Journal of King Saud University – Science 35 (2023) 102924

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Journal of King Saud University – Science

journal homepage: www.sciencedirect.com

Original article

Laboratory evaluation of the effects of *Portunus pelagicus* extracts against *Culex pipiens* larvae and aquatic non-target organisms

Fahd A. Al-Mekhlafi *, Nael Abutaha, Muhammad Farooq, Muhammad A. Wadaan, Mohammed S. Al-Khalifa

Department of Zoology, College of Science, King Saud University, P.O. Box 2455 Riyadh, 11451 Riyadh, Saudi Arabia

ARTICLE INFO

Article history:
Received 1 February 2023
Revised 12 June 2023
Accepted 28 September 2023
Available online 4 October 2023

Keywords:
Culex pipiens
Toxicity
Larvicidal property
Portunus pelagicus
Zebrafish

ABSTRACT

Culex pipiens (Diptera: Culicidae) is a pathogen-bearing mosquito found worldwide. Marine organisms are a promising source to search for mosquito-killing substances. The potential of the different solvent extracts of *Portunus pelagicus* was examined using *Cx. pipiens* 3rd instar. The bio-safety evaluation was carried out using Danio *rerio* (zebrafish) and *Artemia nauplii* (brine shrimp). The larvicidal activity of *Portunus pelagicus* (blue crab) was found in chloroform extracts against *Cx. pipiens* 3rd instars, with LC_{50} value of 109.11 and LC_{90} value of 88.44 ppm after 48 h of treatment. A Biosafety assessment of *P. pelagicus* methanol extract on *D. rerio* and *A. nauplii* revealed that the extract had toxic effects on *A. nauplii* but was safe for *D. rerio*. However, the non-toxicity of the extract towards *D. rerio* may be misleading; further investigation on other aquatic organisms is required. Thus, the extract should be used with caution.

1. Introduction

Marine natural products are unexplored sources of promising compounds of multipurpose applications that have drawn the attention of scientists for the discovery of innovative medications. (Rekha et al., 2018). Many marine organisms are exposed to extreme environmental conditions that permit them to adapt to new environments and produce different secondary metabolites not found in other organisms (Rekha et al., 2018). Several researchers have reported that secondary metabolites extracted or isolated from marine invertebrates possess biological and therapeutic activities such as anti-parasitic, anticancer, antiviral, antibacterial, antifungal, and antioxidant activities (Barzkar et al., 2019). Marine crabs possess active compounds isolated from different tissues and organs, such as chitin (Casadidio et al., 2019), glucosamine (Barrow, 2010), astaxanthin, and phenolic compounds (Rasmussen and Morrissey, 2007).

Culex pipiens (Diptera: Culicidae) is distributed far and wide and may transmit many pathogens, such as the filarial parasite, Rift Valley fever virus, and West Nile virus to humans and animals (Kasai et al., 2008). *Cx. pipiens* can be found in several Saudi Arabian provinces (Alahmed et al., 2019) and was the most promising vector of bancroftian filariasis (Omar, 1996), reported to be resistant to many insecticides deltamethrin, cyfluthrin, bifenthrin, and this resistance is increasing (Al-Sarar, 2010).

Mosquito control is mainly dependent on synthetic insecticides. Insecticides eliminate large numbers of mosquitoes; as a result, it reduces the transmission of disease but causes resistance in mosquitoes, which may spread to mosquitoes from different geographic regions (Demok et al., 2019; Organization, 2018).

Mosquitocide resistance in *Cx. pipiens* has been reported from different areas in Riyadh (Al-Sarar et al., 2005). It has been revealed to have marked resistance to deltamethrin (187.1 folds), Moderate to low resistance to beta cyfluthrin (14 folds), and lambda cyhalothrin (3.8 folds) respectively (Al-Sarar, 2010). *Cx. pipiens* isolated from other countries have also shown high resistance levels to deltamethrin (233– and 453-folds) (Daaboub et al., 2008), moderate and low resistance levels to bifenthrin (38.4-fold), and lambda-cyhalothrin (3.8-fold) (Nazni et al., 2005). However, *Cx. pipiens* was reported to be susceptible to fenitrothion (Al-Sarar, 2010).

Natural products are increasingly used to combat vectors because they are considered safe to use, such as *Azadirachta indica*, *Anacadium occidentale*, *Mangifera indica* and *Cocos nucifera* (Innocent et al., 2014). These natural products, however, can have

* Correspondent author.
E-mail address: falmekhlafi@ksu.edu.sa (F.A. Al-Mekhlafi).

Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.jksus.2023.102924
1018-3647/

a detrimental effect on the non-target organisms. If a natural product extract is to be used for controlling mosquito larvae, there is a possibility that some components may leak into the groundwater and migrate into nearby rivers and streams, causing toxicity to non-target organisms (Cannon et al., 2004).

There is a large body of literature on the biosafety assessment of natural products on non-target organisms such as *D. rerio* and *Artemia nauplii* (Abutaha et al., 2020; Ragavendran et al., 2019). Interesting similarities exist between the genetic make-up of humans and *D. rerio*, including the presence of over 84% of the genes associated with human illnesses. (Truong et al., 2014). *A. salina* is a valuable test organism used for toxicity testing with several toxicology applications (Nunes et al., 2006). This is because *A. salina* assays could replace cytotoxicity assays that contain animal serum (McLaughlin et al., 1998). The cytotoxicity assays are expensive, time-consuming, tedious, and lack simplicity. Currently, *A. salina* tests are used in toxicology (Costa-Lotufo et al., 2005) to screen extracts for drug discovery (Sangian et al., 2013).

Therefore, the effects of natural product extracts on non-target aquatic organisms must be studied in detail before they are recommended for mosquito control programs. The effects of *P. pelagicus* extract on mosquitoes and non-target aquatic species have not been documented.

2. Material and method

2.1. Preparation of solvent extracts

Portunus pelagicus was purchased from the market, washed several times with distilled water (DW), and then oven dried at 50 °C for 72 h. After being pulverized in an electronic blender, 80 g of the substance was extracted using the Soxhlet method in a series of four different solvents: hexane, chloroform, ethyl acetate, and methanol. All the extracts obtained were filtered. The solvents were evaporated separately using a rotovap (Germany). The dried extract from each solvent extract was then dissolved in methanol to obtain two fractions. One of the fractions is methanol-soluble, whereas the other is not (soluble only in hexane). The fractions were evaporated. All the fractions were kept at − 80 °C in glass bottles until use. Stock solutions (100 mg/mL) of methanol soluble fraction were then subjected to larvicidal screening. The insoluble methanol fractions were untested due to their insolubility in water.

2.2. Preparation of aqueous extracts

Portunus pelagicus (70 g) was soaked in 500 mL of DW, sonicated for 30 min, and kept on a shaker for 3 h. Later, the extract was filtered, evaporated using rotovap, and kept at − 80 °C in glass bottles until use. Stock solutions (10 mg/mL) were made after being stored for 24 h, and larvicidal screening was carried out.

2.3. Larva rearing

Eggs, larvae, pupae, and adults of *Cx. pipiens* were collected from the insectary at Zoology Department (King Saud University, Saudi Arabia, Riyadh) at 28 ± 2 °C and under a 12-h photoperiod.

2.4. Mosquito larvicidal bioassay

The recent larvicidal activity of *P. pelagicus* extracts (133–233 ppm) was calculated after 24 and 48 h. *Portunus pelagicus* extracts were individually introduced to sterile six-well plates (Corning Inc., NY, USA), allowed to evaporate, and redissolved in tap water (total volume, 6 mL) to test against the 3rd instar *Cx. pipiens*. The assay was done in triplicate with 20 larvae per concentration. Aqueous control and methanol (Fisher Scientific, Loughborough, UK) control were maintained separately. During the treatment, the larvae were fed TetraMint ground fish meal (Germany). Larval mortality (%) was calculated 24 and 48 h post-treatment and expressed as 50% (LC_{50}) and 90% (LD_{90}) lethal dose values. Probit analysis was used to calculate the LC_{50} and LC_{90} (Finney, 1952).

2.5. Histopathology study

For histological investigations, larvae (treated and control) were fixed in buffered formalin (10%), then processed as reported previously (Al-Doaiss et al., 2021) using an automatic tissue processor (Sakura, Finetek, Japan), embedding station (Sakura) and a rotary microtome (RM2245, Leica Biosystems, Wetzler, Germany). Finally, the sections (5 μm) were stained (hematoxylin and eosin) using an autostainer (5020, Leica Biosystems). The stained sections were photographed using a microscope (BX53, Olympus, Japan).

2.6. Non-targeted organism test

2.6.1. Artemia nauplii

Artemia cysts (BIO-MARINE®, USA) were hatched following the method of Latha et al. (2016). *A. nauplii* eggs (50 mg) were added to 1000 mL seawater (salinity of 30 parts per thousand) in a separatory funnel under 26 ± 2 °C, pH 8.4; and light: dark period of 16:8h. The flask was aerated for 24 h using an air pump. The hatched *A. nauplii* (24 h) were used for toxicity tests using 500, 375, 175,100, and 50 μg/ml of chloroform extract. Freshly hatched eggs were separated into six sets (groups), each containing 50 animals. Seawater was used as a control. The assay was carried out in six-well plates using 3 mL seawater for 24 h to evaluate the toxic effect of *P. pelagicus* extract. The mortality was reported after 24 h, and the experiment was replicated thrice. The LD_{50} value was calculated using the origin software.

2.6.2. Toxicity on Danio rerio

Wild-type AB strain *D. rerio* (1 year) were acquired from the International Resource Center, Oregon University, USA, and kept in fish tanks (10-liter tanks; Tecniplast, Exton, PA, USA) at 28.5 °C and pH 7.5 and fed daily with Zeigler flake food (Zeiglers Bros, USA). Danio *rerio* were used for the non-target organism test, with three fish (three replicates) being tested with 180 and 220 μg/mL extract dissolved in 400 mL of fish water. A control group was also established with 400 mL of fish water. The tests were performed for 24 h, and fish mortality was recorded.

2.7. Statistical analysis

The LC_{50} and LC_{90}% confidence limits and chi-square values of mortality were calculated using probit analysis using SPSS software. All the results were considered statistically significant at $P < 0.05$.

3. Result

The various extraction solvents were found to have significant weight differences in the yield obtained. The lowest percentage yield was found in ethyl acetate (10 mg), hexane (20 mg), and chloroform (1 g) extract, respectively. In contrast, the highest yield was observed in the methanol (12 g) and aqueous (10 g) extracts.

The mortality in the percentage of *Cx. pipiens* larvae post-treatment with different *P. pelagicus* chloroform extracts concentrations is given in Table 1. The promising result were found

Table 1
Larvicidal activity of Chloroform extract of *Portunus pelagicus* against *Culex pipiens*.

Concentration (µg/mL)	% Mortality ± SE	
Time	24 h	48 h
133	00 ± 00d	26.67 ± 3.33c
167	33.33 ± 3.33c	56.67 ± 3.33b
200	80.00 ± 5.77b	100 ± 0.00a
233	100.00 ± 0.00a	0.00a ± 100
Control	00 ± 00d	6.67 ± 3.33d
LC_{50} (µg/mL)	180.05	88.44
LC_{90} (µg/mL)	218.47	109.11
Df	4	4
F-value	236.50	268.00

LC_{50} - lethal concentration 50% mortality, LC_{90} - lethal concentration 90% mortality. One-way ANOVA followed by Tukey's test was used to assess significant differences between the recorded readings (three replicates of 20 each). Data for columns with different letters are significantly different $P < 0.05$.

against the 3rd instar *Cx. pipiens using* the chloroform extract, resulting in LC_{50} values of 180.05 µg/mL after 24 h of treatment (Table 1). The LC_{50} value was 109.11 after 48 h of treatment with the chloroform extract. In control (methanol), all the larvae were live, and no mortality was reported. However, in the treatment group, the dead larvae settled down. The analysis revealed that LC_{50} and LC_{90} values decreased gradually with time. Dose-dependent mortality was also correlated positively with extract concentrations (Table 1).

In the control group, the midgut was lined by a simple cuboidal epithelial broad healthy cell with rounded large nuclei, smooth acidophilic cytoplasm, and well-developed microvilli (brush border). All epithelial cells were closely attached to the basement membrane (basal lamina). A peritrophic membrane-surrounded lumen shields the epithelia from food substances (Fig. 1A–D). In contrast, the histopathological observation of the midgut of the treated larvae exhibited aberrations (Fig. 1. K–N) such as severe damage, structural disorganization, deformation, and maximum degeneration of epithelial cells (De) and edema (Fig. 1[K]). Formation of globular protrusions towards the lumen and epithelial cell blebbing into the gut lumen were observed (Fig. 1[L]). Swelling or elongation and blebbing of the epithelial cells, degradation and destruction of microvilli, and vesicle formation in the cytoplasm of the epithelial cell were also observed (Fig. 1[M]). Moreover, the treated larvae exhibited alterations in cell size and shape (Fig. 1[N]).

The toxicity of *P. pelagicus* chloroform extract on *A. nauplii* after 24 h od exposure is presented in Fig. 2. The control and the 500, 375, 175, 100, and 50 ppm treated groups showed 0, 100, 58, 45, and 1% mortality after 24 h treatment, respectively. However, the calculated LD_{50} value was 242 µg/mL. Based on the result obtained, *P. pelagicus* chloroform extract was unsafe for *A. nauplii* at high concentrations; the LC_{50} of the extract for *A. nauplii* is higher than that for mosquito larvae. The effect of *P. pelagicus* chloroform extract against a non-target organism, *D. rerio*, is shown in Fig. 3. Results indicated that the extract is not toxic to *D. rerio* at LC_{50} (250 µg/ml) and LC_{100} (500 µg/ml) doses.

4. Discussion

Researchers worldwide are interested in finding natural mosquitocidal chemicals that are environmentally safe, and it is preferred for mosquito control more than synthetic mosquitocides (Benelli et al., 2015). Nature is rich in novel bioactive compounds for the welfare of humankind. The literature reveals that the products extracted from marine habitats have more novel bioactive compounds with higher biological activities than those isolated from terrestrial sources (Sujatha and Joseph, 2011). Recently, extracts from marine organisms have been screened for larvicidal activity. Few investigations have described the application of products from marine organisms such as sea squirt (Chio and Yang, 2008; Mohd Hussein et al., 2001), sea lily (Su et al., 2016), and marine sponges (Reegan et al., 2015; Sujatha and Joseph, 2011) as mosquito control agents. Extracts of marine green algae such as *Ulva lactuca* Linn, *Halimeda macroloba* Decsne, and *Caulerpa racemosa* Frosk have also been investigated for their insecticidal activity (Adaikala Raj et al., 2017). However, to our knowledge, no researcher has studied the mosquitocidal potential of *P. pelagicus* extracts.

Histomorphological changes in larvae treated with 90 µg/mL extract indicate the larval death cause. Anatomically, the midgut of *Cx. Pipiens* larvae is divided into three regions. All regions are composed of a single cuboidal epithelial cell layer with apical microvilli (Mahmoud et al., 2019). The release of digestive enzymes and nutrient absorption via the microvilli are essential activities of the midgut in larvae (Yu et al., 2015). Most previous studies have shown that larvicidal substances cause damage to the epithelial cells of the midgut, which is probably where the compounds are absorbed. The injury to the midgut region disrupts its function, causing larval death (Jiraungkoorskul and Jiraungkoorskul, 2015). Histopathological alterations observed in the midgut include edema, protruding cells, microvilli (brush border) destruction swelling or elongation, and blebbing of the epithelial cells, and vacuolization of cytoplasm, and alterations in cell size and epithelial cell shape. These observations agree with the findings of previous studies (Yu et al., 2015) (Abutaha et al., 2022) (Almkehlafi et al., 2018). (Mahmoud et al., 2019) recorded histopathological results and toxicological data in the midgut region of larvae treated by plant extracts (*Piper nigrum*, *Azadirachta indica*, and *Eucalyptus regnans*) and showed that the alterations were epithelial cell vacuolization, microvilli disorganization, and basement membrane detachment. Similarly, Cx. pipiens treatment with Artemisia judaica resulted in cell swelling, epithelial cell vacuolation, the appearance of cell debris in the lumen, and loss of normal features of the midgut (Hamouda et al., 1996). Most histological alterations on Cx. quinquefasciatus were microvilli destruction, swelling, protruding, degeneration, and epithelial cell vacuolization after treatment with *Melia azedarach* or *Matricharia chamomilla* extracts (Al-Mehmadi and Al-Khalaf, 2010) (Almehmadi, 2011). Regardless of the type of natural or chemical larvicidal substance used, the similarities in histological changes in the midgut epithelia indicate that such changes are a response to cellular toxicity (Jiraungkoorskul and Jiraungkoorskul, 2015).

Lowering the mucosal membrane's surface tension is a major function of metabolites resulting in damaging the digestive system, therefore ion transport, osmoregulation, and nutrition absorption (Sina and Shukri, 2016).

A promising mosquitocidal extract should be target-specific. Hence, toxicity testing using the *A. nauplii* is a rapid method to evaluate the toxicity of substances (McLaughlin et al., 1998). Extracts with LC_{50} values higher than 1000 g/mL are non-toxic, whereas those with LC_{50} values of 1000 g/ml are considered harmful, according to earlier (Clarkson et al., 2004) toxicity indices. The biotoxicity of chloroform extract was assessed on the non-target organism *A. nauplii*. According to our findings, the extract was poisonous to A. nauplii, with an LC_{50} value of 242 g/mL.

The effect of the extract on non-target organisms showed that *P. pelagicus* chloroform extracts are safe on *D. rerio* but not on *A. nauplii*. However, further evaluation is required to assess damages and deformities in tested organisms using different methods and parameters.

This suggests that this extract should be used with caution. The non-toxicity of the extract on *D. rerio* may be misleading and lead to problems for other aquatic species. However, further research is

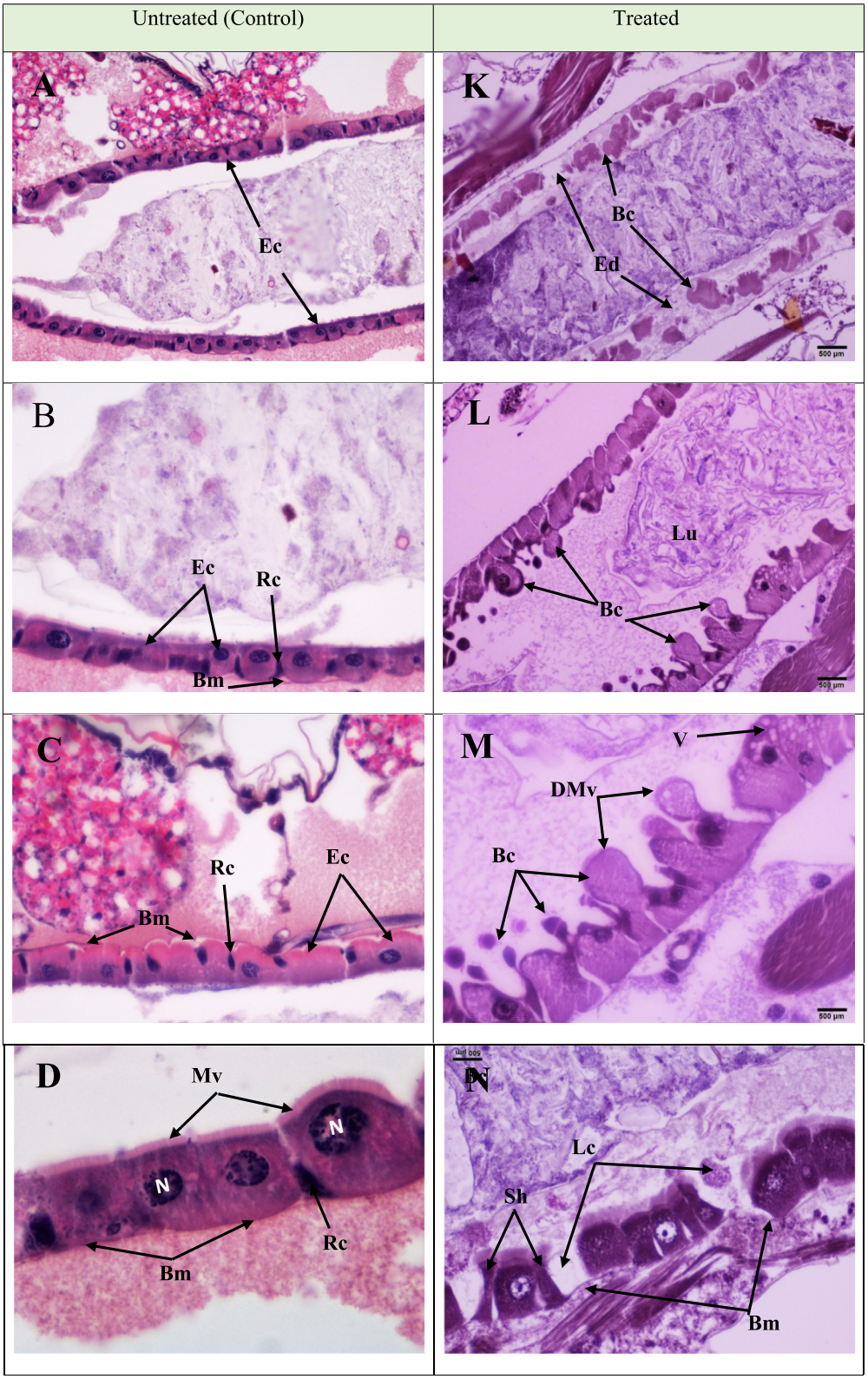

Fig. 1. The longitudinal section in the midgut of *Culex pipiens* larvae showing histopathological alterations induced by *Portunus pelagicus* on the epithelia of the midgut of *Cx. pipiens* larvae. A to D represent longitudinal sections in the midguts of untreated control larvae, showing typical and healthy epithelial cells (Ec), microvilli (Mv), nuclei (N), and regenerative cells (Rc). K to N represent longitudinal sections in midguts of treated larvae, showing affected gut epithelial layer with several lesions. Degenerated epithelial cells (De), edema (Ed), blebbing (Bc), protruding cells into the lumen (Lu), degraded microvilli (DMV), swelling or elongation, and blebbing of the epithelial cells, alterations in cell size and shape of epithelial cells (Sh), irregular basement membrane (Bm), and loss of some cells (Lc).

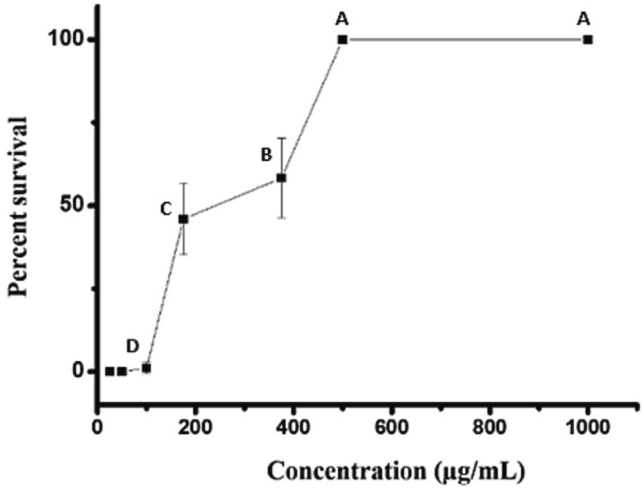

Fig. 2. Toxicity of *Portunus pelagicus* chloroform extract against *Artemia nauplii*.

Fig. 3. Testing the toxicity of *Portunus pelagicus* chloroform extract on *Danio rerio*.

needed to assess its effects on a wide range of non-target organisms to get the complete picture before being considered an alternative to current mosquitocides.

5. Conclusion

The current work documents the larvicidal actions of *P. pelagicus* against *Cx. pipiens* for the first time. The screening results suggest that *P. pelagicus* chloroform extracts are promising for larval control. However, further investigation is required to isolate the active compound/s in the extract and identify their mechanism of action.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors express their sincere appreciation to the Researchers Supporting Project number (RSP2023R112), King Saud University, Riyadh, Saudi Arabia.

Funding

This project was funded by Researchers Supporting Project number (RSP2023R112), King Saud University, Riyadh, Saudi Arabia.

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