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Insecticidal Activity of Harmaline from *Peganum harmala* Against the Larvae of Deltamethrin-Resistant Strain of the Asian Tiger Mosquito, *Aedes albopictus*

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Key words
Harmaline, Larval toxicity, Sublethal effect, *Aedes albopictus*, Deltamethrin-resistant strain, *Peganum harmala*

ABSTRACT

The aim of this study was to explore the potential of harmaline as new, safe and more effective larvicidal of mosquitoes. Lethal and sublethal effects of harmaline were studied against the larvae of deltamethrin-resistant strain of *Aedes albopictus*. Laboratory bioassays were performed to determine the lethal and sublethal effects of harmaline on the larvae of *Ae. albopictus*, according to the standard WHO larval susceptibility test methods. The results indicated that harmaline exhibited strong larvicidal activity against the mosquito larvae, and the lethal effect on larval mortality of *Ae. albopictus* increased in a concentration-dependent manner. The mortality of four instar larvae peaked at 72 h after exposure. Among four instar larvae tested, the first-instar larvae was the most sensitive to harmaline with LC_{50} value of 23.02 mg/L, and the fourth-instar larvae was the most tolerant to harmaline with LC_{50} value of 42.58 mg/L at 72 h after exposure. In addition, sublethal dosage (LC_{10} and LC_{30}) of harmaline could significantly delay the development of larvae and pupae ($P<0.05$). The LC_{30} concentration of harmaline also significantly decreased the pupation and adult emergence rates of larvae treated ($P<0.05$). The present study demonstrated that harmaline has a significant toxic effect against the deltamethrin-resistant strain larvae of *Ae. albopictus*. Particularly, harmaline might still cause markedly sublethal effects to the larvae, even at very low concentration (LC_{10}) of harmaline. It is, therefore, worth further exploring the use of harmaline as a potential larvicide against vector mosquitos. four larval stages of *Ae. albopictus*.

INTRODUCTION

The Asian tiger mosquito, *Aedes albopictus* (Skuse) (Diptera: Culicidae) is one of the most significant pathogen vectors of the twenty-first century. Originating from Asia, it has invaded a wide range of eco-climatic regions worldwide (Bhatt *et al*., 2013; Manni *et al*., 2017; Chen *et al*., 2019). *Ae. albopictus* has proven to be an infectious vector, which can transmit a spectrum of at least 23 human pathogens causing various diseases including dengue virus, West Nile virus and chikungunya virus (Gamez *et al*., 2020; Mitchell, 2020). According to the WHO estimates, about 2.5 billion people live in dengue-risk areas around the world with 50 million dengue infections occurring every year. At present, there are no drugs or vaccines against the main pathogens and parasites transmitted by *Ae. albopictus*. As a result, it is well known that one way to reduce the mosquito populations is targeting mosquito larvae with chemical insecticides, such as pyrethroids, organophosphates, and insect growth regulators. However, repeated use of these chemical insecticides can lead to the development of resistance in mosquitoes or to human health or to undesirable effects on non-target organisms (Dusfour *et al*., 2019; Zhao *et al*., 2020; Deng *et al*., 2021; Montgomery *et al*., 2022). For these reasons, there is an urgent need to develop new insecticides which are more environmentally safe and also biodegradable and target specific against vector

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mosquitoes (Benelli, 2015; Li *et al*., 2021). From this point of view, botanical-based insecticides are promising for mosquito control strategies, since they are rich storehouse of chemicals of diverse larvicide activity, more biodegradable and less hazardous. Particularly, the resistance by vectors against plant-derived insecticides has not been reported so far, and by controlling larval mosquitoes, adults may never become a problem (Dinesh *et al*., 2014; Pleydell and Bouyer, 2019). Indeed, plant extracts and plant-derived compounds belonging to many families have been reported to possess larvicidal properties against *Aedes*, *Culex* and *Anopheles* mosquitoes (Diptera: Culicidae) (Bara *et al*., 2014; Pleydell and Bouyer, 2019; Gou *et al*., 2020).

Harmaline was originally isolated from *Peganum harmala* (Zygophyllaceae), which is a perennial herbaceous and widely distributed in Middle East, India, Mongolia and China (Li *et al*., 2017). This plant is rich in alkaloids and contains about 2% to 6% total alkaloids (dry weight), the alkaloid compounds illustrate well the diversity of pharmacological and biological activities compounds also found in various plants, its active alkaloids include beta-carbolines such as harmaline, harmine and harmalol (Kartal *et al*., 2003; Cao *et al*., 2007; Al-Mazra'awi *et al*., 2009; Mina *et al*., 2015). Modern pharmacology has also revealed that *P. harmala* alkaloids can inhibit acetylcholinesterase (AChE), butyrylcholinesterase (BChE), monoamine oxidase A (MAO-A), interact with-aminobutyric acid (GABA), and induce apoptosis and DNA damage (Khan *et al*., 2013; Mina *et al*., 2015). Currently, studies have shown that harmaline is one of the major active components of beta-carboline alkaloids and has multiple biochemical and pharmacological activities (Di Giorgio *et al*., 2004; Khan *et al*., 2013; Moazeni *et al*., 2017).

At present, some researchers have reported significant insecticide activity of beta-carboline alkaloids and total alkaloid extracts (TAEs) from *P. harmala* against various pests (Al-Mazra'awi *et al*., 2009; Rizwan-ul-Haq *et al*., 2009; Alomar *et al*., 2013; Shang *et al*., 2016; Moazeni *et al*., 2017; Miao *et al*., 2020). Interestingly, in addition to lethal effects, several studies have also revealed that *P. harmala* total alkaloids or its beta-carboline alkaloids could induce sublethal effects on insect development, reproduction, and behavior (Weng *et al*., 2005; Rharrabe *et al*., 2007; Jbilou *et al*., 2008; Al-mazra'awi *et al*., 2009). We have previously evaluated the insecticidal activity of TAEs and its alkaloids against many pests under laboratory and field conditions (Zhao *et al*., 1997; Jiang *et al*., 1999; Li *et al*., 2016; Jiang *et al*., 2023). Although studies investigated the insecticidal activities of some beta-carboline alkaloids and alkaloid extracts from *P. harmala,* but there is little evidence of being used as insecticides for controlling the vector mosquitoes like *Ae. albopictus*. Thus, the aim of this study was to evaluate the lethal and sublethal effects of harmaline against the deltamethrin-resistant strain larvae of *Ae. albopictus*.

MATERIALS AND METHODS

Harmaline and insect

Harmaline was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO) and the highest purity available (>98%), which obtained from the seeds of *P. harmala*. Deltamethrin powder (95% effective content) was purchased from Jiangsu Yangnong Chemical Group Co., Ltd. Tween 80 (Tedia Company, Inc., 1000 Tedia way, Fairfield, OH, USA).

Ae. albopictus, maintained for more than 100 generations without exposure to any known insecticide, was obtained from laboratory colonies in the State Key Laboratory of Pathogen and Biosecurity, Institute of Microbiology and Epidemiology, Beijing. According to following the standard World Health Organization larval susceptibility test methods (WHO, 2005), and the deltamethrin-resistant colony of 15 generations, and the lethal concentration that kills 50% of the fourth instars larvae to deltamethrin (LC_{50}) was 16.87 mg/L when it was used for this experiment. Eggs for the study were obtained by feeding mated 15 generations females with provided 10% sucrose solution for 12 h, and then provided with a rat placed in resting cages (25×25×35 cm) overnight for blood feeding by females. Larvae were fed a diet of dog biscuits, milk powder, beef liver, and yeast powder in a ratio of 2:1:1:1, respectively. The insectary room was maintained at a photoperiod of 14:10 (L/D) h, temperature of 27 ± 2°C and relative humidity of 75%-85%.

Lethal bioassays

The larval mortality bioassays were performed according to the test method of larval susceptibility as recommended by the World Health Organization methods (WHO, 2005). The different concentrations of harmaline solution were prepared with distilled water (0.05% Tween 80 as a carrier solvent), Tween 80 (0.05%) served as a control. Bioassays were performed on first to fourth instars of *Ae. albopictu*s using concentrations of harmine as 20, 50, 80 and 110 mg/L, and thirty randomly-selected larvae per concentration was introduced into all the experiments. For mortality tests, thirty larvae each of the second, third and fourth instar larvae were introduced to a 250 ml glass beaker containing various concentrations of harmaline, and supplemented with 30 mg/L of the mixture food for larvae. A control was also maintained with Tween 80

(0.05%). Each treatment was replicated five times, and each replicated set contained one control for comparison. Larvae were exposed to various concentrations of harmaline at different hours after treatment. Mortality was recorded every 24 h after treatment. The larvae did not respond to the gentle prodding with forceps tip were judged as dead. The mortality rate was corrected when necessary for mortality in the controls using Abbott formula (Abbott, 1925). The lethal concentration (LC_{50} and LC_{90}) and their 95 % confidence limit was calculated using probit analysis (Finney, 1971).

Sublethal bioassays

The LC_{10} and LC_{30} concentrations required to kill 10% and 30% of the second-instar larvae within 72 h, which had already been determined from lethal bioassays, were selected as sublethal concentration of harmaline. Thirty larvae of early second instars were placed in 250 ml beaker and exposed to the LC_{10} and LC_{30} doses of each tested. And 72 h after treatment, dead larvae were counted and only alive larvae were transferred to glass beakers through a small filter, and larvae were provided with the mixture larval food at a concentration of 50 mg/L until pupation (WHO, 2005). For each treatment and control, seven replicates were performed in this experiment. Larval development was monitored daily until all larvae had either pupated or died. Pupae from each treatment were removed daily, and were transferred into a cups with deionized water until adults emerged. The development of mosquito larvae, and pupae and adults emerging each day was recorded.

Statistical analysis

Data from larval mortality tests were subjected to analysis of variance (ANOVA of square root transformed percentages). Differences between the treatments were determined by Tukey's multiple range test to compare differences at $P<0.05$ significance level. The sulethal and lethal dosages of harmaline to the tested larvae were calculated by using probit analysis. And other statistics at 95 % confidence limits of upper confidence limit and lower confidence limit, and Chi-square (χ^2) values were calculated using SPSS 12.0 software. The results with $P < 0.05$ were considered statistically significant to be statistically significant level.

RESULTS

Acute toxicity of harmaline on the larvae of Ae. albopictus

The larval mortality rates of *Ae. albopictus* were gradually increase with the rise in concentrations of harmine, after treating with harmine for 24 h (Fig. 1). In a high-dose treatment (80 and 110 µg/mL), more than 70 % of the observed mortality occurred within the first 24 h, and with a significant level when compared with the control ($P<0.05$). In a low-dose treatment (20 and 50 µg/mL), the mortality rates of all the larval instars were lower. The present investigation also showed that the observed mortality rate of the second-instar larvae of *Ae. albopictus* was higher than that of the third- and fourth-instar larvae in all concentrations examined. Thus, *Ae. albopictus* larval mortality varied in a concentration-dependent manner.

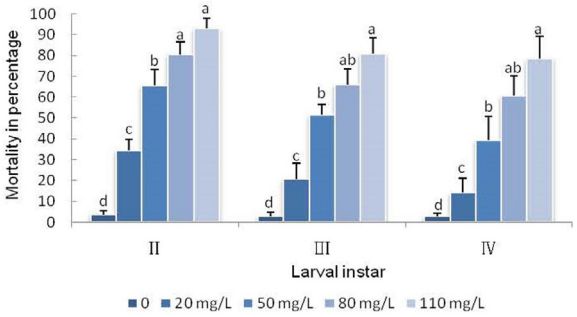

Fig. 1. Percentage mortality of harmaline against the second, third and fourth instar larvae of *Aedes albopictus*. Means (±SE) followed by different letters in bars indicate significantly difference (P<0.05).

The action of harmaline on the early fourth-instars of *Ae. albopictus* was slower at all concentrations examined (Table I). Harmaline did not show that the highest toxicity on the larvae of *Ae. albopictus* until 72 h after experiments. The LC_{50} concentrations at 24, 48, 72, 96 and 120 h were 52.73, 46.01, 41.83, 41.52 and 40.99 mg/L, respectively. Similarly, the LC90 concentrations at 24, 48, 72, 96 and 120 h were 96.55, 86.57, 83.13, 84.27 and 83.63 mg/L, respectively. Thus, the LC_{50} and LC_{90} concentrations obviously were decreased with the time of exposure, and the best time for assessing the susceptibility of *Ae. albopictus* larvae to harmaline was 72 h after treatment.

Susceptibility of four larval stages of Ae. albopictus *to harmaline*

In this experiment, four larval stages of *Ae. albopictus* showed different susceptibilities to harmaline at 24, 48, and 72 h after exposure, respectively (Table II). Among four instar larvae, the first-instar larvae was the most sensitive to harmaline, the LC_{50} values were 27.98, 26.21 and 23.02 mg/L at 24, 48, and 72 h after exposure, respectively. The fourth-instar larvae are the most tolerant to harmaline, the LC_{50} values were 54.12, 47.94 and 42.58 mg/L at 24, 48 and 72 h after treatment, separately. Similarly, the

Table I. Toxicity of harmaline against the fourth-instar larvae of *Aedes albopictus* at different time after treatment.

Treatment time (h)	LC_{50} (mg/L) (95% CL)	LC_{90} (mg/L) (95% CL)	Slope ± SE	χ^2 (df=4)
24	52.73 (42.35-54.63)	96.55 (86.31-102.96)	3.18±0.65	13.09*
48	46.01 (38.52-52.51)	86.57 (79.85-97.45)	3.07±0.24	12.07*
72	41.83 (37.91-48.25)	83.13 (79.71-93.13)	2.17±0.22	11.83*
96	41.52 (36.58-47.21)	84.27 (80.15-91.72)	2.29±0.43	10.87*
120	40.19 (35.86-45.95)	83.63 (78.91-89.79)	2.59±0.58	10.05*

LC_{50} lethal concentration that kills 50% of the exposed larvae; LC_{90} lethal concentration that kills 90% of the exposed larvae; 95%CL is the 95% confidence limits. The means of the five replicates ± SE are shown. χ^2 chi-square, df degree of freedom, each value of slope represented the regression slope of the relationship between larval mortality and lethal time. * Significance at $P<0.05$ level.

Table II. The susceptibility of four larval stages of Aedes albopictus to harmaline at 24, 48 and 72 h after exposure.

T (h)	Instar	LC_{50} (mg/L) (95% CL)	LC_{90} (mg/L) (95% CL)	Slope ± SE	χ^2 (df=4)
24	1	27.98 (24.83-31.67)	52.66 (42.87- 58.01)	3.14±0.38	10.75*
	2	30.91 (26.18-35.16)	57.46 (51.85-64.01)	2.78±0.44	11.87*
	3	43. 77 (36.84-50.31)	82.66 (78.92-90.71)	2.83±0.35	10.02*
	4	54.12 (48.74-61.57)	96.83 (87.26-105.85)	2.01±0.47	12.73*
48	1	26.21 (22.75-31.04)	51.07 (42.35-59.47)	3.49±0.62	10.31*
	2	28.23 (24.86-33.01)	53.84 (46.12-57.63)	2.71±0.33	9.58*
	3	37.68 (31.57-43.61)	70.59 (64.61-76.83)	2.98±0.63	11.06*
	4	47.94 (40.75-53.76)	85.95 (80.57-103.99)	3.02±0.47	12.03*
72	1	23.02 (21.75-30.76)	44.31 (40.62-48.07)	3.29±0.45	9.92*
	2	25.79 (21.02-30.92)	48.39 (43.08-54.62)	2.53±0.29	10.49*
	3	31.09 (26.13-36.18)	65.25 (47.85-73.48)	2.96±0.35	12.45*
	4	42.58 (37.69-50.91)	82.69 (78.66-87.23)	2.87±0.46	11.98*

T is the hours after treatment when larval mortality was evaluated. Instar: 1~4 is representing the first, second, third and fourth instar larvae of *Aedes albopictus*, respectively. χ^2 chi-square, df degree of freedom, each value of slope represented the regression slope of the relationship between larval mortality and lethal time. *Significance at $P<0.05$ level.

Table III. Comparison of development time, pupation and emergence rates of surviving larvae of the second-instar larvae of *Aedes albopictus* in sublethal dosages of harmaline at 72 h post-exposure.

Treatment	Larvae development duration (mean ± SE)			Pupa period (h)	Pupation rate %	Emergence rate %
	Second instar (h)	Third instar (h)	Fourth instar (h)			
Control	37.5 ± 7.2	31.6 ± 6.8	66.2 ± 5.9	102.3 ± 10.5	96.8 ± 2.1	94.1 ± 3.9
LC_{10}	43.4 ± 5.5	45.3 ± 5.7*	95.6 ± 7.1*	130.7± 13.3*	89.7 ± 5.9	83.3 ± 6.3
LC_{30}	56.3 ± 8.1*	62.1 ± 6.1*	104.4 ± 6.3*	149.2± 12.8*	74.5 ± 6.2*	65.4 ± 5.1*

LC_{10} lethal concentration that kills 10% of the exposed larvae; LC_{30} lethal concentration that kills 30% of the exposed larvae; The means of the five replicates ± SE are shown. * Significance at $P<0.05$ level.

third-instar larvae was more tolerant to harmaline than the second-instar larvae. The LC_{50} and LC_{90} values of four instar larvae of *Ae. albopictus* to harmaline decreased with the extension of exposure time. Thus, the results showed that the early larval instars were more sensitive to harmaline than that of the later instars.

Sublethal effects of harmaline on the second instar larvae of Ae. albopictus

After the second-instar larvae of *Ae. albopictus* exposure to sublethal concentration (LC_{10} and LC_{30}) of harmaline for 72 h, the results showed that the development, pupation and adults emergence were affected at different treatments (Table III). Compared with control, the development of larvae exposed to LC_{10} and LC_{30} concentrations had significant influence ($P<0.05$). The developmental durations of larvae tested by LC_{10} and LC_{30} concentrations were longer 48.3 h and 87.5 h from second instar to larvae pupation, and 28.4 h and 46.9 h in pupa duration. Moreover, the LC_{30} concentration of harmaline

could significantly affect the pupation and emergence rates of the survivors (P<0.05), and the pupation and emergence rates of larvae treated by LC_{30} concentration were 23.1% and 38.5% of control, respectively. Therefore, the results suggested that sublethal dosages of harmaline could delay the development of larvae, and decrease the pupation and adult emergence of *Ae. albopictus*.

DISCUSSION

In recent decades, the global use of synthetic insecticides to control mosquitoes has caused environmental pollution and led to the widespread development of insecticide resistance in many mosquito species, including *Ae. Albopictus* (Demok *et al*., 2019; Dusfour *et al*., 2019; Deng *et al*., 2021; Montgomery *et al*., 2021; Li *et al*., 2021). With the increasing demand for more eco-friendly products for mosquito control, plants may be valuable sources for mosquito control products. Plants can produce a vast repository of secondary compounds with a wide range of biological activities such as insecticide activity. Plant alkaloids, either as plant-derived insecticides or as pure compounds, provide unlimited opportunities for new and selective pesticide discoveries because of the multiple insecticidal active targets and unmatched availability of chemical diversity (Benelli, 2015; Baskar *et al*., 2018; Gou *et al*., 2020). Harmaline (7-Methoxy-1-methyl-4,9-dihydro-3H-beta-carboline), is one of a major active compound of β-carboline alkaloids, and has many biological activities, such as cytotoxic effect, DNA intercalation ability and anti-Leishmania activity (Guan *et al*., 2006; Cao *et al*., 2007; Khan *et al*., 2013; Li *et al*., 2017). In this study, the high rate of larval mortality of the different larval stages observed at higher concentrations (80 and 110 μg/mL) of harmaline), within a 24 h exposure indicates the high toxicity of the alkaloid. At lower concentrations (20 and 50 μg/mL) of harmaline, the mortality rates of all the larval instars were lower, and which could lead to morphological malformations of some larvae lived. The lethal effect on larval mortality was dependent on concentration of harmaline. Previous studies have also shown similar results, showing that several beta-carboline alkaloids and alkaloid extracts from the plant of *P. harmala* have significant lethal effects on many pests, such as *Spodoptera litura* (Di Giorgio *et al*., 2004), *Plodia interpunctella* (Rharrabe *et al*., 2007), *Spodoptera exigua* (Rizwan-ul-Haq *et al*., 2009), *Fasciola hepatica* (Moazeni *et al*., 2017), and *Caenorhabditis eleg*ans (Miao *et al*., 2020). In this research, our results also clearly showed that the insecticidal activity of harmaline against fourth-instar larvae increased significantly with exposure time, such as at 24 h, 48 h, 72 h, 96 h and 120 h after exposure, LC_{50} values were 52.73, 46.01, 41.83, 41.52, and 40.19 mg/L, respectively. The results indicated that the best time to assess the susceptibility of *Ae. albopictus* larvae to harmaline was 72 h after treatment. This finding is also supported by our previous investigations (Li *et al*., 2016) and other studies (Rharrabe *et al*., 2007; Shang *et al*., 2016; Miao *et al*., 2020). Interestingly, four larval stages of *Ae. albopictus* larvae showed different susceptibilities to harmaline, and the early larval instars were more sensitive than the later instars in this study. Therefore, the chemical control should be directed against the first and second instar stages, if harmaline is used as larvicide of resistant strain of *Ae. albopictus* larvae in practice.

In addition to the lethal effect, the sublethal concentrations (LC_{10} and LC_{30}) of harmaline on *Ae. albopictus* induced larval development delay, pupation and adult emergence rates decreased in this study. Thus, the results indicated that harmaline, even at very low dosages, might still obvious bioactivity to the larvae of deltamethrin-resistant strain of *Ae. albopictus*. Previous studies have found that harmaline and several beta-carboline alkaloids can induce cells to produce large quantities of singlet oxygen and/or superoxide radical, and these toxic oxygen species have high cytotoxicity, and can attack the membrane to cause unsaturated lipids, as well as destroy the structure and function of the membranes (Weng *et al*. 2005; Khan *et al*., 2013; Li *et al*., 2016). Especially, a study found that *P. harmala* extracts exhibited many sublethal effects on *Tribolium castaneum*, including α-amylase activity, larval development, and progeny production (Jbilou *et al*., 2008). Similarly, another two studies also observed sublethal effects of total alkaloids from *P. harmala* on *Spodoptera exigua* and *Caenorhabditis elegans*, which involved nutrient metabolism and larval development (Rizwan-ul-Haq *et al*., 2009; Miao *et al*., 2020), as confirmed by our previous study (Jiang *et al*., 2023). Thus, these findings suggest that harmaline and other beta-carboline alkaloids have multiple insecticide targets, and imply a possible the mechanisms underlying harmaline-induced changes in mosquito lethal and sublethal trials including effects on acute toxicity, mosquito physiology, larval metabolism and development.

Currently, the growing resistance of *Ae. albopictus* populations to the synthetic pesticides has hindered the efforts to control dengue vector effectively. Thus, there is an urgent need to develop new insecticides that are more environmentally safe, biodegradable and target specific against mosquitoes. Nowadays, biopesticides, including plant-based insecticides, can enhance the control efficiency of insecticide-resistant mosquitoes (Smith *et al*., 2016; Gari1 and Lindtjorn, 2018; Pleydell and Bouyer, 2019). Two recent studies found that the impact of deltamethrin-resistance in *Ae. albopictus* on its fitness cost

and vector competence, such as the resistance prolonged the growth and development of larvae, as well as shorten the life span of resistant *Ae. albopictus* adults (Ngoagouni *et al*., 2016; Gomard *et al*., 2021; Deng *et al*., 2021). Indeed, the biological activity of plant-based insecticides for controlling resistant pests indicates that in addition to lethal effects, potential sublethal physiological effects such as growth inhibition, reproductive interference, repellents, and behavioral effects may also occur. In this study, harmaline offers a potential against the larvae of deltamethrin-resistant strain of *Ae. albopictus*, particularly in its markedly sublethal effects. Therefore, it may provide theoretical information for further research on the mechanisms underlying insecticidal activity of harmaline and other beta-carboline alkaloids, and development of environment friendly pesticides.

CONCLUSION

In this study, harmaline from *P. harmala* seeds against the larvae of deltamethrin-resistant strain of *Ae. albopictus* was studied in the bioassay. The results indicated that harmaline exhibited strong larvicidal activity against the mosquito larvae, and the lethal effect on larval mortality of *Ae. albopictus* increased in a concentration-dependent manner. The mortality of four instar larvae peaked at 72 h after exposure. Interestingly, harmaline could cause markedly sublethal effects to the larvae, even at very low concentrations (LC_{10} and LC_{30}) of harmaline. It is, therefore, worth further exploring the use of harmaline as a potential larvicide against vector mosquitos.

DECLARATIONS

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Statement of conflict of interest

The authors have declared no conflict of interest.

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