and evaporated to obtain the total ethanol extract.

Experimental subjects

Wild fruit fly *Drosophila melanogaster* strain Canton S (CS) was provided by the Kyoto Institute of Technology, Japan.

Investigation of the effect of the extract on growth and development

The investigation of the effect of the extract on the growth and development of fruit flies was based on the research method of Lopez *et al*.[6], Chowański *et al*.[7], and Quiroz-Carreño *et al*.[1] The food treatment medium used for examining the growth and development of fruit fly (testing treatment) consisted of glucose (80 mg/mL), cornstarch (45 mg/mL), yeast (40 mg/mL), agar (8 mg/mL), and 15 mg/mL of ethanol extract of *E. antiquorum* dissolved in ethanol. The control treatment used the same feed composition but without extract. F1 fruit flies developed from these two diet treatments will be used for further investigations. The number of pupae formed was recorded after 10 days, and the number of flies hatching was recorded after 14 days of the survey.

Evaluation of energy storage components
Fifteen female fruit flies were randomly selected to determine the content of basic energy storage components such as carbohydrates, proteins, and total lipids. These components have been shown to have important physiological and growth-related roles in fruit flies.[8,9] Fruit flies were pureed in 500 µL of distilled water to determine carbohydrate and protein content.

Determination of carbohydrate content
Carbohydrates were determined by the method of Nielsen.[10] After homogenizing by the use of a grinding machine (Retsch Mixer Mill MM 400, Germany), the sample solution was centrifuged at 10,000 rpm for 15 min. The supernatant was used for protein determination, whereas the lower residue was washed three times with distilled water by centrifugation at 10,000 rpm for 15 min. Then, 3.2 mL of concentrated (cooled) H_2SO_4 was added, followed by 50 µL of phenol. The mixture was then shaken well and allowed to stand for 30 min. The spectral absorbance of the reaction mixture was measured at 486 nm. Glucose was used as a positive control to construct the standard curve. The carbohydrate content in the sample was determined based on the glucose standard curve equation with different concentrations.

Determination of protein content
The protein determination depended on the method of Bradford.[11] The reaction mixture consisting of 500 µL of sample solution and 1 mL of Bradford reagent was shaken well before allowing to stand for 20 min at room temperature. The spectral absorbance of the reaction mixture was measured at 595 nm. Albumin was used as a positive control to construct the standard curve equation.

Determination of lipid content
Total lipid content was determined by the method of Parkash.[12] The test fruit flies were weighed (w_1) before placing them in a test tube and drying at 60ºC for 48 h (w_2). The dried fruit flies were weighed. Diethyl ether (1.5 mL) was then added and continuously shaken at 200 rpm for 24 h at room temperature. After shaking, the solvent was removed, and the fruit flies were once again dried at 60ºC for 24 h. The final weight was determined (w_3). The relative lipid content was calculated according to the following equation:[12]

$$\% \text{ lipid} = \frac{(w_2 - w_0) - (w_3 - w_0)}{(w_1 - w_0)}$$

Where:
W_0 refers to the weight of the test tube
w_1 refers to the initial weight of flies
w_2 refers to the weight of flies after 48 hours of drying
w_3 refers to the final weight of flies

Evaluation of the inhibitory activity of enzymes belonging to the esterase and phosphatase groups
Adult female fruit flies were used to evaluate the inhibitory activity of enzymes belonging to the esterase and phosphatase groups. Flies were pureed in 500 µL sodium phosphate buffer (20 mM, pH 7.0) to determine the activity of enzymes belonging to the esterase and phosphatase groups.

Determination of acetylcholinesterase (AChE) activity
AChE activity was determined according to the method described by Riaz *et al*.[8] The reaction mixture of 50 µL of sample aliquot, 50 µL of acetylcholine (2.6 mM), and 1000 µL sodium phosphate buffer (20 mM, pH 7.0) was incubated at 25°C for 5 min. Fast blue B salt (0.3%, 400 µL) was then added to the mixture to stop the reaction. The spectral absorbance of the reaction mixture was measured at 405 nm.

Determination of carboxylesterase activity
α-carboxylesterase (α-carboxyl) and *β*-carboxylesterase (*β*-carboxyl) activities were determined according to the method of Riaz *et al*.[8] with some correction. The reaction mixture of 50 µL sample solution, 1000 µL sodium phosphate buffer (20 mM, pH 7.0), 50 µL α-naphthyl acetate, and *β*-naphthyl acetate were added separately to determine the activity of *α*-carboxyl and *β*-carboxyl, respectively. The reaction mixture was then taken to incubate at 30ºC for 20 min. After incubation, 400 µL of Fast blue B (0.3%) (mixed in SDS 3.3%) was added to the mixture to stop the enzymatic reaction and allowed to stand for 15 min at 20°C. The absorbance of the reaction mixture was measured at 430 nm and 590 nm for *α*-carboxylesterase and *β*-carboxylesterase, respectively.

Determination of acid and alkaline phosphatase
Acid phosphatase (AcP) and alkaline phosphatase (AkP) activities were determined according to the method of Riaz *et al*.[8] The reaction mixture, including 50 µL of sample solution, 50 µL of sodium phosphate buffer (50 mM, pH 7.0), and 50 µL of Tris HCl buffer (50 mM, pH 9.0), was separately added to determine the activities of the AcP and AkP, respectively. To both reaction mixtures were added 100 µL of p-nitrophenyl phosphate and incubated at 37ºC for 15 min in a thermostatic cooker. The enzymatic reaction was stopped by the supplementation of NaOH 0.5 N solution. The absorbance of the reaction mixtures were determined at 440 nm.

Quantification of flavonoids and polyphenols
Flavonoid content
Quantification of flavonoids used $AlCl_3$ reagent. Total flavonoid content was determined according to the $AlCl_3$ colorimetric method described by Al-Dalahmeh *et al*.[13] with some corrections. Flavonoid content was expressed in mg quercetin equivalent (QE) per g extract.

Polyphenol content
The Folin-Ciocalteu reagent was used for the determination of polyphenols. The polyphenol content was determined based on the method of Sarker and Oba[14] with some corrections. The polyphenol content is displayed in mg of gallic acid equivalent (GAE) per g extract.

Statistical analysis
The experiment was repeated at least three times. All data were expressed as mean ± standard deviation. The parametric method used for data analysis was the one-way analysis of variance (ANOVA), followed by an unpaired t-test, and a two-tailed t-test to determine significant differences at a confidence level of 95%.

Results and Discussion

Investigating the effect of the extract on the growth and development of fruit flies
Among the pesticides of plant origin, each ingredient can damage any stage of insect development, such as eggs, larvae, and adults, and act on different mechanisms affecting one or more biological systems, including the nervous, respiratory, endocrine, and water balance systems. The results in Figure 1 showed that, at a concentration of 15 mg/mL, the extract was able to inhibit the growth and development of fruit flies. The inhibitory ability was expressed through the reduction by 55.51% compared with the control treatment in the total number of pupae formed after 10 days of investigation in the extract treatment, thereby showing that the extract inhibited the growth of eggs and larvae during pupation development. After 14 days of the survey, more than 80.99% of flies died at the pupal stage and could not develop to the adult stage, while 100% of the pupae developed into adults in the control treatment. Plant secondary metabolites may confer properties similar to synthetic growth regulators, such as teflubenzuron.[15] As insect growth regulators (IGRs), plant compounds

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affect insect reproduction, development, and metamorphosis. These effects can cause irreversible changes in the physiology and behavior of insects.[16] Many bioactive compounds in plant extracts can influence the endocrine regulation of molting and metabolism, thus acting as insect growth regulators.[17] Vinuela *et al*.[18] also investigated that azadirachtin, one of the active metabolites of *A. indica*, had significant effects on insect development, including prolonging the larval or pupal stage and inhibiting the process of molting. These biochemical metabolites induced pupal prolongation and adult emergence by interfering with the biotransformation of the hormone ecdysone by flavonoids affecting cytochrome-P450 involved in the control of the molting process in insects.

Results of the energy storage components

Carbohydrate content

Carbohydrates represent a diverse group of compounds, from simple mono- and disaccharides to the complex organized compounds that makeup cell walls. The relationship between the different carbohydrate components greatly impacts the digestive system and influences the physiology of the gastrointestinal tract.[19]

The carbohydrate content was determined on the basis of linear regression equation y= 0.0011x+0.0974, R^2 = 0.9803. After 14 days, adult fruit fliesraised in a medium supplemented with the extract were significantly reduced by more than 68% of carbohydrate energy storage in their bodies. The carbohydrate content in the fruit flies increased in the medium supplemented with the extract at a concentration of 15 mg/mL with a value of 227±0.91 µg/mL, 3.19 times lower than that of the control treatment, which was 723±1.82 µg/mL (Figure 2). In the study of Zhou *et al*.[20], azadirachtin was found to affect the carbohydrate metabolism of *B. dorsalis* larvae. A significant decrease in the quantity and relative composition of fatty acids and the regulation of carbohydrate metabolism in *B. dorsalis* was observed following exposure to azadirachtin.

Protein content

Proteins participate in all life activities in the organism, from participating in cell structure and tissues to participating in catalytic activity and many other functions.[21]

The physiological effects of azadirachtin include direct inhibition of cell division and protein synthesis.[22] Azadirachtin was also found to severely reduce the protein, glycogen, and lipid content of *Plodia interpunctella*.[23] protein, lipid, and glucose content decreased, while uric acid increased when *Glyphodes pyloalis* larvae were fed neem extract-treated mulberry leaves.[24] These studies clearly show that azadirachtin can affect various biochemical compounds, such as carbohydrates, fatty acids, amino acids, cholesterol, uric acid, and urea. Effect of methanol extract from *Silybium marianum* (L.) on mortality, growth, feeding index, and enzyme activity in the small white butterfly *Pieris rapae* L. was noted in Hasheminia *et al*.[24] The results showed that the glucose and uric acid levels in the 3rd stage larvae treated with the extract increased. At the same time, the total protein and cholesterol decreased. The reduction in compounds such as cholesterol and protein may be due to physiological stress or disruption of the absorption system.[26] In this study, *Euphorbia antiquorum* extract was able to inhibit the protein content of fruit flies by up to 77.71% (Figure 3).

Lipid content

Lipids are the major component of body fat, accounting for more than 50% of the dry weight of tissues.[27]

Adult fruit flies (14-day old) reared in a medium supplemented with extract significantly reduced lipid energy storage components in their body. After adding *E. antiquorum* extract to the fruit fly diet, the lipid content decreased to 4.57±0.17%, while that value in the control treatment was 13.0±0.80% (Figure 4). Azadirachtin may also act at the biochemical level by affecting endogenous metabolites of insects. Azadirachtin can interfere with serotonin-containing regions in the neuroendocrine system of locusts.[28] It significantly reduces the lipid content in body fat.

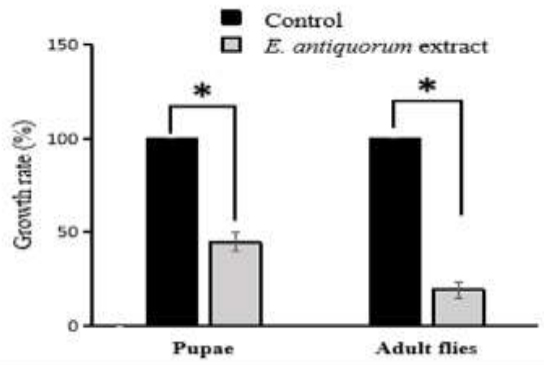

Figure 1: Effect of the *E. antiquorum* extract on growth and development of fruit flies (*, P<0.05)

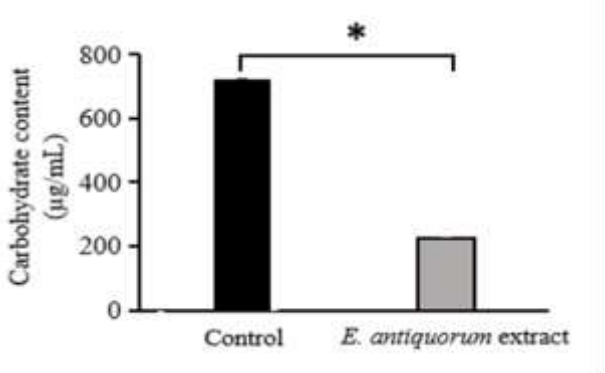

Figure 2: Effect of *E. antiquorum* extract on carbohydrate content (µg/mL) (*, P<0.05)

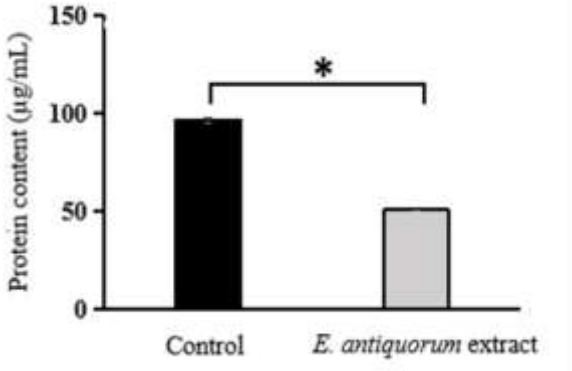

Figure 3: Effect of *E. antiquorum* extract on protein content (*, P<0.05)

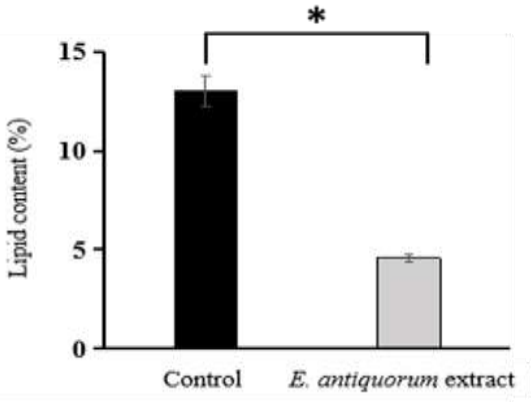

Figure 4: Effect of *E. antiquorum* extract on lipid content (*, P<0.05)

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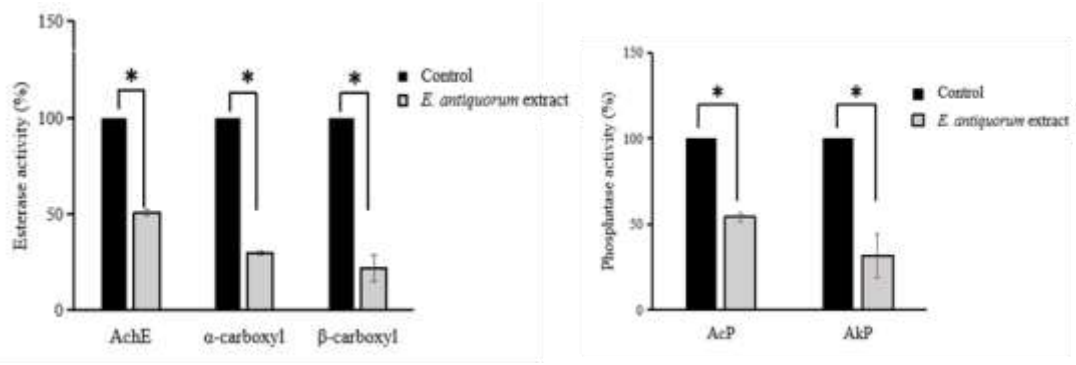

Figure 5: Inhibitory activity of *E. antiquorum* extract on enzyme content of fruit flies: (A) Enzyme esterase; (B) Enzyme phosphatase (*, $P < 0.05$)

In the study of Spochacz *et al*.[29], at a concentration of 1%, *Solanum nigrum* fruit extract reduced the lipid content in body fat by 0.57 ± 0.02 mg/mg, whereas that number in the control treatment was 0.69 ± 0.02 mg /mg.

Evaluation result of inhibitory activities of enzymes belonging to esterase and phosphatase groups

Acetylcholinesterase is a serine hydrolase that catalyzes acetylcholine's hydrolysis. This enzyme is the target of organophosphate and carbamate insecticides, which phosphorylate, or carbamylate serine, the active site preventing hydrolysis of the neurotransmitter acetylcholine when the postsynaptic membrane remains depolarized and transsynaptic conduction cannot take place leading to insect death.[30] Approximately 70% of the world's insecticide market depends on synthetic AChE inhibitors (organophosphates, carbamates, and neonicotinoids), including those that act on voltage-controlled sodium channels (especially pyrethrins).[31] In recent years, a new area of biopesticide development has been detecting less harmful natural compounds (to humans, other mammals, and the environment) that act on insect nicotine acetylcholine receptors (nAChR) by inhibiting their AChE activity.

The toxic effects of bioactive plant compounds are complex and depend on the chemical composition, insect pest type, and insect development stage.[32] In particular, the toxicity of essential oils or extracts is mainly related to action targets such as receptors and channels in the insect nervous system.[33,34] In this study, at a concentration of 15 mg/mL, the extract was able to inhibit the activity of the enzymes AchE, α-carboxyl, β-carboxyl, AcP, and AkP with values of 51,0±1.65%; 29.8±0.88%; 22.0±6.94%, 54.1±2.66%, and 31.6±12.7%, respectively; compared with the control treatment (Figure 5). This result is completely consistent with the previous study by Riaz *et al*.[8] used fruit flies to evaluate the toxicity of extracts of weeds, namely *Euphorbia prostrata, Parthenium hysterophorus, Fumaria indica, Chenopodium murale,* and *Azadirachta indica*. The study results showed that the extract was able to minimize the activity of enzymes including AchE, α-carboxyl, β-carboxyl, AcP, and AkP in fruit flies. Another study by Attaullah *et al*.[35] on the evaluation of the insecticidal activity of *P. harmala, D. stramonium, A. indica, T. terrestris* and *C. murale* against the 2nd stage larvae of *M. domestica* also indicated that they could reduce the activity of AChE, ACP, AKP, α-carboxyl, and *β*-carboxyl. Similarly, the enzyme inhibitory activity of plant extracts was reported by Zibaee and Bandani[36] and demonstrated that increasing concentrations leads to enhanced inhibition of target enzymes.

Quantitative results of flavonoids and polyphenols

The content of polyphenols and flavonoids in *Euphorbia antiquorum* extract was determined based on the linear regression equation of the standard substance of gallic acid ($y = 0.0778x + 0.0255$, $R^2 = 0.9975$) and quercetin ($y = 0.0046x + 0.0218$, $R^2 = 0.9832$).

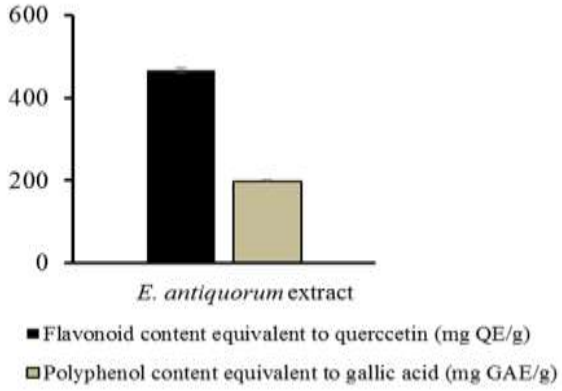

Figure 6: Total flavonoid and polyphenol content in the extract

Conclusion

E. antiquorum extract inhibits fruit fly growth and development through lethality at the pupal stage and reduces energy storage components such as carbohydrates, proteins, and lipids. Besides, the study also recorded the ability to inhibit enzymes of the esterase group (AchE, α-carboxyl, and β-carboxyl) and phosphatase (AcP and AkP). It can be concluded that the *E. antiquorum* is a potential plant species in the study of active ingredients applied in the production of insecticides in the direction of inhibiting the growth and development process.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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