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Larvicidal effectiveness of acethonilic and methanolic *Ipomoea cairica* extract using two extraction methods and its effects on the morphology of *Culex quinquefasciatus* Say mosquito

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ABSTRACT

Plants produce a rich source of chemicals, drugs and secondary metabolites that can act as insect killer. The larvicidal activity of various part of *Ipomoea cairica* extracts was evaluated against *Culex quinquefasciatus*. The highest percentage of yield extract was given by Soxhlet with acetone solvent for all of the different plant parts. While, maceration technique only provided 1/3 of the yield compared to Soxhlet technique. Using 24 h larvicidal bioassay technique, third instar larvae of *Cx. quinquefasciatus* were exposed to concentrations ranging from 10 to 600 ppm of the acetone and methanol extracts of *I. cairica* from leaves, stem and flower bud. Acethonilic extract of *I. cairica* leaves was extracted using a Soxhlet apparatus which exhibited the most promising larvicidal activity against *Cx. quinquefasciatus* larvae with significantly lower LC_{50} value of 110.65 ppm, meanwhile methanolic leaves extract exhibited LC_{50} value of 143.47 ppm. Larvae exposed to the *I. cairica* extracts for 24 h showed morphological malformation that included a detachment of thorax, disruption of the digestive tract and pigmentation of cuticle on their death. Thus, suggested that *I. cairica* plant extract, especially acethonilic leaves extract can be considered as one of plant-derived insecticides for control of *Cx. quinquefasciatus,* a vector of filariasis.

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Biological control; *Culex quinquefasciatus*; larvicidal efficacy; mosquito; plant extract

Introduction

Mankind has been plagued by mosquito nuisance and mosquito-borne diseases since ages, resulting in various health problems and economic losses. Mosquito transmits some of the deadliest diseases known to man, such as

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malaria, yellow fever, dengue, encephalitis, filariasis and a hundred or so other infectious diseases, claiming many millions of lives. The risk of infection exists all year and can be fatal if left untreated. *Culex quinquefasciatus* Say is one the most abundant tropical house mosquito (AbuHassan & Che Salmah 1990) and it is also one of the major vectors for human lymphatic filariasis, caused by nematode *Wuchereria bancrofti* (Harwood & James 1979) An estimated 120 million people in 73 countries are currently infected, and an estimated 1.393 billion live in areas where filariasis is endemic (WHO 2012). Due to the lack of awareness among people, early detection and complete treatment of this disease are very difficult.

Vector control is still one of the primary weapons to combat mosquito spread diseases in endemic areas. Synthetic chemical larvicides and adulticides are still the major sources in controlling the mosquito's population in most parts of the world (Rahuman et al. 2009). The primary environmental difficulty with the use of chemical control is that often pesticides are applied directly into residential areas and into sensitive natural environments. The risks of pesticide are also associated with the acute and chronic pesticide impacts to humans, wildlife and other non-target species, the persistence of certain pesticides in the environment and the transport of pesticides outside target areas, which can cause unintended environmental damage. The intensive use of insecticides has also resulted in the emergence and spread of resistance to organophosphorus (OP) (Karunaratne & Hemingway 2001), carbamate (Vaughan et al. 1998) and pyrethroid insecticides (Mebrahtu et al. 1997; Brengues et al. 2003) and this resistance problem shifted the mosquito control programme to the use of environmental friendly, biodegradable and safer alternative insecticides of plant origin.

The search for natural products as an alternative to synthetic insecticides for the control of destructive insects and vectors of diseases is desirable due to the prevalent occurrence of vector resistance, toxic and non-biodegradable residues contaminating the environment and undesirable effects on non-target organisms (Jantan et al. 2005). Botanical insecticides are safe and degradable, and effective alternatives to conventional synthetic insecticide (Nathan et al. 2004). Many studies have reported the application of the potential plant extracts or essential oil on mosquito larvae. The extract of *Vitex trifolia* (Kannathasan et al. 2007), the petroleum ether–acetone extract of *Calotropis gigantean* (Neraliya & Srivastava 1996), the methanolic extracts of *Solanum suratense*, *Azadirachta indica*, and *Hydrocotyle javanica* (Muthukrishnan et al. 1997), the essential oil of *Zingiber officinalis* Linn (Zingiberaceae) (Pushpanathan et al. 2008) exhibited larvicidal activity against the larvae of *Cx. quinquefasciatus*. *Rhinacanthus nasutus* dried root powder methanol extracts (Rongsriyam et al. 2006), petroleum ether extract of *Solanum xanthocarpum* fruit (Rahuman & Venkatesan 2008), leaf extract of *Ageratina. Adenophora* (Mohan & Ramaswamy 2007) showed acute toxicity against the larvae of *Ae. aegypti* and *Cx. quinquefasciatus*. Acetone and methanol extracts of *Canna indica*

L also have been reported to have larvicidal activity against *Cx. quinquefasciatus* (Rahuman et al. 2009).

Regardless, researchers are often confronted with the challenge of extracting the plant material in order to study on biological activities associated with a plant or a plant extract. Sample preparation is a crucial first step in the analysis of plant materials. Different extraction techniques have been developed to exploit plant material resources and to obtain such valuable products (Chemat et al. 2004). The process of extraction of active substances from a plant material by means of a solvent generally occurs in two main stages: first, dissolution of material near the surface (so-called washing or fast extraction) and second, diffusion of the solute from the porous plant residue into the solution (so-called slow extraction) (Smelcerovic et al. 2006). The extraction relies primarily on the solubilisation of metabolites and by increasing the temperature to favour solubilisation (Jones & Kinghorn 2006). The right choice of extraction procedure is necessary to extract the desired chemical components from the studied plant for further analysis.

Therefore, the present study aimed to explore two commonly employed plant extraction methods; maceration and Soxhlet with two different solvents; acetone and methanol, in order to evaluate the larvicidal activity of various parts; leaves, stems, and flower buds of *Ipomoea cairica* Linn (Convolvulaceae) crude extract against *Cx. quinquefasciatus* larvae. The yield percentage of plant extracts based on the above methods and solvents were also determined.

Materials and methods

Mosquito colonies

Culex quinquefasciatus larvae were collected from stagnant water area of Bagan Dalam, Penang, Malaysia (5° 24′ 00″N, 100° 23′00″E). The larvae were kept in enamel trays containing dechlorinated tap water. In order to have standard age and size of third instar larvae used in this study, we need to culture the mosquitoes until F1 generation. The larval density was maintained at first larva to 20 mL dechlorinated tap water per tray. Larvae were fed with a fine powder mixture of dog biscuit, beef liver, yeast and milk powder in the ratio of 2:1:1:1 by weight. Pupae were transferred into plastic containers and were maintained in a mosquito cage to emerge. Adults were continuously provided with 10% sucrose solution in a 50 mL conical flask with a cotton wick. On day five, the adults were given a blood meal of laboratory blood mice overnight. A bowl with 50 mL of chlorine free water was kept inside the cage for oviposition. Egg rafts were observed to hatch and were transferred to enamel trays. Larval rearing and all experiments were conducted under laboratory conditions at a temperature (28 ± 2 °C) and (80 ± 10%) relative humidity (RH). This project has been approved by the Animal Ethic Committee, Universiti Sains, Malaysia. However, the animal care permit is not requires for this kind of study.

Plant collection

Leaves, stems and flower buds samples of *I. cairica* were collected from residential areas in Relau, Penang, Malaysia (5°25′00″N 100°19′00″E) by utilising a random sampling method and were identified by the Botanical Department of Universiti Sains, Malaysia. All of the plant parts were air dried at room temperature for 1–2 weeks until its weight remained constant. The dried plant parts were then powdered mechanically using electrical stainless steel blender (Panasonic: MX-899TM). This increases the surface area for extraction, hence increasing the rate of extraction (Elango et al. 2011). The sample was then kept under room temperature in a labelled airtight container. Two different extraction methods were used in this experiment; maceration and Soxhlet with two different solvents; methanol and acetone.

Maceration extraction

An amount of 60 g of dried leaves powder was measured and placed in a beaker. One litre of either methanol or acetone solvent were added into the beaker and the sample was left to macerate for 7 d, until the cellular structure of the plant sample was softened, penetrated by the solvent and the soluble components were dissolved. The plant sample in the solvent was occasionally stirred to facilitate the speed of extraction. After the procedure was done, the extract obtained will be a mixture of solvent, extract and sample waste. The extract was then filtered with Whatman filter paper No. 1 to remove the sample waste and then evaporated in a rotary vacuum evaporator in order to have crude extract. The same procedures were repeated once to give a total of 120 g of plant powder macerated in 2 L of solvent and the process was also repeated for dried powdered stems and flower buds.

Soxhlet extraction

For extraction using a Soxhlet apparatus (Favorit, Malaysia), 120 g of dried plant powder was weighed and placed into the paper thimble. Small stones were placed in between the powdered plant sample, to enhance the extraction as it allowed better filtration. As a precaution method cotton wool was placed on top and bottom part of the extraction flask to prevent the sample from entering other parts of the apparatus. Methanol and acetone were used as chemical solvent as these solvents exist as a polar liquid at room temperature was experimented separately. Two litres of solvent were placed in a round-bottom flask with the heating mantle underneath. The round-bottom flask and the extractor were covered with muslin cloth for better heat absorption. The solvent was heated and refluxing solvent repeatedly washed the fine grind plant material extracting the desired compound into the round-bottom flask. The extraction in the Soxhlet apparatus with the boiling point at 70 °C was done for about 3 h until the solution of the extract and solvent in the siphon arm becomes clear which indicates the sample has

been extracted entirely. The extract was then evaporated to dryness in the rotary vacuum evaporator. The same procedures were repeated using dried, powdered stems and flower buds of the studied plant.

Drying the extract

In order to remove the solvent from the crude extract, the extract collected in the round-bottom flask was then evaporated in a rotary vacuum evaporator. The boiling point of methanol and acetone were 66 and 50.5 °C, respectively. The temperature of the water bath was set to 55 °C with 100 rounds per minute. The extract collected was then poured into a petri dish and placed in the oven at 37 °C for 24 h to evaporate any leftover solvent and the crude extract obtained was in the form of a paste. The weight of the crude extract was measured to determine the percentage of yield and was then preserved in petri dishes at 4 °C until further use.

Larvicidal bioassay

The larvicidal bioassay was done according to the WHO (2005) standard procedures. A stock solution was prepared by dissolving one gram of crude extract in 100 ml of solvent and subsequently serial dilutions were done to prepare concentration ranging from 10 to 600 ppm. A total of 25 numbers of late third instar larvae was placed in paper cups with the different concentration the plant extract with a total of 250 ml (declorinated water and plant extract). About 1 ml of 10% solvent in 249 ml distilled water was served as control. The larval mortality was observed after 24 h post-treatment. Dead larvae are those that are insensitive to probe with a needle and moribund larvae are those incapable of rising to the surface for respiration (WHO 2005). The experiment was replicated four times for each concentration.

Statistical analysis

We used MANOVA to test the significant effects of different plant parts, concentrations, methods of extractions and solvents on *Cx. quinquefasciatus* larval mortality using SPSS version 16.0. Data were tested for normality using Kolomogorov–Smirnov test and were log-transformed (y + 1) prior to analysis to satisfy the assumption of ANOVA. Results of larval mortality on different concentration were tested using probit analysis in order to have lethal concentration 50 (LC_{50}) and lethal concentration 95 (LC_{95}). Data were log-transformed (log 10) in order to satisfy the assumption of probit analysis.

The percentage of the yield in this study was determined by the formula,

$$\text{Percentage of yield} = \frac{\text{Paste (g)}}{\text{Dried plant powder (g)}} \times 100\%.$$

Results

Different extraction techniques and solvents used to obtain the crude extract of *I. cairica* plant parts, showed significant effect on the mortality of *Cx. quinquefasciatus* larvae ($F = 32.56$, df = 1, $p < 0.05$; Table 1). Soxhlet had proven to provide higher extraction efficiency compared to maceration based on the percentage yield of *I. cairica* crude extracts (Figure 1). The highest yield was obtained from the leaves

Table 1. Effects of different plant parts, solvents and extraction methods studied on *Ipomoea cairica* crude extracts against *Culex quinquefasciatus* larvae.

Source	df	MS	*F*-ratio	Sig.
Plant parts (*P*)	2	1.149	24.989	< **0.001**
Solvent (*S*)	1	8.812	191.638	< **0.001**
Concentration (*C*)	10	58.418	1270.451	< **0.001**
Extraction method (*E*)	1	3.463	75.303	< **0.001**
P × *S*	2	0.092	2.001	0.137
P × *C*	20	0.129	2.808	< **0.001**
P × *E*	2	0.671	14.597	< **0.001**
S × *C*	10	0.593	12.889	< **0.001**
S × *E*	1	1.497	32.556	< **0.001**
C × *E*	10	0.348	7.574	< **0.001**
P × *S* × *C*	20	0.055	1.194	0.255
P × *S* × *E*	2	0.474	10.312	< **0.001**
P × *C* × *E*	20	0.238	5.176	< **0.001**
S × *C* × *E*	10	0.239	5.204	< **0.001**
P × *S* × *C* × *E*	20	0.072	1.564	**0.058**
Error	396	0.046		
Total	528			

Note: df-degree of freedom; MS-mean square, Significant values are in bold.

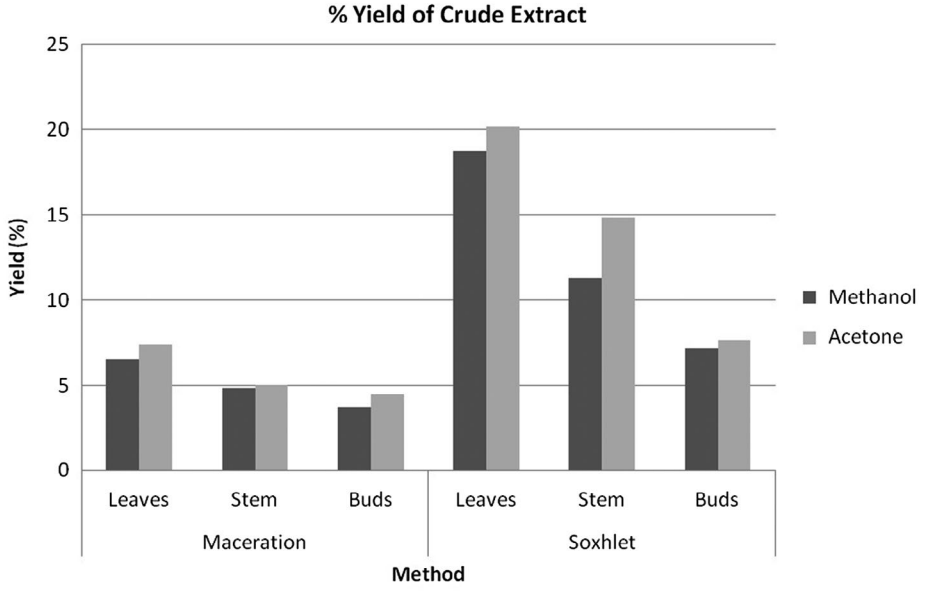

Figure 1. Percentage of yield obtained from different plant parts of Ipomoea cairica crude extract using different method of extractions and solvents.

of the *I. cairica* using the Soxhlet technique with acetone as solvent at 20.16%, and followed by methanol at 18.75%. The maceration method only extracted 7.43 and 6.54% for acetone and methanol, respectively from the leaves of the studied plant (Figure 1). Subsequently, for the extraction of stem and flower buds, Soxhlet technique also gives better yield compared to the maceration method, but the yield was lesser than leaves for both solvent used; acetone and methanol.

Considerable differences in larvicidal efficacy of the plant extracts obtained from the different extraction methods were indicated by the results from this study, extracts obtained from Soxhlet found to be more active than extracts from maceration method (Figures 2 and 3). The study also revealed that for both the extraction method; maceration and Soxhlet, acetone extract of leaves indicated the highest larvicidal activity towards *Cx. quinquefasciatus* larvae with significantly lower LC50 values at 129.564 and 110.653 ppm, respectively ($F = 32.56$, df = 1, $p < 0.05$; Table 1) after 24 h exposure. While, acetone extract of flower buds

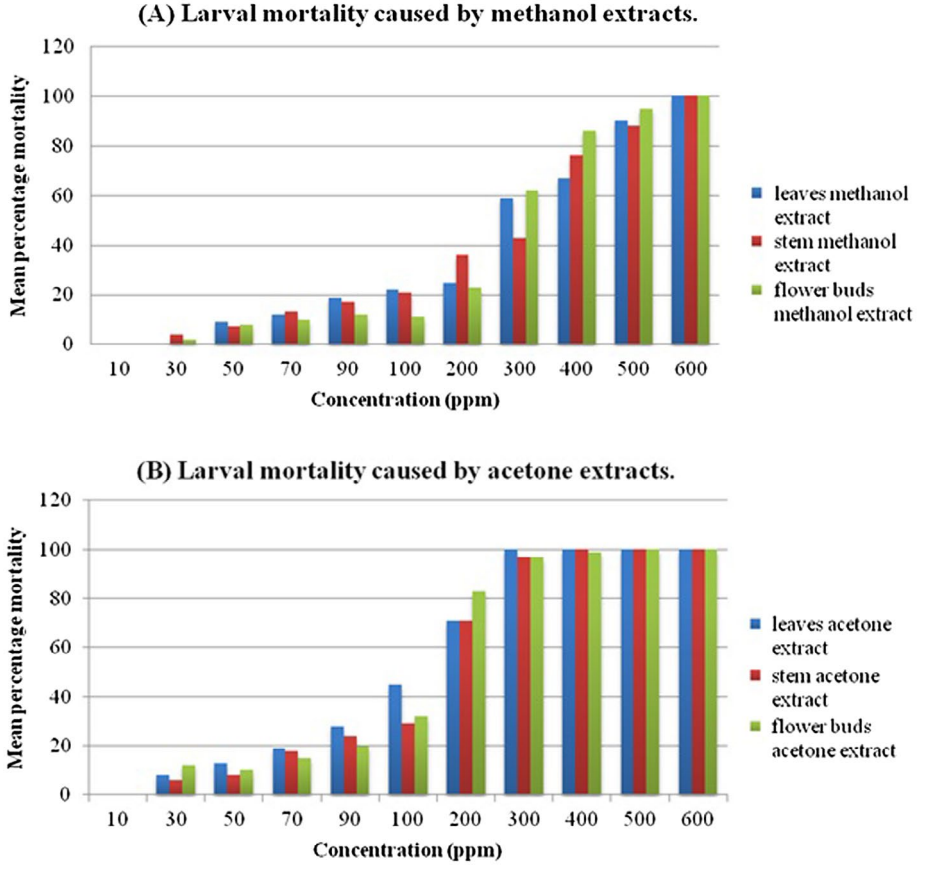

Figure 2A–B. Mean percentage mortality of Culex quinquefasciatus larvae against different concentrations of extracts; A, Methanol extracts; B, Acetone extracts, obtained from maceration method.

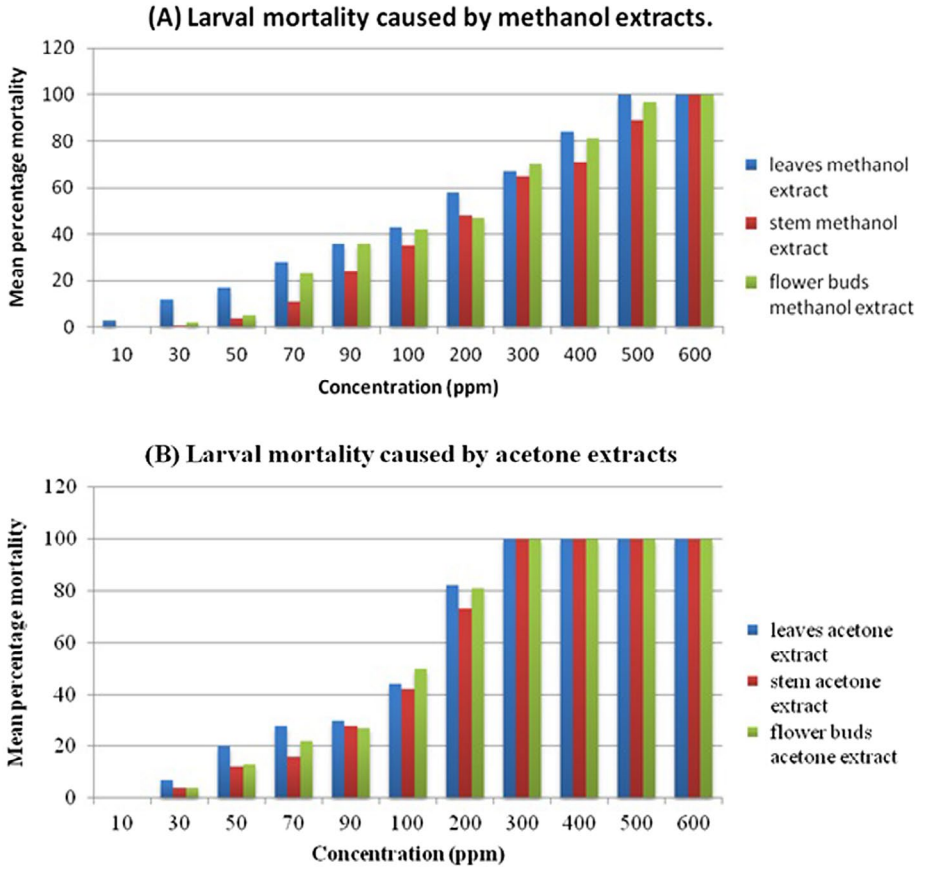

Figure 3A–B. Mean percentage mortality of Culex quinquefasciatus larvae against different concentrations of extracts; A, Methanol extracts; B, Acetone extracts, obtained from Soxhlet technique.

Table 2. Lethal concentrations of *Ipomoea cairica* crude extract of different plant parts against *Culex quinquefasciatus* larvae.

		Solvent used			
		Methanol		Acetone	
Methods of extraction	Plant parts used	LC50 (ppm)	LC95 (ppm)	LC50 (ppm)	LC95 (ppm)
Maceration	Leaves	234.601	1217.475	129.564	554.834
	Stem	235.548	1259.032	144.533	551.401
	Buds	236.604	939.873	131.899	501.685
Soxhlet	Leaves	143.466	1133.378	110.653	422.115
	Stem	191.494	1018.312	127.989	421.348
	Buds	157.116	1259.382	113.382	336.077

Notes: ppm: parts per million; LC_{50} – Lethal concentration required to kill 50% of the population exposed; LC_{95} – Lethal concentration required to kill 90% of the population exposed.

obtained from Soxhlet technique presented low LC95 value at 366.077 ppm. In general, acetone extracts of the various plant parts studied gives lower LC50 and LC95 values compared to methanolic extracts (Table 2).

The different plant parts and concentrations of extracts tested also have significant effects on *Cx. quinquefasciatus* larval mortality ($F = 2.81$, $df = 10$ $p < 0.05$; Table 1). The mean percentage mortality calculated clearly shows that with increasing extract concentration from 10 to 600 ppm, the larval mortality rate also increase (Figures 2 and 3). At 300 ppm of concentration, leaves acethonilic extract obtained from both Soxhlet and maceration methods showed 100% mortality. In which, the larvicidal activity was higher compared to other plant part extracts tested as it effectively kills *Cx. quinquefasciatus* larvae even at lower concentrations. In other hand, acethonilic extract of stem and flower buds obtained from Soxhlet also exhibited larvicidal properties by causing mortality of the larvae which was observed to be directly proportional to the tested concentrations (Figures 2B and 3B). The methanol extracts of the different plant parts also exhibited larvicidal activity at different concentrations, but it was observed to be less effective compared to acetone extracts tested (Figures 2 and 3). No mortality was recorded in the control treatment (distilled water with 1% methanol or 1% acetone).

Observations under laboratory condition revealed that after 24 h treatment with the all extracts tested, the treated larvae expressed behavioural modifications and severe malformations. The treated larvae were found to be restless, wriggled up and down and tend to curl up. These behavioral patterns persisted, causing the larvae to eventually slow down movement, unable to reach the surface and finally resulted in larval knockdown. Observation under stereomicroscope (Olympus EX41), showed dark pigmentation of the cuticle (Figure 4B and C), disrupted digestive tract (Figure 4B) and disruption of the membranous cervix causing detachment of the thorax (Figure 4C) of the treated larvae, as compared to the control larvae (Figure 4A).

Discussions

Our study revealed that the acetone extract of *I. cairica* leaves obtained using Soxhlet extraction imposes the most effective larvicidal properties against *Cx. quinquefasciatus* larvae compared to maceration method. Using Soxhlet extraction, higher percentage of crude extract yield with greater efficacy of larvicidal ability to kill *Cx. quinquefasciatus* larvae can be produced. Soxhlet method had been found to be more effective in extracting larvicidal components for both *Azadirachta indica* and *Artemisia annua* Linn compared to reflux and hot extraction methods (Tonk et al. 2006). Comparison of accelerated solvent extraction (ASE) and Soxhlet extraction with conventional maceration showed that the active compound contents were higher using the former two, partly because of the constant higher temperatures used, which result in accelerated diffusion through the plant material (Smelcerovic et al. 2006). The advantages of plant extraction using Soxhlet also include the displacement of transfer equilibrium by repeatedly bringing fresh solvent into contact with the plant powder thus, maintaining a relatively high extraction temperature with heat from the distillation flask (Luque

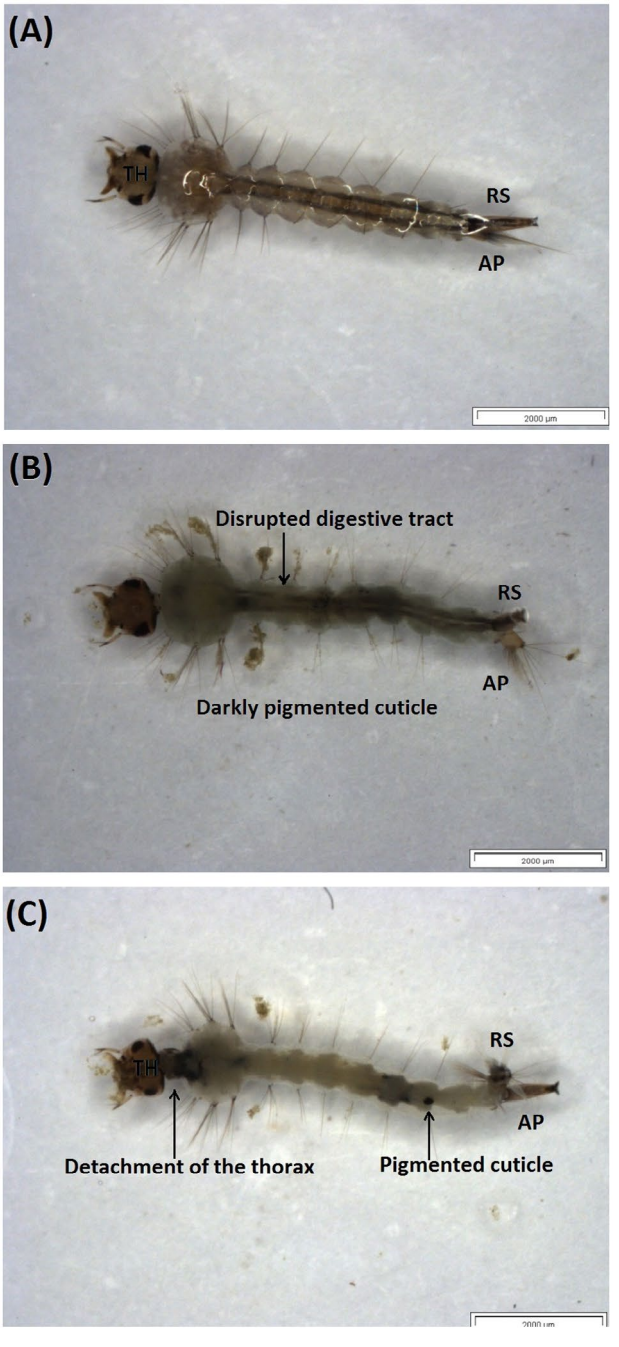

Figure 4A–C. Morphological malformations in Culex quinquefasciatus larva. A, Culex quinquefasciatus larva observed in control solution; B, Larva with disrupted digestive tract and darkly pigmented cuticle; C, Larva with detachment of the thorax and pigmented cuticle observed after exposure to plant extracts tested.
Note: THthorax; AP- anal papillae; RS- respiratory siphon.

de Castro & Garcia-Ayuso 1998). Other advantages include shortened extraction time and give a better extract yield.

Contrarily, maceration process can be quite time consuming, taking up to several weeks (Jones & Kinghorn 2006; Trusheva et al. 2007) Exhaustive maceration can also consume large volumes of solvent and can lead to the potential loss of metabolites from plant material. Also, according to the results obtained in the present study, it is shown that less composition of active compound will be extracted and will reduce the efficacy of plant extract as a larvicide. Some compounds may not be extracted efficiently if they are poorly soluble at room temperature. On the other hand, as the extraction is performed at room temperature, maceration is less likely to lead to the degradation of thermolabile metabolites (Jones & Kinghorn 2006). However, maceration method is as well simple, easy to conduct and relatively cheap since it does not require advance tools.

Yield of crude extract obtained from Soxhlet and maceration methods was different based on the solvents used. Different solvents resulted in different percentage of yield extract and compositions of the active compound (Umuzewa 2003; Zarnowski & Suzuki 2004). Different organic solvents provided different polarity gradients in dissolving the toxic components present in the plant materials, and also extract greater measures of bioactive components responsible for the lethal effect (Sasidharan et al. 2010). In this study, acetone appeals to be an effective and best solvent in extracting larvicidal compounds from of *I. cairica* plant. Crude extract obtained from this solvent caused higher mortality of the larvae at lower concentration suggested that the most active compound was extracted. The active compound of the studied plant might be more soluble in acetone than methanol as solvent. Similar results utilised acetone as solvent to extract *Lantana camara* L. has been found efficient to cause 100% mortality towards *Cx. quinquefasciatus* larvae at the dose of 1 mL/100 ml (Dwivedi & Karwasara 2003).

Higher percentage of yield was obtained from *I. cairica* leaves, in addition to better larvicidal activity. This suggested that the phytochemical responsible for the toxic effect on *Cx. quinquefasciatus* larvae may be highly concentrated in the leaves of the studied plant, causing higher mortality of *Cx. quinquefasciatus* larvae even at lower concentration. It has been reported in studies done previously, that the bioactivity of phytochemical against mosquito larvae can vary significantly depending on the plant species and their climatic or geographical areas, plant parts, age of plant part, solvent used in the extraction and mosquito species (Tawatsin et al. 2001; Shaalan et al. 2005). The plant materials are not completely homogeneous, which may be one reason for the variations in active compound contents in different extracts from the same plant material (Smelcerovic et al. 2006). Nevertheless, based on the results from the current study, extracts obtained from the stem and flower buds of the studied plant also exhibited larvicidal activity but need a higher concentration to elicit the efficacy effect. Therefore, the active compound responsible for the larvicidal effects can be obtained from various parts of *I. cairica* plant for the control of *Cx. quinquefasciatus* larvae.

The treated larvae were observed to show signs of neurotoxicity, such as ceaselessly moving, curling up, blackening of the abdomen and finally led to death. Effects of seed extract of *Seseli diffusum* against *Ae. aegypti,* whereby the larvae exposed to the seed extract also showed similar reactions of restlessness, pigmentation and performed self-biting to the anal papillae with their mouth parts and formed a ring shape (head to siphon) (Kabir et al. 2013). The behavioural changes and morphological malformations in the treated larvae showed the presence of a neurotoxic compound in the plant. The active compound present in the studied plant may have interfered with the metabolic activities of the larvae causing general disruption in the development of larvae and eventually resulted in death.

Phytochemical studies have revealed the presence of the naturally occurring lignanolides, arctigenin and trachelogenin in *I. cairica* (Lima & Braz-Filho 1997; Fuss 2003; Umezawa 2003; Meira et al. 2012). Lignans are secondary plant metabolites that occur in all parts of plants, such as heartwood, bark, roots, rhizomes, stems, leaves, flowers, fruits and seeds, as well as in secreted products such as resins and they may be found in the variable content (Ríos et al. 2002). The lignans plays an important part in plant defence mechanism by affording protection against predators such as insects (Fürstenberg-Hägg et al. 2013). Lignan toxicity differs from that of conventional neurotoxic insecticides, as it is more chronic rather than acute (Bernard et al. 1995). Lignan secondary metabolite from phenylpropanoid precursors influence the physiological function including endocrine system of insects (Harmatha & Dinan 2003).

Essential oil of *I. cairica* has been found to induce 100% mortality in larvae of *Cx. quinquefasciatus, An. stephensi, Ae. aegypti* and *Cx. tritaeniorhynchus* (Thomas et al. 2004). It can be persisted in water and gave an effectiveness up to 21 days (Thiagaletchumi et al. 2014). Essential oils distilled from *I. cairica* also acted as a promising repellent against *An. stephensi* and did not cause skin irritation, hot sensations or rashes on the arms of the test volunteers during the study period (Rajkumar & Jebanesan 2007). Hence, with the following results from the current study, it is sufficient to consider *I. cairica* for further study focusing on isolation of active principles compound that responsible for the larvicidal properties and assessment in the field for the development of a new alternative mosquito larvicide.

On the whole, the plant extraction using Soxhlet and acetone as solvent gave prominent yield of crude extract with a higher degree of active compound. Among extracts of various parts of *I. cairica*, the acethonilic leaves extract was found to be the most effective in causing *Cx. quinquefasciatus* larvae death. This study also provided further clarification that *I. cairica* plant has larvicidal properties, but its potential as larvicide against mosquito larvae should be researched in depth. The isolation of crude extract to identify the active components and subsequent field trials are required for development of new biopesticides as one of the alternative control methods for insect vectors. These findings will contribute to the practical control of mosquito larvae in the polluted aquatic ecosystem, potable waters and other artificial breeding sites.

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Disclosure statement

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