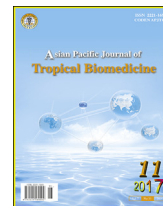




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Adulticidal, larvicidal, pupicidal and oviposition deterrent activities of essential oil from *Zanthoxylum limonella* Alston (Rutaceae) against *Aedes aegypti* (L.) and *Culex quinquefasciatus* (Say)

Mayura Soonwera¹, Siriporn Phasomkusolsil^{2*}¹Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand²Department of Entomology, Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand

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ABSTRACT

Objective: To evaluate adulticidal, larvicidal and oviposition deterrent response of the essential oil from dried *Zanthoxylum limonella* (*Z. limonella*) fruit against *Aedes aegypti* (*Ae. aegypti*) and *Culex quinquefasciatus* (*Cx. quinquefasciatus*).

Methods: *Z. limonella* oil was tested by biological assays at 1%, 5% and 10% concentrations in ethanol. Adulticidal efficacy was tested against the 2–3 day old adult females. Larvicidal activity was tested against immature stage of mosquitoes. Oviposition deterrence of the oil was evaluated on gravid females.

Results: The adult mortality was observed after 24 h with the LC₅₀ of 6.0% for *Ae. aegypti*, and 5.7% for *Cx. quinquefasciatus*. Larvicidal bioassay was carried out with the 10% *Z. limonella* oil against immature stages of *Ae. aegypti* and *Cx. quinquefasciatus*, which caused 100% mortality after 12 h and 24 h. In the larvicidal experiment, *Z. limonella* showed effective result at 1%, 5% and 10% concentrations with the values of LT₅₀ *Ae. aegypti* = 9.78, 5.61, 0.24 h for larvae and LT₅₀ = 64.08, 21.23 h for pupae; *Cx. quinquefasciatus* had LT₅₀ = 28.46, 20.25, 1.01 h for larvae and LT₅₀ = 67.52, 27.96, 4.11 h for pupae, respectively. Oviposition deterrence of the oil was evaluated on gravid females. In the study, 10% *Z. limonella* showed 100% repellency for *Ae. aegypti* and 99.53% for *Cx. quinquefasciatus*. Likewise, oviposition activity indexes of these oil concentrations were all negative values ranging from –0.89 to –1.00 for *Ae. aegypti* and –0.64 to –0.99 for *Cx. quinquefasciatus*. The oviposition activity indexes values revealed that *Z. limonella* oil has deterrent effect, and it caused a remarkable negative response resulting in very few eggs.

Conclusions: This result indicates that *Z. limonella* oil can be used as an effective adulticide, larvicide and oviposition deterrent against *Ae. aegypti* and *Cx. quinquefasciatus*.

1. Introduction

Mosquitoes are the most important insect pests that affect the health and wellbeing of humans and domestic animals worldwide. *Aedes aegypti* (*Ae. aegypti*) (yellow fever mosquito), the principal vector for dengue and yellow fever, bites primarily during the day and at dawn and dusk [1]. Dengue fever and dengue hemorrhagic fever cause 390 million infections every year, of which 96 million manifest clinically [2]. Dengue fever has now spread around the world, and is endemic in most parts of Asia, including India and South East Asia. Unfortunately, currently no effective vaccine is available for

*Corresponding author: Siriporn Phasomkusolsil, Department of Entomology, Armed Forces Research Institute of Medical Sciences, 315/6 Ratchawithi Road, Bangkok 10400, Thailand.

Tel: +66 (0) 26962817

Fax: +66 (0) 23547885

E-mail: msiriporn@hotmail.com (S. Phasomkusolsil).

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the prevention of dengue fever. *Culex quinquefasciatus* (*Cx. quinquefasciatus*), more commonly called the southern house mosquito, is the principal vector of lymphatic filariasis caused by *Wuchereria bancrofti* and a potential vector of *Dirofilaria immitis* [3]. It is one of the most widespread mosquitoes in the world. Lymphatic filariasis is probably the fastest spreading insect-borne disease of humans in the tropics. The disease has a focal distribution, and it is estimated that currently over 2.5 million people are at risk of acquiring filariasis [4]. Both diseases not only cause mortality and morbidity among humans but also cause social, cultural, environmental and economic loss to the society [5].

One of the methods to prevent these diseases is to control the vectors in an attempt to interrupt disease transmission. Chemical insecticides play a major role in efforts to manage mosquito populations. Although chemicals have been used successfully as components of pest management strategies, many of these synthetic insecticides are limited in mosquito control programmes. This limitation is largely as a result of environmental contamination and insecticide resistance to DDT and almost all classes of pesticides used for control of disease vectors include pyrethroids, carbamates, and organophosphates [6]. This has necessitated the need for searching and development of environmentally safer, target specific and cost effective insecticides against mosquitoes. An alternative and recent approach for mosquito control is the use of natural products of plant origin, known as botanical derivatives. Many botanical natural products are effective, environment-friendly, easily biodegradable and inexpensive. They have no negative effects on non-target organisms and have varied novel modes of action [7]. Among botanical natural products are various highly volatile essential oils currently used in food, perfume, cosmetic and pharmaceutical industries [8]. Essential oils can be synthesized by all plant organs (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and root) and can therefore be extracted from these parts [9]. They are mainly formed by mixtures of monoterpenes, sesquiterpenes, phenylpropanoids and metabolites that confer the mixtures with organoleptic characteristics and biological activities [10]. In general, essential oils have been considered as important natural resources to act as insecticides [11], with low mammalian toxicity and degrading rapidly in the environment [12]. Essential oils derived from various plants show varied bioactivities against mosquito species. These activities range from toxicity with ovicidal, larvicidal, pupicidal [13,14] to adulticidal activities that include oviposition deterrence and repellent actions [15–17]. Some of the plant families known as excellent sources of essential oils with insecticidal properties include the Rutaceae family. In family Rutaceae, the genus *Zanthoxylum* provides a variety of secondary metabolites, including alkaloids, aromatic and aliphatic amides, lignans and coumarins with important phytochemical and biological activities [18,19]. The genus *Zanthoxylum* comprises over 200 species distributed worldwide especially in Eastern and Southeast Asia, America and Africa [20]. Various species of this genus are used for medicinal purposes such as stomachache, toothache, intestinal worms, rheumatism, scabies, snakebites, fever and cholera [21].

In Asia, *Zanthoxylum limonella* (*Z. limonella*) (Dennst.) Alston is locally known in northern region of Thailand as one of the spices, namely ma-kwaen and has been traditionally used in food and traditional medicine. It is a tree that grows up to 50 m high. The different parts of *Z. limonella* have been used in Thai traditional medicine. The fruits are used as a spice for flavoring

traditional food while essential oil is extracted from the fruits. *Z. limonella* fruit has been used in a traditional medicine [22] and its essential oils have stimulation effect on reducing muscle strain [23]. Several research groups have extracted oil from *Z. limonella* fruit. The fruits are used as appetizers as well as for treatment of cholera, asthma, bronchitis, heart troubles, piles and toothache, and for relief of hiccup. It is also used as food supplements to protect against emergent diseases such as cardiovascular problems, cancer and diabetes [24]. The oil extracts from *Z. limonella* fruits are reported to possess herbicidal and biological activities including antimalarial and anti-tuberculosis [25]. The essential oil from these fruits is effective against earthworms, tapeworms and hookworms [26]. However, studies on mosquitocidal properties of *Z. limonella* are limited. In the present study, the essential oil from dried *Z. limonella* or ma-kwaen fruit was evaluated for their adulticidal, larvicidal and oviposition deterrence activities against the medically important vector mosquitoes, *Ae. aegypti* and *Cx. quinquefasciatus*.

2. Materials and methods

2.1. Mosquito rearing

Ae. aegypti (L.) and *Cx. quinquefasciatus* (Say) mosquitoes were reared in the Laboratory of Entomology, Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. The colonies of mosquitoes were maintained and all the experiments were carried out at 30–35 °C and 60%–80% relative humidity with a photoperiod of 12 h light followed by 12 h dark (12L:12D). Larvae were reared in white plastic trays (30 cm × 35 cm × 5 cm and containing 2 500 mL tap water). Fish food (OPTIMUM®) was added (0.1 g for 1st and 2nd instar larvae, 0.3 g for 3rd instar larvae, and 0.5 g for 4th instar larvae each day) to each tray for two weeks until pupation of all larvae. Newly formed pupae were transferred from the trays to cups containing water and placed in screened cages (size 30 cm × 30 cm × 30 cm) until they emerged as adults. The adults were continuously provided with 5% sucrose solution on saturated cotton pads. On day 5 after emergence, the adult females were deprived of sugar for 12 h then provided with human blood by artificial membrane feeding technique [27] for 30 min. Two days after blood feeding, plastic cups filled with tap water were placed inside the cage for oviposition which occurred by day 3 or 4 after blood feeding.

2.2. Plant materials

Fruits of *Z. limonella* commonly known as ma-kwaen were collected from Den Chai District, Phrae Province, Thailand. Fruit identification was authenticated by a plant taxonomist from Department of Plant Production Technology, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. Essential oil was extracted from plant material by water distillation [28]. During this process, one kilogram of the air-dried fruits was powdered using an electric blender and placed in an extraction column connected to a round bottomed distillation flask containing distilled water. The flask was heated to about 100 °C and the extraction was stopped after the distillation time reached 5 h. After the water distillation process, the essential oils settled at the bottom layer

of the separatory funnel were separated several times until no oil was left in the funnel. The essential oil was stored in airtight bottle and kept at 4 °C for later experiments. Essential oils were dissolved in 70% ethanol and evaluated at three concentrations of 1%, 5% and 10%.

2.3. World Health Organization (WHO) susceptibility test

Knockdown and mortality were evaluated using the WHO susceptibility test according to the latest published guidelines [29]. Whatman No 1. filter papers (12 cm × 15 cm) were impregnated with 2 mL of testing sample. After dipping, the papers were allowed to dry and were then placed in the plastic tubes of WHO test kits (125 mm in length and 44 mm in diameter). A piece of blank paper consisting of only 70% ethanol was used as a control. For the WHO assay, 25 females used in the bioassays were from batches of non-blood-fed mosquitoes (2–3 d after emergence). They were introduced into holding tubes then transferred to the exposure tube with a piece of treated filter paper. After 1 h in the exposure tube, mosquitoes were returned to the holding tube and provided a 5% sugar solution. Cypermethrin was used as positive control. Knockdown after the 1-h exposure and mortality at 24 h were recorded. As per WHO definition, a mosquito was scored in the assay as alive if it was able to fly, irrespective of the number of legs still intact, and dead or knocked down, if immobile or incapable of flying or standing in a coordinated manner [29]. The mortality percentage was calculated using the following formula:

$$\% \text{ Mortality} = \frac{\text{Number of dead adults}}{\text{Number of adults introduced}} \times 100$$

2.4. Larvicidal bioassay

The larvicidal assay was conducted according to the guidelines of the WHO [30] with slight modifications. Evaluation of larvicidal activity was conducted in 250 mL plastic cups. An aliquot (1 mL) of essential oil dissolved in ethanol (1%, 5% and 10% concentrations) was added to 99 mL of distilled water. Ten immature stages (early fourth-larval stage and pupal stage) of *Ae. aegypti* and *Cx. quinquefasciatus* were transferred to the test medium and mortality recorded at 12, 24 and 48 h time periods and mortality percentage was calculated. Five replicates for each concentration were carried out. The dead and moribund larvae/pupae were recorded. The larvae were touched gently with the help of a glass rod and were considered dead in the absence of any signs of movement. Pupa was considered dead if it did not move when prodded repeatedly with a soft brush. Any moribund larvae/pupae were added to the dead larvae/pupae for the purpose of calculating mortality percentages [31]. For comparison, a commercial formulation of temephos larvicide (0.012 mg/L) was used as positive control.

For characterizing and quantifying morphogenetic anomalies, dead larvae, pupae and adults were removed daily and preserved in 70% ethanol. The dead larvae, pupae or incompletely enclosed adults were counted and inspected under a stereoscopic microscope. The criteria for determining the effects of the oils on mosquitoes were as follows: (1) normal larvae; (2) deformed dead larvae where stages of larval development and often the

pupae were visible inside the larval cuticle; (3) pre-pupae death during emergence observed from the larval exoskeleton; (4) pupae completely escaped the larval exoskeleton but remained unmelanized until death or white pupa; (5) pupae were abnormally shaped or deformed pupa; (6) dead pupae were melanized and brown normal in appearance; (7) adult had partly emerged from the pupal case before dying. The proportion (%) of the occurrence of a given type of the morphogenetic aberration was calculated for each stage.

2.5. Oviposition deterrence bioassay

Fifteen gravid 5–7 d old females were transferred in a bioassay cage (30 cm × 30 cm × 30 cm) containing two plastic cups (250 mL in capacity), filled with 99 mL of well water and placed diagonally at opposite corners of the cage. An aliquot (1 mL) of essential oil (1%, 5% and 10% concentrations) dissolved in 70% ethanol was added to one cup while an equivalent quantity of ethanol was added to the second (control). A piece of filter paper strip was placed on the internal surface of each cup to provide a support for oviposition. The paper was placed in each cup so that the lower half of the paper was submerged in water. The bioassay was conducted in a laboratory using Soonwera [32] method with slight modification. After 48 h, the eggs laid in each cup were counted after removal of the oviposition paper. Five replicates were performed for oviposition test. The oviposition experiments were expressed as mean number of eggs and oviposition activity index, which was calculated using the following formula [33].

$$\text{OAI} = \frac{\text{NT} - \text{NC}}{\text{NT} + \text{NC}}$$

where OAI represents oviposition activity index, NT is the total number of eggs in the test solution, and NC is the total number of eggs in the control solution. The oviposition activity index ranges from –1 to +1, with 0 indicating neutral response. The positive index values indicate that more eggs were deposited in the test cups than in the control cups, and that the test solutions were attractive. Conversely, more eggs in the control cups than in the test cups result in negative index values and the test solutions were a deterrent.

The effective repellency percent for each essential oil was calculated using the following formula [34].

$$\text{ER}\% = \frac{\text{NC} - \text{NT}}{\text{NC}} \times 100$$

where ER is effective repellency, NC is the total number of eggs in the control solution and NT is the total number of eggs in the test solution.

The ratio of numbers of tested eggs to effective repellency percentage was calculated as follows:

$$\text{REER} = \frac{\text{Numbers of tested eggs laid per female}}{\text{ER}\%}$$

where REER is the ratio of numbers of tested eggs to effective repellency percentage, and ER is effective repellency.

2.6. Statistical analysis

50% knockdown time (KT₅₀), 50% effective concentration (EC₅₀), 50% lethal concentration (LC₅₀) and 50% lethal time

(LT₅₀) values were calculated using probit analysis. The adult and larval mortality data were subjected to be analyzed by Duncan's multiple range test via SPSS program for Windows (version 23.0). The mean number of eggs deposited in test and control cups was analyzed using a paired *t*-test. Each bioassay evaluated relationships of EC₅₀ of adulticidal effects, the larval and pupal mortality rates, and the relationship between the ratio of numbers of tested eggs to effective repellency percentage and concentrations by regression analysis.

3. Results

3.1. WHO susceptibility test

The knockdown rates and KT₅₀ of *Z. limonella* oil as determined using impregnated papers with WHO test kits against *Ae. aegypti* and *Cx. quinquefasciatus* are shown in Table 1 and results were compared with cypermethrin paper. The result showed that the knockdown time decreased with increased concentration, and KT₅₀ was recorded as 128.4 min, 121.8 min and 7.2 min against *Ae. aegypti* and 130.4 min, 129.7 min and 10.7 min against *Cx. quinquefasciatus* at 1%, 5% and 10% concentrations, respectively. The highest degree of female's knockdown rate with 10% *Z. limonella* was shown at 68.7% (*Ae. aegypti*) and 92.5% (*Cx. quinquefasciatus*), respectively. KT₅₀ values of 1%, 5% and 10% cypermethrin impregnated papers were 4.1 min, 1.0 min and 0.8 min against *Ae. aegypti* and 4.5 min, 1.3 min and 0.9 min against *Cx. quinquefasciatus*. Results of regression analysis revealed that the EC₅₀ values of *Z. limonella* are negatively correlated with exposure time, and had a regression coefficient close to 1 in each species (Figure 1). In Table 2, the mortality values increased depending on the increasing essential oil concentration. The statistical data LC₅₀, 95% confidence limits and regression equation were also calculated. The result of adult susceptibility test revealed that 10% *Z. limonella* exhibited the highest activity for *Ae. aegypti* (LC₅₀ = 6.0%) and *Cx. quinquefasciatus* (LC₅₀ = 5.7%). It showed that mortality rates were at 100% and their corresponding regression equations are $y = -1.76 + 0.19x$ (95% CI = 5.5–6.6, $R^2 = 0.863$, $\chi^2 = 71.811$, $df = 28$) and $y = -1.45 + 0.15x$ (95% CI = 5.1–6.3, $R^2 = 0.691$, $\chi^2 = 78.810$, $df = 28$), respectively. This suggests that this essential oil does not possess insecticidal properties at lower doses or at less than 10% concentration.

3.2. Larvicidal bioassay

The results of the larvicidal activity of *Z. limonella* against the larvae of *Ae. aegypti* and *Cx. quinquefasciatus* are presented

in Table 3. After 12 h-exposure, 67% mortality of *Ae. aegypti* was noted at larvae exposed to 1% *Z. limonella* oil, whereas it increased to 84% mortality at 5% concentration. Mortality increased to 100% at 10% concentration. The LT₅₀ values of 1%, 5% and 10% concentrations *Z. limonella* oil against *Ae. aegypti* larvae were as follows: LT₅₀ values were 9.78 h, 5.61 h and 0.24 h and pupae were 64.08 h and 21.23 h, respectively. The mortality of *Cx. quinquefasciatus* larvae after the treatment of *Z. limonella* oil at 1%, 5% and 10% concentrations were noted as 24%, 37% and 100% for larval stage after 12 h-exposure. The highest degree of LT₅₀ values of *Z. limonella* oil was shown at 28.46 h, 20.25 h and 1.01 h for larval stage, respectively. The results of the pupicidal activity of *Z. limonella* against *Ae. aegypti* and *Cx. quinquefasciatus* pupae are presented in Table 4. *Z. limonella* oil exhibited toxicity against *Ae. aegypti* pupae after 24 h-exposure. Mortality of 18% was noted at pupal stage treated with 1% concentration, which increased to 100% at 10% concentration. In addition, the mortality of *Cx. quinquefasciatus* pupae after the treatment of *Z. limonella* oil at 1%, 5% and 10% concentrations were noted as 7%, 59% and 100% for pupal stage after 24 h-exposure. The highest degree of LT₅₀ values of *Z. limonella* oil was shown at 67.52 h, 27.96 h and 4.11 h for pupae, respectively. When data were pooled for the three concentrations (1%, 5% and 10%), larvicidal and pupicidal activities of *Z. limonella* oil showed a positive relationship between mortality rates and exposure periods which was significant ($R^2 = 0.963$ 2 and 0.984 9 for *Ae. aegypti* larvae and pupae and $R^2 = 0.986$ 6 and 0.987 2 for *Cx. quinquefasciatus* larvae and pupae) (Figures 2 and 3).

The frequency of occurrence of different morphogenetic aberrations and mortality rates of *Ae. aegypti* and *Cx. quinquefasciatus* is shown in Table 5. The distribution of mortality rates and morphogenetic abnormalities of *Ae. aegypti* and *Cx. quinquefasciatus* larvae are shown in Figure 4. The results showed that majority of the morphogenetic changes of *Ae. aegypti* larvae were identified as normal larvae and deformed larvae. When larvae were exposed to 1%, 5% and 10% concentrations, the greatest mortality was due to normal larvae ($P < 0.05$). Meanwhile, *Cx. quinquefasciatus* larvae were identified as normal larvae, deformed larvae, dead pre-pupae during emergence from the larval exoskeleton and white pupa. For *Cx. quinquefasciatus* larvae exposed to 1% and 5% concentrations, the majority mortality was due to white pupae. At 10% *Z. limonella* oil caused the greatest mortality as deformed larvae ($P < 0.05$). Figure 5 shows the occurrence of different morphological aberrations and mortality rates among *Ae. aegypti* and *Cx. quinquefasciatus* pupae. Deformed pupa was identified as pupa with attached larval exuvium at the caudal end, brown pupa and adult attached to the pupal case. At concentrations of 1% and 5% of *Z. limonella* oil caused morphological abnormalities among *Ae.*

Table 1

Knockdown rates and KT₅₀ of *Ae. aegypti* and *Cx. quinquefasciatus* adults to *Z. limonella* oil and cypermethrin.

Mosquito species	<i>Ae. aegypti</i>						<i>Cx. quinquefasciatus</i>					
	<i>Z. limonella</i>			Cypermethrin			<i>Z. limonella</i>			Cypermethrin		
Concentrations, (%)	1	5	10	1	5	10	1	5	10	1	5	10
Knockdown in 1 h exposure (%)	1.5	11.0	68.7	76.4	91.6	92.5	1.1	18.2	58.6	75.9	89.7	92.2
KT ₅₀ (min)	128.4	121.8	7.2	4.1	1.0	0.8	130.4	129.7	10.7	4.5	1.3	0.9
Constant	-2.896	-1.517	-1.170	-0.551	-1.144	-0.682	-3.088	-1.090	-1.252	-0.648	-1.144	-0.682
Slope ± SE	0.023 ± 0.004	0.012 ± 0.002	0.164 ± 0.009	0.134 ± 0.009	0.858 ± 0.168	0.759 ± 0.161	0.024 ± 0.005	0.008 ± 0.002	0.117 ± 0.007	0.146 ± 0.010	0.858 ± 0.168	0.759 ± 0.161
R ²	0.010	0.456	0.864	0.715	-	-	0.125	0.532	0.860	0.840	-	-

KT₅₀ = 50% knockdown time; R² = regression coefficient.

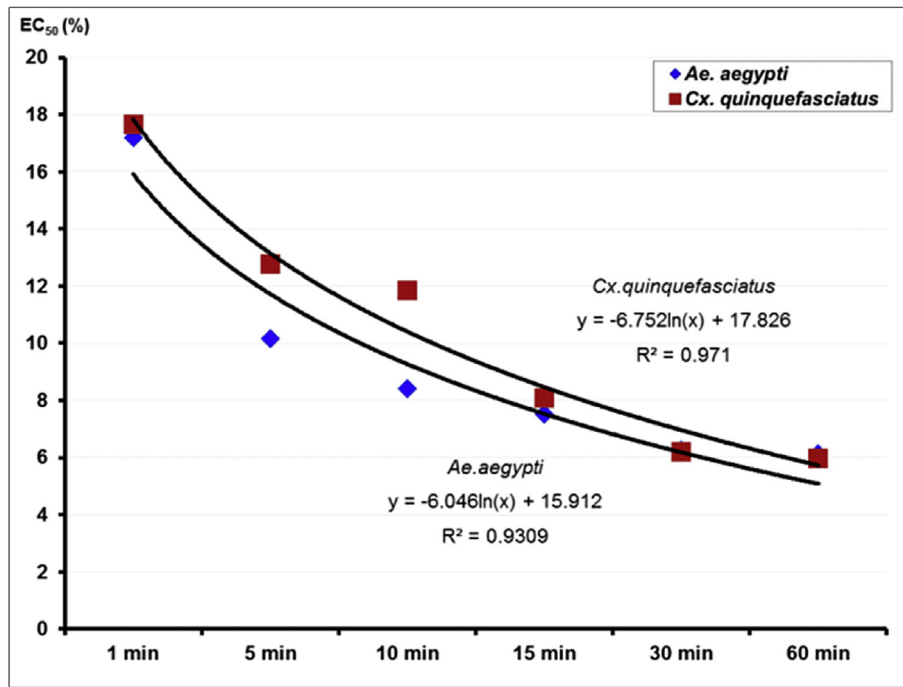


Figure 1. Relationship of EC₅₀ of adulticidal activity of *Z. limonella* oil against *Ae. aegypti* and *Cx. quinquefasciatus* adults.

aegypti adults emerging from the pupal case. Almost all mortality of *Ae. aegypti* and *Cx. quinquefasciatus* pupae occurred as brown pupae at 10% *Z. limonella* oil, and the rates were 82% and 89% respectively.

3.3. Oviposition deterrent bioassay

The oviposition deterrence activity of *Z. limonella* oil against *Ae. aegypti* and *Cx. quinquefasciatus* is presented in Table 6. The

Table 2

Mortality rate for *Ae. aegypti* and *Cx. quinquefasciatus* adults to *Z. limonella* oil (mean ± SD).

Mosquito species	Concentrations (%)	Mortality at 24 h (%)
<i>Ae. aegypti</i>	1	6.0 ± 2.1
	5	20.4 ± 3.5
	10	100.0 ± 0.0
<i>Cx. quinquefasciatus</i>	1	10.4 ± 4.3
	5	24.0 ± 3.3
	10	100.0 ± 0.0

results showed that the different concentrations of *Z. limonella* oil reduced number of eggs deposited by gravid females *Ae. aegypti* of treatment at 1%, 5% and 10% concentrations. The mean number of eggs laid by *Ae. aegypti* mosquito in *Z. limonella* oil showed effective results as (37.33 ± 15.7), (3.33 ± 5.77) and 0.00 eggs per cup respectively and was significantly different from controls ($P < 0.05$). The effectiveness percentage shown by *Z. limonella* oil against oviposition was 94.34% in 1% concentration, 99.38% in 5% concentration and 100% in 10% concentration. The range of oviposition activity index of *Z. limonella* oil at three concentrations compared with a control group was from -0.89 to -1.00. *Z. limonella* oil oviposition deterrence activity against *Cx. quinquefasciatus* gravid female mosquito showed effective results. There were significant differences between test and control by paired *t*-test ($P < 0.05$). The mean number of *Cx. quinquefasciatus* eggs laid in the three concentrations of *Z. limonella* oil was (134.00 ± 52.26), (45.67 ± 12.10) and (2.33 ± 3.21) eggs per cup respectively. The OAI range of -0.64 to -0.99 values indicated that the test solutions were deterrents and the percentage of effective deterrence of *Z. limonella* oil against oviposition was 77.94%, 91.31% and 99.53%

Table 3

LT₅₀ and mortality rates at 12 and 24 h for *Ae. aegypti* and *Cx. quinquefasciatus* larvae and pupae to *Z. limonella* oil.

Mosquito species	Stage	Concentrations (%)	Mortality (%)		LT ₅₀ (h)	95% Confidence limit		R ²	χ ²	df
			After 12 h	After 24 h		LCL	UCL			
<i>Ae. aegypti</i>	Larvae	1	67.0 ± 8.2	71.0 ± 5.7	9.78	8.02	12.09	0.44	185.373	98
		5	84.0 ± 7.0	92.0 ± 9.2	5.61	4.23	7.40	0.48	440.387	98
		10	100.0 ± 0.0	100.0 ± 0.0	0.24	0.19	0.29	0.77	39.817	98
<i>Cx. quinquefasciatus</i>	Larvae	1	24.0 ± 9.7	30.0 ± 9.4	28.46	24.60	34.17	0.48	78.541	98
		5	37.0 ± 11.6	50.0 ± 14.9	20.25	18.20	22.91	0.66	96.986	98
		10	100.0 ± 0.0	100.0 ± 0.0	1.01	0.78	1.27	0.50	316.316	98

Values were based of three concentrations and five replications ± SD; UCL = upper confidence limit, LCL = lower confidence limit; R² = regression coefficient; df = degree of freedom.

Table 4

LT₅₀ and mortality rates at 24 and 48 h for *Ae. aegypti* and *Cx. quinquefasciatus* pupae to *Z. limonella* oil.

Mosquito species	Stage	Concentrations (%)	Mortality (%) ± SD		LT ₅₀ (h)	95% Confidence limit		R ²	χ ²	df
			After 24 h	After 48 h		LCL	UCL			
<i>Ae. aegypti</i>	Pupae	1	18.0 ± 7.9	27.0 ± 4.8	64.08	54.52	80.58	0.64	26.023	48
		5	49.0 ± 9.9	100.0 ± 0.0	21.23	19.34	23.45	0.68	30.839	48
		10	100.0 ± 0.0	100.0 ± 0.0	–	–	–	0.68	10.102	48
<i>Cx. quinquefasciatus</i>	Pupae	1	7.0 ± 4.8	24.0 ± 5.2	67.52	57.83	84.58	0.78	25.196	48
		5	59.0 ± 12.9	81.0 ± 8.8	27.96	25.60	30.62	0.78	55.507	48
		10	100.0 ± 0.0	100.0 ± 0.0	4.11	3.55	4.67	0.96	3.489	48

Values were based of three concentrations and five replications ± SD; UCL = upper confidence limit, LCL = lower confidence limit; R² = regression coefficient; df = degree of freedom.

respectively. The relationship between the numbers of tested eggs to effective repellency percentage and the concentrations of *Z. limonella* oil are shown in Figure 6. There is negative correlation between the ratio of numbers tested eggs to effective repellency percentage and the concentration of *Z. limonella* oil against *Ae. aegypti* and *Cx. quinquefasciatus* with a regression coefficient close to 1 in each species and corresponding regression equations of $y = -0.379\ln(x) + 0.3705$ and $y = -1.624\ln(x) + 1.7382$, respectively.

4. Discussion

In this work, the essential oil was isolated from the seed and pericarp of the dried fruit of *Z. limonella* (ma-kwaen). The seeds contain fatty oil, while the essential oil is concentrated in pericarp. The value of loss on drying was high (17.90% w/w) due to the contents of volatile oil (9.63% w/w) and water (9.18% w/w) [35]. Essential oil of *Z. limonella* is a highly volatile and bioactive compound with high toxicity against mosquitoes. The high volatile vapor is responsible for repelling gravid females from egg laying and also shows insecticidal activity.

The high range of maximum and minimum of volatile oil content obtained may be attributed to the age and geographical area of each *Z. limonella* source. The result of the current study shows remarkable adulticidal toxicity of *Z. limonella* oil against the two mosquito vectors tested. Larvicidal and oviposition deterrent activities against mosquitoes have been reported from *Z. limonella*. The secondary metabolites present in this plant constitute a defense system against insect/pest attacks. Itthipanichpong *et al.* [36] reported the chemical compositions of the essential oil distilled from the fruit of *Z. limonella* in Thailand and found the presence of 33 chemical components. Limonene (31.1%), terpinene-4-ol (13.9%) and sabinene (9.1%) were found to be the major monoterpenes components. Monoterpenes were predominant in the essential oil from ma-kwaen fruit. Limonene which is the most abundant component in the *Z. limonella* oil belongs to a class of natural compounds known as limonoid terpenes [37]. Limonoids are secondary metabolites produced in plants found in the family Rutaceae and have exhibited a range of biological activities like insecticidal, insect antifeedant and growth regulating activity on insects [38]. Moreover, limonoids

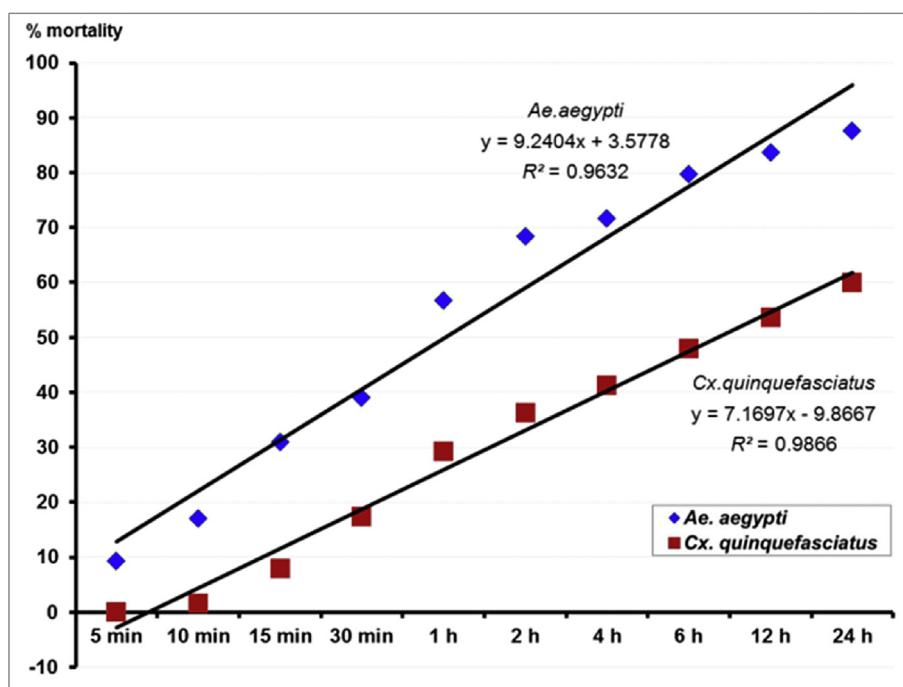


Figure 2. Larvicidal effects of *Z. limonella* oil against *Ae. Aegypti* and *Cx. quinquefasciatus* larvae expressed as linear regression.

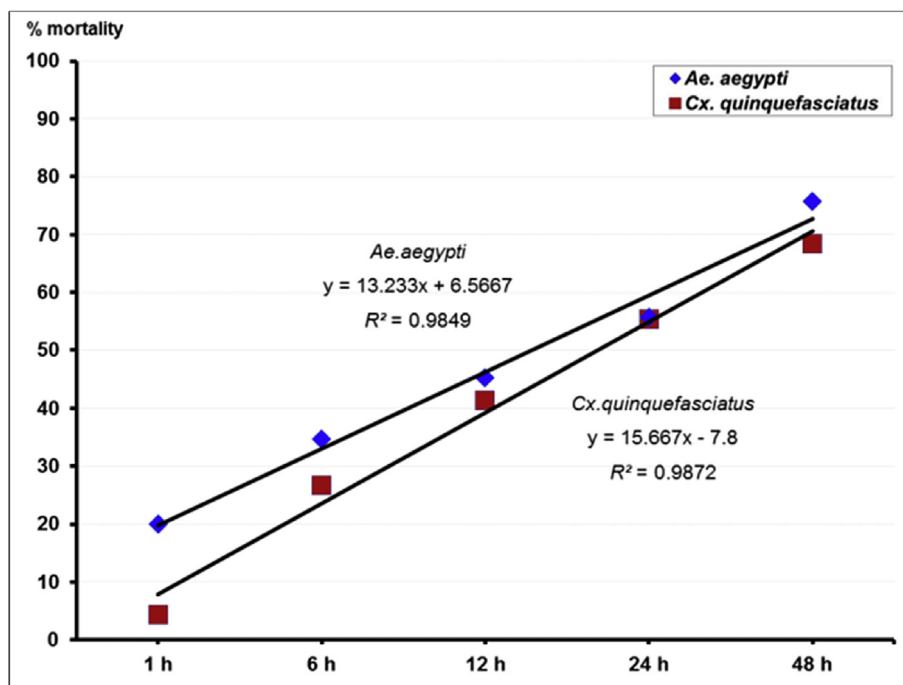


Figure 3. Pupicidal effects of *Z. limonella* oil against *Ae. aegypti* and *Cx. quinquefasciatus* pupae expressed as linear regression.

affect the egg laying of insects due to nutritional disruption which ultimately induce antifeedant effects [39].

In this study, *Z. limonella* oil showed a toxic effect towards mosquitoes. The highest activity with 100% adult mortality was found in 10% concentration against the adults of *Ae. aegypti* and *Cx. quinquefasciatus* with LC₅₀ 6.0% and 5.7% respectively. Few reports are available that indicate adulticidal property of *Zanthoxylum* genus. In another study, the effect of *Zanthoxylum heitzii* bark extracts (LD₅₀ = 102 ng/mg female) on adult females of the mosquito *Anopheles gambiae* were recently investigated by Overgaard *et al.* [40]. Essential oil from *Zanthoxylum beecheyamum* fresh leaves showed adulticidal effect against *Culex pipens* and *Cx. quinquefasciatus* [41].

Nowadays, mosquito control programmes are focused more on the elimination of mosquitoes at larval stage at their breeding sites with larvicides since adulticides may reduce the adult population only temporarily. Therefore, a more efficient approach to reduce the population of mosquitoes would be targeted at the

larvae [42,43]. Our results clearly indicate that *Z. limonella* oil was most effective against immature stages of both *Ae. aegypti* and *Cx. quinquefasciatus*. In the larvicidal assay, *Z. limonella* oil demonstrated efficacy in both *Ae. aegypti* and *Cx. quinquefasciatus* was dose dependent. When exposed to the higher oil concentrations, more larvae showed toxic symptoms that led to an increase in mortality. In the present study, the LT₅₀ of 10% *Z. limonella* oil were recorded as 0.24 h against *Ae. aegypti* and 1.01 h and 4.11 h respectively against *Cx. quinquefasciatus* larvae and pupae. In a study conducted under laboratory conditions to monitor the possible site of action of *Z. limonella* on killing *Ae. aegypti* and *Cx. quinquefasciatus* larvae and pupae, the treated larvae tended to show toxic symptoms and die earlier at increasing oil concentrations. These findings suggest that concentrations of *Z. limonella* oil affects degree of toxicity, time to mortality, and mortality rates. Earlier studies reported a few cases of larvicidal activity from the genus *Zanthoxylum*. Rabha *et al.* [44] reported

Table 5

Mortality rates of larvae after 24 h treatment and pupae after 48 h treatment of *Ae. aegypti* and *Cx. quinquefasciatus*.

Mosquito species	Stage	Concentrations (%)	Stages at death (%)							Total mortality (%)	Stage (%)
			NL	DL	PP	WP	DP	BP	PA		
<i>Ae. aegypti</i>	Larvae	1	63.0	8.5	–	–	–	–	–	71	29
		5	77.0	16.0	–	–	–	–	–	92	8
		10	94.0	6.5	–	–	–	–	–	100	–
	Pupae	1	–	–	–	–	0.7	–	26.0	27	73
		5	–	–	–	–	3.5	–	97.0	100	–
		10	–	–	–	–	18.0	82.0	–	100	–
<i>Cx. quinquefasciatus</i>	Larvae	1	1.4	3.5	4.6	21.0	–	–	–	30	70
		5	0.9	4.5	6.6	38.0	–	–	–	50	50
		10	37.0	51.0	6.5	6.0	–	–	–	100	–
	Pupae	1	–	–	–	–	6.6	1.9	16.0	24	76
		5	–	–	–	–	14.0	6.0	61.0	81	19
		10	–	–	–	–	11.0	89.0	–	100	–

NL: normal larva, DL: deformed larva, PP: pre-pupa and pupa not completely out of the larval exoskeleton, WP: white pupa, DP: deformed pupa, BP: dead normal brown pupa, PA: adult attached to the pupal case, A: normal adult.

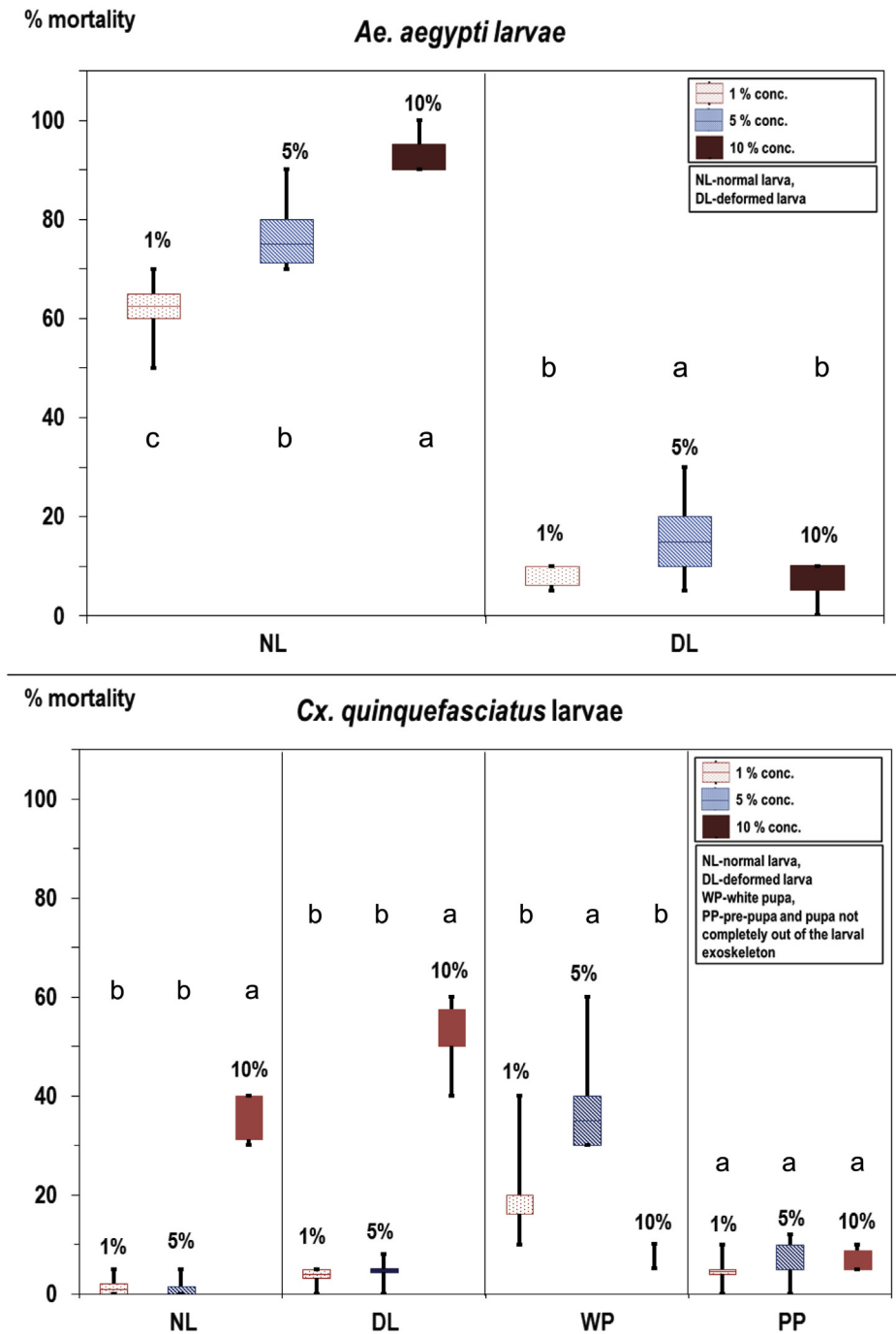


Figure 4. Box plot distributions of mortality rates and morphogenetic abnormalities of *Ae. Aegypti* and *Cx. quinquefasciatus* larvae. In each categorized aberrations, different letters indicate statistically significant differences ($P < 0.05$, Duncan's multiple range test).

the hydrolate of *Z. limonella* was most effective for larvicidal activity against two laboratory reared mosquito species *Aedes albopictus* and *Cx. quinquefasciatus* with LC_{50} 11% and 15.5% (v/v). The pericarp extract of *Z. limonella* was found to possess the most effective larvicidal activity against *Ae. albopictus* and *Cx. quinquefasciatus* with LC_{50} at 0.01 ppm and 0.02 ppm and LC_{90} at 0.47 ppm and 0.73 ppm respectively [45]. The aromatic compounds are stored in the pericarps while oil and unsaturated fatty acids accumulate in the seed [46]. Tennyson *et al.* [47] found that the hexane, diethyl ether and dichloromethane extracts of *Z. limonella* at 1 000 ppm showed 100% mortality of *Cx. quinquefasciatus* larvae at 48 h bioassay. Tiwary *et al.* [48] tested the essential oil of *Zanthoxylum armatum* against three

species of mosquitoes and reported *Cx. quinquefasciatus* to be the most susceptible from oil with an LC_{50} value of 49 ppm followed by *Ae. aegypti* and *Anopheles stephensi* with LC_{50} values in the range of 54–58 ppm.

Oviposition is one of the most important events in the life cycle of mosquitoes. Therefore, gravid mosquito females show a high degree of preference in selecting oviposition sites [49]. This preference may be due to the presence of oviposition pheromones or oviposition attractants and repellents in natural habitats [33,50]. Application of oviposition repellent is an effective strategy to control mosquito populations since controlling the egg and larva is easier compared to targeting at the free-flying adult. Essential oils have effective chemicals for repelling gravid females from egg

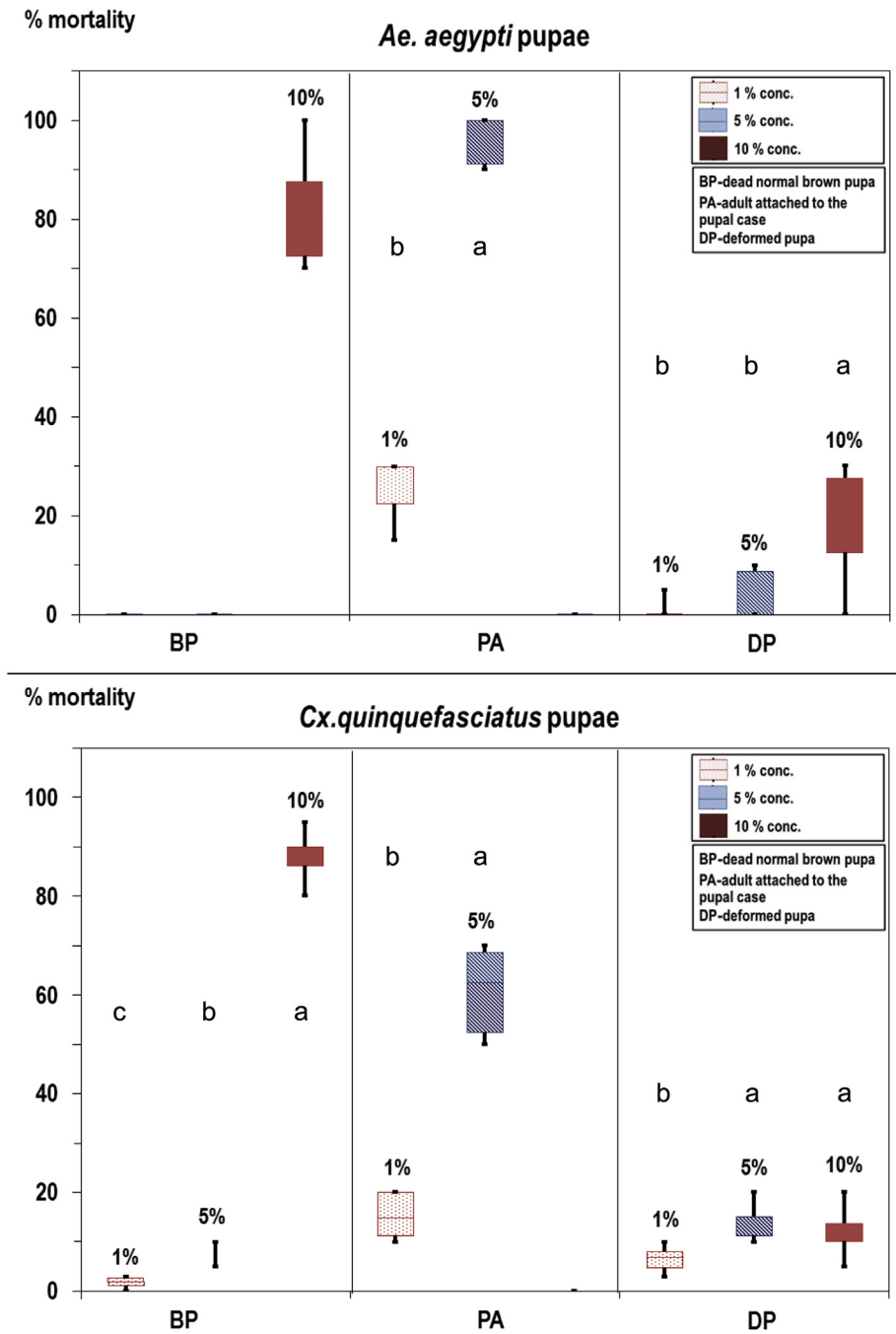


Figure 5. Box plot distributions of mortality rates and morphogenetic abnormalities of *Ae. Aegypti* and *Cx. quinquefasciatus* pupae. In each categorized aberrations, different letters indicate statistically significant differences ($P < 0.05$, Duncan's multiple range test).

laying because gravid female mosquitoes are highly sensitive towards volatile compounds. The antennae of mosquito are replete with chemoreceptors that enable the insect to detect air-borne stimuli and assist in locating suitable sites for oviposition [51]. Female mosquitoes find oviposition sites but gravid females move away from oviposition repellent breeding site without laying egg [52]. Studies on oviposition deterrence activity of insect derived from plant extracts are scarce. In this study, essential oil of *Z. limonella* has been implicated as an oviposition deterrent, and GC/MS analysis revealed that limonene was the main component [36]. Tripathi *et al.* [53] have shown this compound have insecticidal properties with the application of limonene suppressing oviposition and reducing eggs, threatening adult survival in species of stored product pest. Limonene was

also found to be the major component of the essential oil of *Anethum sowa* that was shown to deter oviposition in *Callosobruchus maculatus* females [54]. The results obtained from the present study confirm the oviposition deterrence potential of *Z. limonella* oil for control and management of *Ae. aegypti* and *Cx. quinquefasciatus* population as determined by the numbers of eggs deposited in test containers compared to control containers. Lower numbers of eggs deposited in test containers indicate that more gravid mosquitoes were repelled by the test oil. Furthermore, repellency of *Z. limonella* increased with the increasing concentration and showed 78%–100% oviposition deterrent activity at all the tested concentrations (1%, 5% and 10% concentrations) against *Ae. aegypti* and *Cx. quinquefasciatus* adult females. Previously, studies reported the ovicidal toxicity

Table 6Oviposition deterrent of *Z. limonella* oil in three concentrations against mosquitoes.

Mosquito species	Treatments	Concentrations (%)	Total number of eggs \pm SD		OAI	ER%	No. of tested eggs laid per female (n)	REER (%)
			Tested	Control				
<i>Ae. aegypti</i>	<i>Z. limonella</i>	1	112.0 \pm 15.7*	1 930.0 \pm 94.1	-0.89	94.34	37.3	0.395
		5	10.0 \pm 5.8*	1 724.0 \pm 49.8	-0.99	99.38	3.3	0.033
		10	0.0*	1 706.0 \pm 31.8	-1.00	100.00	0.0	0.000
<i>Cx. quinquefasciatus</i>	<i>Z. limonella</i>	1	866.0 \pm 84.6	1 290.0 \pm 43.4	-0.21	32.35	288.7	8.924
		5	402.0 \pm 52.3*	1 809.0 \pm 19.0	-0.64	77.94	134.0	1.719
	Temephos	5	137.0 \pm 12.1*	1 565.0 \pm 96.8	-0.84	91.31	45.7	0.500
		10	7.0 \pm 3.2*	1 545.0 \pm 27.0	-0.99	99.53	2.3	0.023
	Temephos	1	1008.0 \pm 52.0	1 262.0 \pm 34.3	-0.11	19.65	336.0	17.099

*Significant differences between tested and control by paired *t*-test ($P < 0.05$); OAI = Oviposition active index; ER = Effective repellency; REER = Ratio of numbers tested eggs to effective repellency percentage.

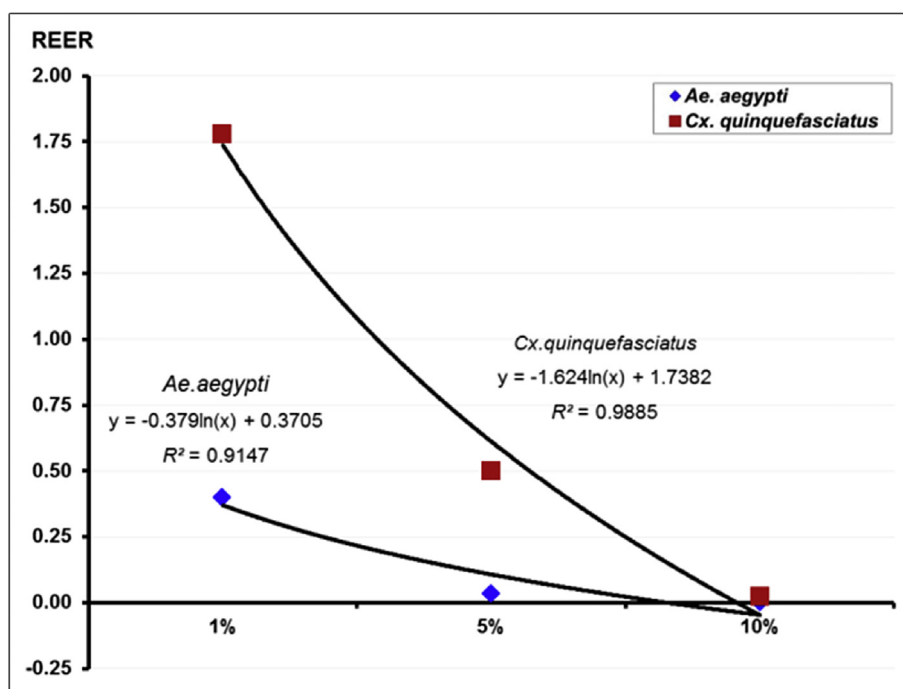


Figure 6. Relationship between ratio of numbers of tested eggs to effective repellency percentage and concentrations of *Z. limonella* oil against *Ae. aegypti* and *Cx. quinquefasciatus*.

REER = Ratio of numbers tested eggs to effective repellency percentage.

effect of some *Zanthoxylum* against vector mosquitoes. The result corresponded with those of Marr and Tang [55], who reported the ovicidal properties of some *Zanthoxylum* essential oils.

The biological properties of *Z. limonella* oil not only act as effective adulticide, larvicide and oviposition deterrent, but are also used against mosquitoes as repellent. *Z. limonella* oil has been reported to have repellency activity. Trongtokit *et al.* [56] investigated the repellency of 10% and 50% concentration in alcohol and undiluted *Z. limonella* oil against *Cx. quinquefasciatus*, *Anopheles dirus* and *Ae. aegypti*, and found that the undiluted oil was the most effective and provided 2 h complete repellency. Furthermore, *Z. limonella* exhibited better protection against bites of *Ae. albopictus* in mustard oil than in coconut oil and gave protection time of 4–5 h [57]. In another study, Maji and Hussain [58] reported successful microencapsulation of *Z. limonella* oil in glutaraldehyde crosslinked gelatin in order to improve mosquito repellent properties.

In conclusion, the present study clearly proved that essential oil of *Z. limonella* has remarkable adulticidal, larvicidal, pupicidal and oviposition repellency properties against *Ae.*

aegypti and *Cx. quinquefasciatus*. Herbal products are one of the best alternatives to chemical control for mosquito control. This study recommends that this plant could form safe and eco-friendly alternative to synthetic pesticides. These results are encouraging for developing new natural mosquitocidal products from plant oil thereby offering an alternative to synthetic products.

Conflict of interest statement

We declare that there is no conflict of interest.

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