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LARVICIDAL ACTIVITY OF HEXANE-EXTRACTS OF *Cinnamomum cassia* and *Zingiber officinale* AND THEIR COMBINATION AGAINST *Culex pipiens* (DIPTERA: CULICIDAE)

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ABSTRACT

Botanical products are encouraged for mosquito control because of their low cost, rapid degradation, and low toxicity. The hexane extracts of *Cinnamomum cassia* (C) and *Zingiber officinale* (Z) were assessed alone or in combination on the 3rd instar of *Culex pipiens*. The LC_{50} values of *C. cassia* and *Z. officinale* against *Cx. pipiens* were 317.61 and 269.4 μg mL^{-1}, respectively. The Z75%: C25% ratio was the most promising among the different combinations tested, with IC_{50} value of 251.76 μg mL^{-1}. The histological sections of extract-treated larvae (C25%: Z75%) induced degeneration of epithelial cells, degradation of microvilli, and loss of nuclei. Antioxidant activity and phenolic content of *Z. officinale* hexane extract showed a linear relationship. *Z. officinale* extract at 60.5 μg mL^{-1} possessed the highest hemolytic activity (73.9 ± 0.5%). Our results revealed that the combined hexane extracts of *C. cassia* and *Z. officinale* provided promising larvicidal action against *Cx. pipiens.*

Keywords: *Cinnamomum cassia*, *Culex pipiens*, combination effect, hemolysis, larvicide, *Zingiber officinale*

INTRODUCTION

Mosquito-transmitted diseases such as yellow fever, zika chikungunya, dengue fever, filariasis, and malaria cause significant public health issues resulting in millions of deaths annually (Gubler, 2009). *Culex pipiens* is the most widespread group of mosquitoes globally (Vinogradova, 2000). *Cx. pipiens* has been reported from several areas in Saudi Arabia (Al Ashry *et al.*, 2018). This species is considered one of the most common and widely distributed mosquito species. The larvae of *Cx. pipiens* are more dominant in surveyed areas of the country, such as Tabuk (Al Ashry *et al.*, 2018), Hail (Mo'awia *et al.*, 2020), Asir (Al Ashry *et al.*, 2014), and Jeddah (Alghamdi, 2021).

Female *Cx. pipiens* feed on human and animal blood, transmit diseases from human to human and from animal to humans and cause millions of mortalities annually (Takken and Knols, 1999). Moreover, *Cx. pipiens* causes many diseases, such as Encephalitides, West Nile virus, filariasis, etc. (Thenmozhi *et al.*, 2014), and can induce local dermatitis (Peng *et al.*, 2004). There are no vaccines available to humans to protect against such transmitted diseases (Rehman *et al.*, 2019). Mosquito management is an important method to prevent and control mosquito-borne diseases. On the other hand, the long use of synthetic insecticides has led to the development of resistance in mosquito populations, non-biodegradability, and environmental pollution (Gill and Garg, 2014). Therefore, there is an urgent need to find a promising strategy to manage the diseases transmitted by mosquitoes (Jones *et al.*, 2021).

The use of botanical products having mosquitocidal potential has become the focus to combat resistance development in mosquitoes (Ghosh *et al.*, 2018, Senthil-Nathan, 2020). In contrast to the synthetic mosquitocides, botanical products possess low cost and toxicity to humans, rapid degradation, and promising control of targeted organisms (Mdoe *et al*., 2014; Shoukat *et al.*, 2020). The combination potential of botanical extracts is widely reported (Al-Mehmadi and Al-Khalaf, 2010, Younoussa *et al*., 2020, Shoukat *et al*., 2020). Their combined use increases the efficacy as compared to single plant extract use, prevent the mosquito resistance issues (Mohan *et al.*, 2010; Younoussa *et al.*, 2020). Herbalists always prefer a combination of plant extracts over single extract because the positive interaction between such herbal extracts. As in any kind of positive interaction between insecticides or drugs, synergism can exist between the components of a single extract as well as a blend of herbs; since herbalists have always recommended the use of whole plant extracts and their combinations rather than single active compounds (Williamson, 2001). To search the development of larvicidal agent from botanical extracts and prevent mosquito resistance, the present study was aimed to evaluate the larvicidal and combination effects of *Cinnamomum cassia* and *Zingiber officinale* hexane extracts against *Cx pipiens* under laboratory conditions.

MATERIALS AND METHODS

Extraction of plant

The bark of *Cinnamomum cassia* (family - Lauraceae) and rhizome of *Zingiber officinale* (family - Zingiberaceae) were purchased from a herbal shop in Riyadh, Saudi Arabia. The collected plant (20 g) was powdered in a commercial blender and then extracted in hexane (1:10, solid to solvent ratio) using an ultra-sonicator (WISD, Seoul, Korea) for 10 min at 40°C. Freshwater was poured to maintain the temperature at 40°C. The extract was then kept in a shaker for 1 h and filtered through a filter paper. The process was repeated twice. The filtrate was combined, and the rotary evaporated (Buchi Rotavapor, Germany) under a controlled vacuum. Stock solution (20 mg mL^{-1}) was prepared and kept at 4°C till further analysis. The binary blends of hexane extracts of *C. cassia* (C) and *Z. officinale* (Z) in proportions of C25%: Z75%, C50%: Z50%, and C75%: Z25% were prepared.

Total phenolic content and antioxidant activity

The total phenolic content of the extracts was determined by using Folin-Ciocalteu (FC) reagent as described by Abutaha *et al*. (2020). The DPPH (1,1-diphenyl-2-picrylhydrazyl, 0.008% w/v) radical scavenging activity (RSA) of hexane extracts of C. *cassia* and *Z. officinale* extracts was carried out as per Abutaha *et al*. (2020). A mixture of 195 µL DPPH working solution and 5 µL of different concentrations of sample dilutions (10 to 100 mg mL^{-1}) was incubated for 30 min at 25ºC. Methanol was used as a blank, and quercetin (1 mg mL^{-1}) as a positive control. The absorbance was read at 520 nm using a plate reader (ChroMate, England). The RSA was calculated as:

$$\text{Scavenging\%} = \frac{A540\text{control} - \text{A540extract}}{\text{A540control}} \times 100$$

Mosquito collection and rearing

The adults and larvae of *Cx. pipiens* were kept in the Insectary of Bio-Product Research, King Saud University, Riyadh (Saudi Arabia), since December 2018. The larvae were kept in a plastic rearing tray with tap water at 30°C, with 12 h day/night cycle. The emerged larvae were provided with grounded fish flakes (DAJANA, China). The trays were kept in a rearing cage after pupal formation. After 2 days, adult mosquitoes were transferred to other wooden rearing cage via an electrical aspirator. A beaker having tissue paper soaked in 10% sugar solution was provided to adult mosquitoes. For blood-feeding, the female mosquitoes were fed on albino mice blood over-night in rearing cage. The work was approved by the Animal Ethic Committee of the university.

Larvicidal bioassay
The larvicidal potential of hexane extracts of *C. cassia* and *Z. officinale* were tested alone and in combination against 3rd instar of *Cx. pipiens* as per Almekhlafi (2021). The binary blends of hexane extracts of *C. cassia* (C) and *Z. officinale* (Z) in proportions of C25%: Z75%, C50%: Z50%, and C75%: Z25% were prepared. Plant hexane extracts individually or in combination were dissolved in methanol, and concentrations of 125, 250, 500, and 1000 ppm were prepared in 8 mL in 6 well plates. The negative control (methanol) was added to the well, left until evaporated completely, and then the larvae were added. Twenty larvae of 3rd instar were shifted into each test concentration, and each concentration was tested in triplicate. Mortality was reported 24 h of post-treatment. The larva was confirmed dead if no movement was observed after touching with a needle.

Histology analysis
Fixed midguts of larvae from control and treatments were dehydrated in different concentrations of ethanol (70-100%) and embedded in paraffin. The sections were cut into 3 μm thickness, stained with hematoxylin and eosin (Almekhlafi, 2021). The stained sections were imaged using an Olympus microscope (Olympus BX60, USA).

Hemolytic activity
In vitro hemolytic activity of extracts was performed as per the protocol of Lum *et al.* (2015). In brief, mice blood was collected by cardiac puncture from albino mice maintained under standard conditions (Abutaha *et al.*, 2021). A 3% (v/v) suspension of erythrocytes was incubated with various extract concentrations in a 96-well plate at 37°C for 30 min. After 30 min, the erythrocytes were pelleted at 4000 g for 5 min (Centurion Scientific K3, UK) at 4°C. The supernatant was collected and OD_{540} read spectrophotometrically (ChroMate, USA). Phosphate buffer saline (PBS) was used as a negative control while Triton X-100 as a positive control for complete cell lysing. The percent hemolysis was calculated using the following formula:

$$\text{Percent hemolysis } = \frac{A\text{ extract} - \text{APBS}}{\text{A triton} - \text{APBS}} \times 100$$

Statistical analysis
The results were reported as means ± standard deviation. The analysis was carried out using one-way ANOVA, followed by Tukey's test. Values of $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The yield obtained from *Z. officinale* hexane extracts (1.5%, w/w) was higher than that of *C. cassia* (0.85%, w/w). The phenolic and antioxidant activities were detected only in *Z. officinale* hexane extract. The antioxidant potential of hexane extracts was assessed by DPPH test and correlated to its TPC (Fig. 1). The free radical scavenging activity (RSA) enhanced with increase in concentration. The linear relation between antioxidant activity and phenolic content showed that the phenol in extract may be the main contributor for RSA of extract. The present study revealed that *Z. officinale* is a promising source of naturally occurring antioxidant phenolic compounds. Phenolic compounds are commonly found in plants, and their bioactive properties have been correlated to their phenolic contents. The phenolic compounds from different types of natural products have been used for controlling the mosquitos (Thomas *et al.*, 2017; de Carvalho *et al.*, 2019). These toxic phytocompounds ingested orally or absorbed through cuticle might affect the physiology of insects (Nisbet, 2000) by interfering with ligand-ligated chloride channel or by blocking receptors (cholinergic or octopamine), the target sites of insect control (Ffrench-Constant *et al.*, 2016).

Larvicidal activity
The percent mortality of *Cx. pipiens* larvae treated with hexane extracts of *C. cassia* and *Z. officinale*

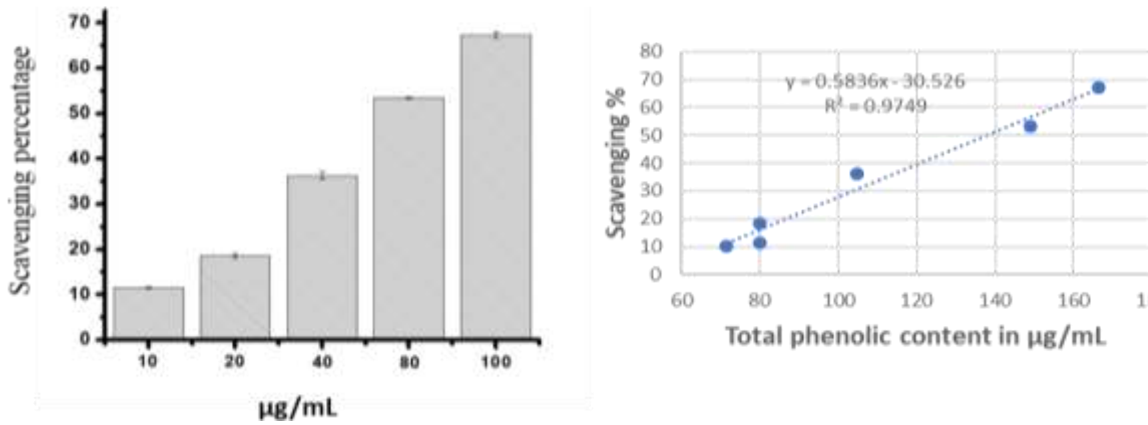

Fig. 1: A) The radical scavenging activity of *Zingiber officinale* hexane extract represented by percentage of inhibition. B) Correlation between radical scavenging activity and total phenolic content of *Z. officinale* hexane extract. Coefficient of determination, $R^2 = 0.974$ and correlation coefficient, r = 0.996. Regression equation (C2 = 7.52 + 0.5979 C1)

individually or in combination 24 h post-treatment is given in Table 1. The hexane extracts of two plants tested singly showed promising larvicidal activity against larvae, and the effective-ness was improved with the increase in concentration. The highest larvicidal activity was found in *Z. officinale*

Table 1: Mortality (%) of *Cx. pipines* 3rd instar treated with *Zingiber officinale* (Z) and *Cinnamomum cassia* (C) alone and in combination 24 h post exposure as well as LC_{50}, LC_{90} and LC_{95} values ($\mu g\ mL^{-1}$)

Treatment	Concentration ($\mu g\ mL^{-1}$)	Mortality (%) ± SE[a]	LC_{50} ($\mu g\ mL^{-1}$)	LC_{90} ($\mu g\ mL^{-1}$)	LC_{95} ($\mu g\ mL^{-1}$)	df	F
Z100%	0	0.00 ± 0.00^c					
	125	4.67 ± 2.33^c					
	250	25.00 ± 5.86^b	269.42	443.72	495.50	4	215.98
	500	100.00 ± 0.00^a					
	1000	100.00 ± 0.00^a					
C25%: Z75%	0	0.00 ± 0.00^c					
	125	2.33 ± 2.33^c					
	250	76.00 ± 2.00^b	271.19	434.72	455.16	4	1105.52
	500	100.00 ± 0.00^a					
	1000	100.00 ± 0.00^a					
Z50%: C50%	0	0.00 ± 0.00^c					
	125	2.33 ± 2.33^c					
	250	47.00 ± 4.04^b	292.57	450.36	470.09	4	406.49
	500	100.00 ± 0.00^a					
	1000	100.00 ± 0.00^a					
C75%: Z25%	0	0.00 ± 0.00^c					
	125	2.33 ± 2.33^c					
	250	62.67 ± 11.33^b	251.76	420.53	441.63	4	70.97
	500	100.00 ± 0.00^a					
	1000	100.00 ± 0.00^a					
C100%	0	0.00 ± 0.00^c					
	125	7.00 ± 4.04^c					
	250	60.33 ± 13.33^b	317.61	471.17	490.16	4	43.47
	500	98.00 ± 2.00^a					
	1000	100.00 ± 0.00^a					

[a]The mean of mortality ± SE within a column followed by the same letter do not significantly differ from each other as per Tukey test ($P < 0.05$), df = degree of freedom; F = F-value

with LC_{50} value of 269.42 µg mL^{-1}, followed by *C. cassia* with LC_{50} value of 317.61 µg mL^{-1} at 24 h (Table 1). The mixture of C75%: Z25% caused high mosquito mortality (in the range of 2.33% (125 µg mL^{-1}) to 100% (500 µg mL^{-1}) as compared to the other combinations. *Z. officinale* extracts (LC_{50} = 269.42 µg mL^{-1} and LC_{90} = 443.72 µg mL^{-1}) was most potent as compared to *C. cassia* extract (LC_{50} = 317.61 µg mL^{-1} and LC_{90} = 471.17 µg mL^{-1}). However, the blending of two extracts enhanced their effectiveness by decreasing the LC_{50} values to 251.76, 292.57 and 271.19 µg mL^{-1} for the combination of C75%: Z25%, C50%: Z50%, and C25%: Z75%, respectively as compared to LC_{50} of *C. cassia* (LC_{50}: 317.61 µg mL^{-1}) tested singly. Table 2 presents the combined effects of hexane extracts of *C. cassia* and *Z. officinale* against the larvae of *Cx. pipiens*, 24 h post-treatment. The combination of 25% *C. cassia* extract with 75% of *Z. officinale* extract (C25%: Z75%) resulted in IC_{50} value of 271.19. Similarly, the combinations of *C. cassia* 75% and *Z. officinale* 25% (IC_{50} = 251.76) lowered the IC_{50} value against the larvae of *Cx. pipiens* 24 h post-treatment.

Table 2: Larvicidal activity of individual and combined extracts of *Cinnamomum cassia* and *Zingiber officinale* against 3rd instar of *Culex pipiens*

Extracts used	Combination of extract	LC_{50} (µg mL^{-1})
C. cassia alone	C 100%	317.61
Z. officinale alone	Z 100%	269.42
C. cassia & Z. officinale	25%: 75%	271.19
C. cassia & Z. officinale	50%: 50%	292.57
C. cassia & Z. officinale	75%: 25%	251.76

Integrated mosquito management relies on a combination of common-sense practices and cost-effective methods to control mosquitoes with the most negligible toxicity to man and the environment. The biodegradability, low cost, efficacy, and target-specificity make botanical extracts a vital source in integrated mosquito management plans (Al-Mehmadi and Al-Khalaf, 2010) and, thus, may be more advantageous than other synthetic insecticides. Blending plant's secondary metabolites could reduce the LC_{50} value of a single plant extract and, therefore, becomes more effective in controlling mosquitoes and resistant mosquitos to larvicidal agents. Several factors play an essential role in the development of resistance, such as biological factors (genetics), behavioral factors (movement and exposure), and operational factors (use of insecticides) (NRC, 1986). Our results are similar to the previously published studies that reported the larvicidal potential of combined effect of essential oils and methanol extracts of *Callistemon rigidus* and *Eucalyptus camaldulensis* against *Anopheles gambiae* larvae (Younoussa *et al.*, 2020). A similar observation was seen when the extract of *Sophora alopecuroides* extract was found to be more effective than a single active compound of its active ingredients against *Ae. albopictus* (Shoukat *et al.*, 2020).

Botanical-derived insecticides have been reported for their toxic effects on the mosquito's midgut. For example, the combined extract of *Matricaria chamomilla* and *Foeniculum vulgare* led to the destruction of microvilli and degeneration of epithelial cells in the midgut of *Cx. pipiens* larvae (Al-Mekhlafi *et al.*, 2021). Similarly, the ethanolic extract of *Melia azedarach* that caused microvilli damages, cell vacuolization, cell lysis on the midgut of *Cx. quinquefasciatus* larvae (Al-Mehmadi and Al-Khalaf, 2010). The observed histopathological effects of the *C. cassia* and *Z. officinale* extract combination on the midgut of *Cx. Pipiens* larvae agreed with the results of *Cx. pipiens* treated with *Artemisia judaica* that showed loss of normal appearance of epithelium cells, vacuolation, and loss of microvilli (Hussien and Shoukry, 1997, Assar and El-Sobky, 2003). Conversely, when, asafoetida and garlic extract were combined they showed antagonistic action compared to their individual application against of *Cx. pipiens* larvae (Muturi *et al.*, 2018). Similar antagonistic observation was also reported when combined extract of *Cymbopogon nardus* and *Eucalyptus citriodora* against *A. gambiae* larvae (Obame-Engonga *et al.*, 2017).

In negative control the histological observations showed that the midgut of 3rd instar of *Cx. pipiens* was composed of one layer of columnar epithelium cells with a microvilli-covered surface surrounded by a peritrophic membrane (Fig. 2A,B). The peritrophic membrane was regular and smoothly attached to the epithelium. The histological section showed marked morphological changes in the extract-treated larvae as compared to the control. The larval exposure to C25%: Z75% extract at

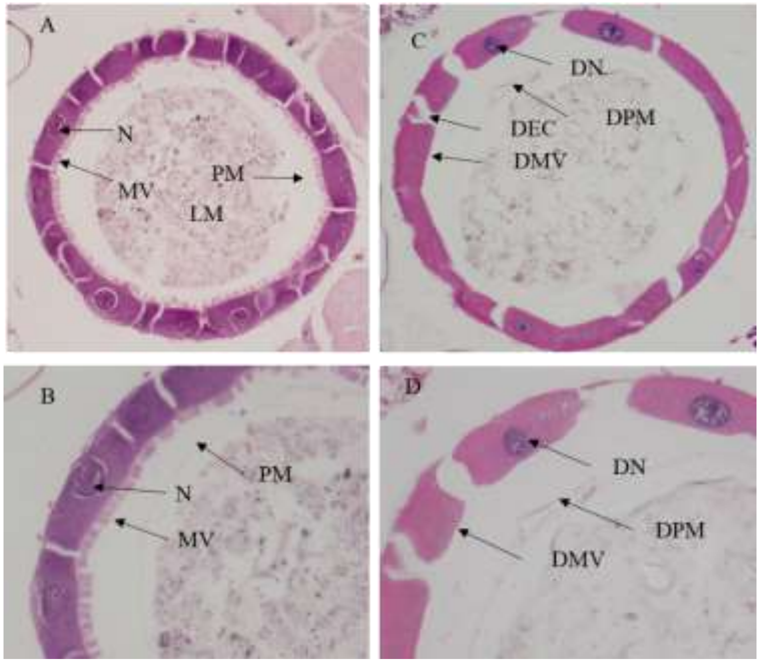

Fig. 2: Photomicrograph of the midguts of *Culex pipiens* third instars treated with *C. cassia* 75% and *Z. officinale* 25% hexane extract (LC_{50}:251.76) at 24 h post-treatment. (A, B) Cross-sections of the midgut of untreated larvae. (C, D) Cross-sections of the midgut of treated larvae. Degraded microvilli (DMV); degenerating epithelial cells (DEC); degenerating peritrophic membrane (DPM); degenerating nuclei (DN); Nuclei (N); Microvilli (MV); Peritrophic membrane (PM); lumen (LM)

LC_{50} level induced degeneration of epithelial cells, degradation of microvilli, loss of nuclei, and partial destruction of peritrophic membrane. The muscle tissue was also affected leading to its detachment from the basement membrane (Fig. 2C,D). Histological observations showed that the midgut of the *Cx. pipiens* larvae was altered after treatment with the extract. The midgut cells, regenerative cells, and entero-endocrine cells play an important role in metamorphosis (Nishiura *et al*., 2003) and homeostasis (Brown *et al*., 1985). Once the midgut cells are affected in treated larvae, the organ remodeling during pupation is prevented and homeostasis disrupted. The damage to the larval midgut of *Cx. pipiens* may get impaired in the process of digestion and absorption; thus disrupting the larval development leading to the death (Procópio *et al*., 2015).

Hemolytic activity

The hemolytic effect of individual extract of *C. cassia*, *Z. officinale,* and their combination in the ratio of Z75:C25 was screened against mice erythrocytes. These extracts exhibited a hemolytic effect toward mice erythrocytes. *Z. officinale* extract at dose 60.50 µg mL^{-1} showed highest hemolytic activity (73.99 ± 0.5%) whereas *C. cassia* and combination of Z75:C25 at dose 60.5 µg mL^{-1} had a lower hemolytic activity of 28.62 ± 0.4% and 29.03 ± 0.9%, respectively (Fig. 3). All the samples showed hemolytic effect toward mice erythrocytes at high concentrations. However, the hexane extract of *Z. officinale* exhibited maximum hemolytic activity. The lysis effect may be attributed to the effect of extracts on the erythrocyte membrane. Human erythrocyte membrane is structurally similar to the horse, monkey, dog, rabbit, rat, and mouse membrane (Virtanen *et al*., 1998). However, further study is required on human RBCs since compositions differ between species (Namdee *et al*., 2015).

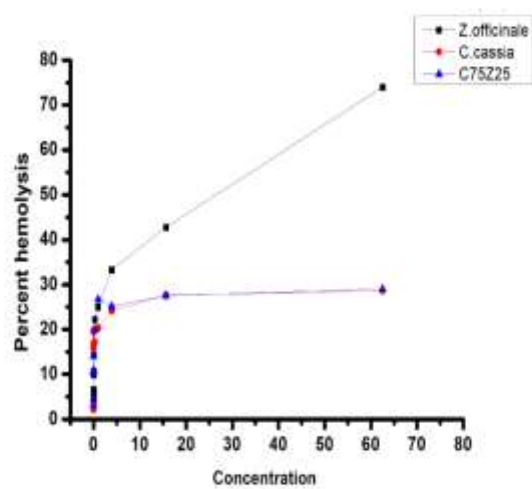

Fig. 3: The hemolytic effect of the individual extract of *Cinnamomum cassia, Zingiber officinale,* and their combination in the ratio of C75:Z25 on albino mouse red blood cells. HC_{50} (*Z. officinale*: 72.6 µg mL^{-1}) is the concentration that resulted in 50% of hemolysis of red blood cells after 30 min of incubation.

Conclusion: Our results demonstrated that combining the hexane extracts *C. cassia* and *Z. officinale* extract provides promising larvicidal activity against *Cx. pipiens* that could be associated with the presence of phenols. *Z. officinale* extract showed the highest radical scavenging activity and hemolysis compared to other extracts tested. DPPH radical scavenging activity increased dose-dependently with the increase of total phenolic content. The result of hemolytic activity revealed that all the extracts tested possessed hemolytic activity at the highest concentrations used. However, further studies are required to investigate the toxicity on non-target organisms.

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Conflict of Interest: The authors have declared that they have no conflicts of interest.

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