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RESEARCH ARTICLE

ADULT EMERGENCE INHIBITION AND SUBLETHAL EFFECTS OF CRUDE EXTRACT OF *CYPERUS AROMATICUS* (FAMILY: CYPERACEAE), AGAINST DENGUE VECTOR MOSQUITOES

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ABSTRACT

The growth inhibition activity and sublethal effects (EI_{50}) of the crude extract of *Cyperus aromaticus* Mattf and Kukenth (Cyperaceae), containing juvenile hormone III (JH III) was evaluated against the 3rd instar larvae of two *Aedes* mosquito species, namely *Aedes aegypti* (Linnaeus) and *Aedes albopictus* Skuse under laboratory conditions. A steam distillation system was used to prepare the crude extract from whole parts of natural plant which collected from different parts of Penang, Malaysia. The crude extract was analyzed by High-Performance Liquid Chromatography (HPLC). Laboratory evaluation was carried out against late 3rd instar larvae of the Vector Control Research Unit (VCRU) strains of *Ae. aegypti* and *Ae. albopictus* using the standard WHO method. Bioassay tests presented the remarkable growth inhibition activity of the extract of *C. aromaticus* against the two test *Aedes* mosquitoes. *Ae.albopictus* was significantly more susceptible to the crude extract with the lower EI_{50} value of 30.37 µg/ml, followed by *Ae. aegypti* with EI_{50} values of 39.52 µg/ml. The reproductive potential, growth period, adult size and longevity of the mosquitoes were significantly affected following exposure to sublethal dosage (EI_{50}) of the extract, but the sex ratio of the adult population was not significantly affected . The present study revealed the bioefficacy of the crude extract of *C. aromaticus* (Whole plant) in controlling *Aedes* mosquito populations in the effort to reduce the transmission of dengue fever.

INTRODUCTION

Mosquitoes are vectors of pathogens of several infectious diseases like malaria, dengue fever, lymphatic filariasis, Japanese encephalitis, yellow fever and chickungunya (Nauen 2007). They are also important pests of man, causing allergic reactions, including local skin and systematic reactions such as urticaria and angioedema (Peng *et al*., 1999). Presently mosquito borne diseases have become a global health problem with serious social and economic implications especially in tropical and subtropical countries (Gubler, 2002, Sachs and Malaney, 2002). *Aedes aegypti* (Linnaeus) and *Aedes albopictus* Skuse are considered as the two main vectors of dengue fever in Malaysia (Lam, 1993, Rozilawati *et al*., 2007). Dengue fever is endemic and found mostly in the urban and suburban areas in Malaysia (Chen *et al*., 2006). The morbidity and mortality rates of dengue fever (DF and DHF) in this country are on the rise since 1999. In 2010, 46171 cases of dengue fever with 134 deaths were reported (WHO, 2011).

Different approaches have been used to reduce the mosquito borne diseases in endemic zones of the world. Chemical control of mosquito vectors being a main strategy. The use of insecticides is an effective way of reducing vector population, but wide-scale application of synthetic insecticides has many drawbacks such as adverse effect on non-target organisms, environmental contamination (Lapcharoen *et al*., 2005) and the development of insecticide resistance among vector species. Even resistance to bioinsecticides such as *Bacillus thuringiensis* (Georghiou and Wirth, 1997, Regis *et al*., 2001) and *B. sphaericus* (Rodcharoen and Mulla, 1994, Silva-Filha *et al*., 1995, Mulla *et al*., 2003) as well as insect growth regulators such as methoprene (Shemshedini and Wilsont, 1990, Cornel *et al*., 2002) were reported. Thus the search for newer insecticides that are effective without any harmful effects on non-target organisms as well as environmentally friendly, is important (Markouk *et al*., 2000, Redwane *et al*., 2002). Natural resources from tropical rain forest are a great source of biologically active compounds that act as insecticides, larvicides, growth inhibitions, antifeedants, insect repellents

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and IGRs (Jacobson, 1982, Patil *et al*., 2006, Binder *et al*., 1991). Malaysia is a tropical country which is rich in natural resources. *Cyperus aromaticus* Mattf and Kukenth is a rhizomatous sedge, a member of the Cyperaceae family which grows in tropical and subtropical regions including Malaysia. It is known as navua sedge or 'rumput ganda' in Malaysia (Henderson, 1954). The presence of insect JH III in *C. aromaticus* (Toong *et al*., 1988) makes the plant a potential candidate for consideration in mosquito control. The aim of this study is to investigate the bioactivity of whole plant extract of *C. aromaticus* against *Ae. aegypti* and *Ae. albopictus* and their consequent sublethal effects on some biological and morphological parameters in the two *Aedes* mosquito species during two generations.

MATERIALS AND METHODS

Preparation of crude extract and HPLC assay

Samples of *C. aromaticus* were collected from different parts of Penang, Malaysia (table 1). Whole parts of natural plant were washed and dried under shade. A steam distillation system was used to prepare the crude extract from the mother plant (Chan *et al*., 2004). The crude extract was stored in 10 ml bottles, sealed and covered with aluminium foil and kept in the freezer for further bioassay tests on mosquito larvae. For high resolution separation and detection of JH III, the crude extract was analyzed by High-Performance Liquid Chromatography (HPLC) (Chan *et al*., 2010).

Table 1 Plants collected from the natural habitats used for bioassays against *Aedes* mosquito species

Common Name	Scientific Name	Family	Habitat	Source of Collection	Collection Time
Rumput ganda	*Cyperus aromaticus*	Cyperaceae	Damp and wet places	Sungai Tiram and Bayan Lepas	From November to June (9 – 12 Am)

Laboratory-cultured mosquito strains

The Vector Control Research Unit (VCRU) strains of *Ae. aegypti* and *Ae. albopictus* were used in this study. The VCRU susceptible mosquito strains were originally from Penang, Malaysia and they have been maintained in the laboratory of the Vector Control Research Unit in Universiti Sains Malaysia since the 1980s and have not been exposed to any insecticides. The eggs of the two *Aedes* mosquito species were obtained from the VCRU. They were placed separately in different culture trays containing seasoned water and kept under laboratory condition at a temperature of 27°C ± 2 and 80% ± 5 relative humidity. Once hatched, the emerged larvae were fed daily with larval food made up of a mixture of milk powder, ground beef liver, yeast and dog food in a ratio of 1:1:1:2. The late 3rd instar larvae of each species were picked and used for bioassays.

Emergence inhibition activity of Cyperus aromaticus extract

Laboratory evaluation was carried out against the late 3rd instar larvae of *Ae. aegypti* and *Ae. albopictus* using the standard WHO guideline (WHO, 2005) with some modification. A 2% stock solution was prepared by dissolving 400 mg of the crude extract in 20 ml acetone. In all tests, 20 late 3rd instar larvae of each mosquito were placed in 200 ml disposable plastic cups containing 100 ml seasoned water treated with the required amount of the stock solution of the crude extract. Activity range finding tests were conducted to determine the maximum and minimum doses which caused more than 0% and less than 100% growth inhibition. Then 6 and 7 concentrations of the crude extract within the activity range were tested on *Ae. albopictus* and *Ae. aegypti* mosquitoes respectively. Each concentration was replicated three times and three controls treated with 1 ml of acetone. All tests were repeated three times on different occasions. All tests were carried out in a temperature controlled laboratory (27°C ± 2 and relative humidity of 80% ± 5). An appropriate amount of larval food (10 mg/larva) was added to each cup after the larvae were introduced into the water both test and control cups. All the cups were covered with netting to prevent adults from escaping. Mortality at the larval, pupal and adult stages was recorded. Moribund larvae and pupae, as well as adult mosquitoes which did not separate completely from the pupal case, were recorded as "affected". The number of successfully emerged adults was assessed by counting and removing empty pupal exuviae for each replicate. The experiments ended when all the larvae or pupae in the controls died or emerged as adults.

Sublethal effects of the crude extract of Cyperus aromaticus on Aedes mosquitoes

The sublethal impacts of *C. aromaticus* extract on some biological and morphological aspects of two *Aedes* mosquito species were evaluated using the EI_{50} dose of the crude extract. Initially a total of 300 - 500 late 3rd instar larvae of each mosquito, *Ae. aegypti* and *Ae. albopictus* were treated with the crude extract of *C. aromaticus* at the concentration of the EI_{50} doses (39.52 and 30.37 µg/ml respectively). The controls were treated with 1 ml acetone. Larval food was provided until pupation. The containers were then placed separately in foldable emergence cages and were kept under controlled laboratory conditions (27°C ± 2 and relative humidity of 80% ± 5). Adults were allowed to emerge. Emerged adults were fed by giving access to cotton wick soaked with 10% sucrose, and kept for further experiments.

Fecundity and fertility assays

To study the effect of sublethal dose (EI_{50}) of the plant extract on the fecundity of the two *Aedes* mosquito species, two cohorts of 4-5 day old females (mated) from the control and treated group of each mosquito species were separately placed in foldable experimental cages. A white mouse restrained within a piece of wire-netting was placed in each cage during the day for the *Aedes* mosquitoes as a source for blood meal. The number of blood–engorged females of the mosquito species were recorded a day after. Twenty blood fed of each *Aedes* mosquito species were transferred into individual paper cups with a wet cone shape filter paper as oviposition substrate. The top of the cups were covered by a piece of mesh cloth and 10% sucrose solution was provided using cotton pads placed on top of the mesh cloth. The number of eggs produced by the

blood fed female mosquitoes was counted daily using a stereo microscope at a magnification of 10× for a maximum period of seven days. The eggs collected from the fecundity tests were used for studying the effect of sublethal dose of crude extract on the hatching performance mosquito species. The filter papers containing eggs were kept wet for two more days and then allowed to dry. Ten days after egg laying, three filter papers were submerged in a culture tray containing 1 liter of seasoned water and held at standard rearing conditions for 48 hours for hatching. The eggs were examined and counted for hatching using a stereo microscope at a magnification of 10×. The hatchability percentage was calculated by the number of eggs hatched. A second blood meal was provided on the eighth day for the female mosquitoes in the experimental cages, and the data mentioned earlier were recorded for the second gonotrophic cycle.

Growth period and sex ratio assays

The effects of sublethal dose of crude extract of *C. aromaticus* on developmental period (time taken for pupal formation and adult emergence) and also sex ratio of two *Aedes* mosquito species were evaluated. A total of 300 late 3rd instar larvae of each mosquito species *Ae. aegypti* and *Ae. albopictus* were separately placed in 200 ml disposable plastic cups containing 100 ml seasoned water (10 larvae per cup) at the concentration of the EI_{50} doses (39.52 and 30.37 µg/ml respectively), and 200 larvae were treated with only 1 ml of acetone as the control (10 larvae/ cup). More larvae were used for treatment with the crude extract at EI_{50}, because it was expected that half of them die. The cups were covered with a piece of mesh cloth and kept at standard culturing condition. Larval food was provided during larval development. The number of live and dead larvae, pupae, adults and also emerged males and females were recorded every 12 hours until either all the mosquitoes emerged or all individual which failed to emerge died. For the F1 generation, 300 larvae that hatched from the eggs produced by treated individuals (plant extract and control) were transferred to culture trays (50 larvae /tray) containing 1 liter of seasoned water. Larval food was provided during larval development. The number of live and dead larvae and pupae were observed and recorded every 12 hours, and the emerged pupae were transferred into plastic cups containing 100 ml seasoned water (10 pupae/ cup) and covered with a piece of mesh cloth. Adult emergence (male and female) was observed and recorded every 12 hours.

Adult size

The length of the adult wing as an indicator of body size (Clements, 1999) was used to study the sublethal effect of the crude extract of *C. aromaticus* on the adult size of the *Aedes* mosquito species. After adult emergence, 30 males and 30 females of each treated mosquito species (plant extract and control) were randomly aspirated out of the emergence cage and maintained in separate cages and fed only on seasoned water for 24 hrs. All individuals were anesthetized with chloroform and their right wings detached. Each wing was mounted in a drop of physiological saline on a glass slide. The wing length from the axial incision to the apical margin (excluding the fringe) was measured using a stereo microscope at a magnification of 100×.

Adult mosquito longevity

To determine the impact of a sublethal dose of the crude extract of *C. aromaticus* on the longevity of adult mosquito species, 40 males and 40 females of the emerged adults of each mosquito species treated at the 3rd instar larval stage (plant extract and control) were maintained in foldable cages separately (20 male and 20 female per cage). Mosquitoes were fed with sucrose using cotton pad and kept at standard rearing conditions. Mortality was recorded daily until the death of the last individual. All sublethal experiments were repeated three times on different occasions and all mentioned tests were conducted on the F1 generation. The F1 larvae were not exposed to any chemical.

Statistical analysis

Abbott formula was used and the survival percentages were corrected if necessary. The emergence inhibition (EI) values obtained at each concentration were calculated (WHO, 2005). Then all EI values were subjected to probit regression analysis using SPSS Software to determine EI_{50} and EI_{90} values and their 95% confidence intervals. Data from sublethal studies were subjected to analysis of variance (ANOVA). An arc sine transformation of the data was made before using ANOVA, for analysing the percentage data from egg hatchability studies. The sex ratio data were compared by non parametric Kruskal Wallis test.

RESULTS

HPLC assay

In the present study, according to the HPLC analysis, the JH III content in the crude extract of *C. aromaticus* was 284.11± 7.17 µg/g dried weight.

Emergence inhibition activity of Cyperus aromaticus extract

In this study, mosquito larvae of *Ae. aegypti* and *Ae. albopictus* displayed different susceptibilities to the crude extract. Based on the EI_{50} values, *Ae. albopictus* was significantly more susceptible to the crude extract of *C. aromaticus* with an EI_{50} value of 30.37 µg/ml, followed by *Ae. aegypti* mosquito with EI_{50} values of 39.52 µg/ml (Table 2).

Table 2 The adult emergence inhibition and slope values of the crude extract of *C. aromaticus* on the 3rd instar larvae of the two *Aedes* mosquito species

Mosquito Species	EI_{50} (µg/ml) (95% CI)	EI_{90} (µg/ml) (95% CI)	Slope ± SE
Ae. aegypti	39.52 (33.82 – 45.01)	86.32 (71.09 – 120.46)	3.77 ± 0.28
Ae. albopictus	30.37 (23.24 – 37.21)	70.73 (53.69 – 129.91)	3.49 ± 0.28

EI: Emergence Inhibition , **CI:** Confidence Interval , **SE:** Standard Error

Sublethal effects of the crude extract of C. aromaticus on Aedes mosquito species

The sublethal dose (EI_{50}) of the crude extract of *C. aromaticus* significantly ($p \leq 0.05$) reduced the mean number of eggs oviposited by parental generation of *Ae. aegypti*, during the

first and second gonotrophic cycles and the mean number of eggs oviposited by parental generation of *Ae. albopictus* during the first gonotrophic cycle as well. The reduction effects for *Ae. aegypti* were 10.9 and 21.5 % respectively and for *Ae. albopictus* was 11.8%. (Table3). The reduction effects of sublethal dose of the crude extract on the mean number of eggs produced by F1 generation of both *Aedes* mosquitoes during the first and second gonotrophic cycles were not significant at $p \geq 0.05$ (Table 3). The sublethal dose of the crude extract significantly ($p \leq 0.05$) reduced the hatching percentage of eggs produced by the parental generation of both *Ae. aegypti* and *Ae. albopictus* during the first gonotrophic cycle by 17.38% and 25.71% respectively. The negative effects of this extract on the hatching percentage of eggs produced by the parental generation of both *Aedes* during the second gonotrophic cycle were not significant at $p \geq 0.05$. There were also no significant effects of the sublethal dosage of the crude extract of *C. aromaticus* on the hatching percentage of eggs produced by the F1 generation of two mosquitoes during the first and second gonotrophic cycles at $p \geq 0.05$ (Table 3).

Table 3 Number of eggs and percentage of egg hatch (Mean ± SE) laid by *Aedes* mosquito species for the parental (treated with the crude extract of *C. aromaticus*) and the F1generation

Mosquito Species	G	GC	No of Egg Control	No of Egg Treated	ER (%)	Egg hatch (%) Control	Egg hatch (%) Treated
Ae. aegypti	P	1st	118.4 ± 3.3	105.5 ± 2.9*	10.9	96.36 ± 0.7	79.98 ± 1.9*
		2nd	117.8 ± 3.6	92.4 ± 4.2*	21.5	95.20 ± 0.4	89.41 ± 5.5
	F1	1st	125.9 ± 2.8	122.9 ± 3.2	2.4	96.66 ± 0.3	92.16 ± 3.7
		2nd	123.3 ± 2.6	118.6 ± 4.3	3.8	97.52 ± 0.0	95.96 ± 0.7
Ae. albopictus	P	1st	114.1 ± 4.0	100.6 ± 3.7*	11.8	97.54 ± 0.6	71.83 ± 4.0*
		2nd	96.6± 3.6	89.6 ± 3.4	7.3	94.82 ± 2.4	82.94 ± 0.8
	F1	1st	112.6 ± 3.8	109.2 ± 3.4	3.0	93.38 ± 1.6	91.08 ± 1.9
		2nd	114.7 ± 4.2	110.8 ± 4.0	3.4	91.04 ± 2.3	93.94 ± 1.8

*Mean values within a row are significantly different at $p \leq 0.05$ **G** Generation , **GC** Gonotrophic cycle, **P** Parental, **ER** Effective Reduction of egg production

Larvae of *Ae. aegypti* and *Ae. albopictus* exposed to the sublethal dose of the crude extract of *C. aromaticus* at the late 3rd instar stage, showed a significant ($p \leq 0.05$) delay in pupal formation by 0.32 and 0.44 days respectively, compared with the controls. Whereas the effects of the crude extract of *C. aromaticus* on the pupal formation time for F1 generation of the two *Aedes* mosquitoes were not significant at $p \geq 0.05$ (Table 4). Adult emergence of the 3rd instar larvae of *Ae. aegypti* and *Ae. albopictus* exposed to the sublethal dose of the crude extract of *C. aromaticus*, were significantly ($p \leq 0.05$) longer than that for the control population. The extensions in time were 0.32 and 0.51 days in the females and 0.38 and 0.4 days in the males respectively. The effects of the sublethal dose of the crude extract of *C. aromaticus* on adult emergence time for the F1 generation of females of the two *Aedes* mosquitoes as well as males of *Ae. albopictus* were not significant at $p \geq 0.05$. Whereas the adult emergence time for the F1 generation of males of *Ae. aegypti* was significantly delayed by 0.1 days ($p \leq 0.05$) (Table 4).

Analysis of data obtained indicated that the sex ratio of *Ae. aegypti* and *Ae. albopictus* (either parental or F1 generation) was not significantly affected by the exposure of the 3rd instar larvae of each mosquito to the EI_{50} dose of the crude extract of *C. aromaticus* at $p \geq 0.05$ (Table 5).

Table 4 Pupal formation and adult emergence time (Mean ± SE) for the parental of the *Aedes* mosquito species (treated with the crude extract of *C. aromaticus*) and the F1 generation

Mosquito Species	G	Pupation time (Day) Control	Pupation time (Day) Treated	Adult Emergence time (Day) Female Control	Adult Emergence time (Day) Female Treated	Adult Emergence time (Day) Male Control	Adult Emergence time (Day) Male Treated
Ae. aegypti	P[1]	2.49 ± 0.01	2.81±0.01*	4.56±0.02	4.88±0.03*	4.03±0.01	4.41±0.02*
	F1	7.03 ± 0.01	7.07±0.01	9.72±0.03	9.69±0.03	9.42±0.02	9.52±0.3*
Ae. albopictus	P	2.57±0.01	3.01±0.02*	5.02±0.02	5.53±0.04*	4.48±0.02‘	4.88±0.04*
	F1	7.45±0.02	7.45±0.01	10.91±0.031	0.89±0.031	0.30±0.031	0.36±0.02

*Mean values within a row are significantly different at $p \leq 0.05$.
G Generation, **P** parental generation, **1** Days from day of treatment, **F1** F1 generation

The sublethal dose of the crude extract of *C. aromaticus* significantly reduced the wing length of emerged males and females of *Ae. aegypti* by 0.04 and 0.08 mm compared to the untreated controls at $p \leq 0.05$. Similarly the wing length of

Table 5 Adult sex ratio (Male: Female) of the parental of the *Aedes* mosquito species (treated with the crude extract of *C. aromaticus*) and the F1 generation

Group	Sex Ratio (Male : Female) *Ae. aegypti* P	*Ae. aegypti* F1	*Ae. Albopictus* P	*Ae. Albopictus* F1
Control	1.18 ± 0.12	1.12 ± 0.01	1.15 ± 0.16	1.03 ± 0.07
Treated	1.24 ± 0.11	1.05 ± 0.06	0.92 ± 0.27	1.17 ± 0.06

P Parental generation, **F1** F1generation

the males of *Ae. albopictus* significantly was decreased by 0.03 mm. Whereas there was no significant effect of the extract on wing length of the females of *Ae. albopictus* as well as the wing length of F1 generation of both *Ae. aegypti* and *Ae. albopictus* (males and females) which emerged from non exposed larvae compared to the controls at $p \geq 0.05$ (Table 6).

Table 6 Adult wing length and adult longevity (Mean ± SE) for the parental (treated with the crude extract of *C. aromaticus*) and the F1 generation of the *Aedes* mosquito species

Mosquito Species	G.	Ge.	Wing Length (mm) Control	Wing Length (mm) Treated	Time of Adult Survival (Day) Control	Time of Adult Survival (Day) Treated
Ae aegypti	P	F	2.64 ± 0.01	2.56 ± 0.01*	49.82 ± 1.45	40.20 ± 1.38*
		M	2.03 ± 0.01	1.99 ± 0.01*	24.57 ± 0.87	20.73 ± 0.87*
	F1	F	2.98 ± 0.01	2.95 ± 0.01	54.24 ± 1.83	52.36 ± 1.59
		M	2.20 ± 0.01	2.19 ± 0.01	21.40 ± 0.99	18.65 ± 0.81*
Ae. albopictus	P	F	2.50 ± 0.01	2.47 ± 0.01	44.85 ± 1.74	39.20 ± 1.53*
		M	2.10 ± 0.00	2.07 ± 0.00*	25.43 ± 1.07	21.11 ± 0.98*
	F1	F	2.61 ± 0.01	2.61 ± 0.01	43.18 ± 1.30	39.15 ± 1.39*
		M	2.16 ± 0.00	2.18 ± 0.01	17.31 ± 0.70	17.69 ± 0.75

*Mean values within a row are significantly different at $p \leq 0.05$.
G generation, **Ge.** Gender, **P** parental, **M** Male, **F** Female

The mean longevity of adult *Aedes* mosquito species (males and females) which emerged from the 3rd instar larvae exposed to a sublethal dose (EI_{50}) of the crude extract of *C. aromaticus* was significantly shorter than that for the control population. Between the females of the two mosquito species, the mean longevity of *Ae. aegypti* exposed to a sublethal dose of the crude extract was significantly more affected by reduction of 9.62 days. Whereas between the males of the two mosquito species treated with a sublethal dose of the crude extract, the longevity of *Ae. albopictus* was significantly more decreased by 4.32 days ($p \leq 0.05$). Likewise the mean longevity of males of F1 generation of *Ae. aegypti* and females of *Ae. albopictus* were significantly decreased by 2.75 and 4.03 days respectively. Whereas there were no significant effects of the sublethal dose of the crude extract on the mean longevity of the

females of the F1 generation of the *Ae. aegypti* as well as males of the F1 generation of *Ae. albopictus* ($p \geq 0.05$) (Table 6).

DISCUSSION

Since the past few years, there are extreme interests in plant or plant-based products as potential sources of natural insect control agents due to the numerous problems associated with the use of synthetic insecticides. Botanical products are generally pest-specific and often slow-acting compounds which are less hazardous and more environmentally friendly and easily biodegradable. These compounds are thought to be able to provide effective, sustainable and cheap control against mosquito larvae (Adeyemi, 2010). *Cyperus aromaticus* is a rhizomatous sedge which contains JH III. According to the HPLC analysis, the JH III content in the crude extract of *C. aromaticus* (whole plant) was 284.11± 7.17 µg/g dried weight. The JH III isolated from *C. aromaticus* collected from the field (mature mother plant) had the same 10 R configuration as insect JH III (Chan *et al*., 2004). Similarly JH III was found in the mature plant and also roots of *C. iria* at the concentration of 151 and 27.2 ± 3.3 µg/g fresh weight respectively (Bede *et al*., 1999, Toong *et al*., 1988). Whereas the JH levels detected in most insect species ranged between 0.1-100 ng/g FW(Toong *et al*., 1988).

In the present study the adult emergence activity of the crude extract of *C. aromaticus* against two vector mosquito species was demonstrated. A number of plant derivatives have been evaluated against various vector mosquito species as insect growth regulators. There are differences between the bioefficacy of phytoextracts against vector mosquito species depending on the plant species, part of the plant, age of the plant part, solvent used for extraction and the mosquito species (Shaalan *et al*., 2005). Different species of mosquito larvae show different susceptibilities toward even the same botanical extracts. The 50% adult emergence inhibition (EI_{50}) resulting from ≤ 39.52 µg/ml of the extract of *C. aromaticus*, on the two *Aedes* mosquito species reported in the present study confirmed its remarkable potential for control of mosquito population. The growth inhibition effect of this extract was noticeably higher than those reported for some other plant extracts in the literature. For example treatment of *Anopheles stephensi*, *Ae. aegypti*, and *Culex quinquefasciatus* with 500 µg/ml of *Abutilon indicum* leaf extracts decreased the adult emergence by 49.45 , 53.2 and 73.0%, respectively (Arivoli and Tennyson, 2011b). It was also reported that 500 µg/ml of whole plant crude extracts of *Citrullus colocynthis* reduced the adult emergence of three species of vector mosquito, *An. stephensi*, *Ae. aegypti* and *Cx.quinquefasciatus* by 53.0, 55.8 and 70.4%, respectively (Arivoli and Tennyson, 2011a). Likewise, 250 µg/ml of the acetone extract of the Egyptian plant *Cupressus sempervirens* leaves exhibited a reduction in the adult emergence of the mosquito *Culex pipiens* by 40.0% (EL-Sheikh *et al*., 2011). A 50% adult emergence inhibition was demonstrated using aqueous leaves extract of *Calotropis procera* at 277.90 and 183.65 µg/ml against *Anopheles arabiensis* and *Cx. quinquefasciatus*, respectively (Elimam *et al*., 2009).

In this study, the reproductive potential (fecundity and fertility) of both *Aedes* mosquitoes was decreased by a sublethal dose of the crude extract of *C. aromaticus* during the first two gonotrophic cycles. Several studies have addressed the reproductive responses of mosquito species towards plant derived extracts. Most of them have presented similar findings of reduction in egg production in different mosquito species, following exposure to different concentrations of various phytoextracts. The methanolic extracts of the leaves and seeds of *Melia azedarach* markedly decreased the fecundity of *An. stephensi* when the mosquito larvae were treated with different concentrations (ranging from 0.25 to 2 %) of both extracts (Nathan *et al*., 2006b). In addition, noticeable reduction of egg production in *An. stephensi* was observed following treatment the mosquito larvae with different concentrations (ranging from 0.1 to 1%) of methanol extracts of leaves of *Dysoxylum malabaricum* (Nathan *et al*., 2006a). Similarly, 0.048 µg/ml of the n-hexane fraction of acetone extract from the leaves of *Catharanthus roseus* (Kuppusamy *et al*., 2009) and different concentration of ethanol and methanol extracts of *Andrographis paniculata* (Kuppusamy and Murugan, 2010) decreased the egg production in this mosquito. The finding of the current study, as well as previous researches showed that phytochemicals with insect growth regulators can significantly inhibit normal ovarian maturation and egg production in mosquitoes and therefore decrease the vector mosquito population. Possibly, variation in egg production of mosquitoes in response to chemical exposure is due to factors which are involved in the regulation of egg production in mosquitoes such as genetical factors as well as hormonal and nervous system stimulations (Attardo *et al*., 2005, Gulia-Nuss *et al*., 2011).

The results of this study also revealed the reduction of egg hatchability (fertility) in the parental generation of both *Aedes* species following treatment with a sublethal dosage of the crude extract of *C. aromaticus*. Similar results have been documented in other studies using different phytochemicals on various mosquito species and other insects. For instance, using sublethal dose (EI_{50}) of the ethyl acetate fractions of *Calophyllum inophyllum* seed and leaf, *Solanum suratense* and *Samadera indica* leaf extracts and the petrol ether fraction of *Rhinocanthus nasutus* leaf extract, resulted in a significant decrease in the fertility of *Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti* (Muthukrishnan and Pushpalatha, 2001). After exposing *Ae. aegypti* larvae to 10 µg/ml of the acetone fraction of the petroleum ether extract of the seeds of *Argemone mexicana*, a 100% failure in egg hatch in the mosquito population was observed (Sakthivadivel and Thilagavathy, 2003). Also using several plant extracts including *Lantana camara* (leaves), *Pelargonium zonale* (leaves), *Cupressus macrocarpa* (leaves), *Cyperus rotundus* (whole plant) and *Acacia nilotica* (seeds) powders, showed a highly significant decrease in the percentage of egg hatch in the house fly, *Musca domestica* (Elkattan *et al*., 2011). Variability among the anti fertility effect of active compounds of various plant extracts, could cause different effects on the reproduction potential (Muthukrishnan and Pushpalatha, 2001). The extract of *C. aromaticus* might exert their effects on fecundity and hatchability in mosquito species through its influence on the endocrine system due to the JH III content. Both embryogenesis and embryonic ecdysis are inhibited in eggs of

many insects species exposed to IGRs with JH activity in the female body or after the egg deposition (Staal, 1975).

Using sublethal dosage of the crude extract of *C. aromaticus* against *Ae. aegypti* and *Ae. albopictus*, showed significant prolong in pupal formation time and adult emergence period in both *Aedes* treated mosquito species. The current findings were in agreement with results of several previous researches. Application of different concentrations of the crude leaf extracts from several plants such as *Murraya koenigii* (Arivoli and Tennyson, 2011c), *Citrullus colocynthis* (Arivoli and Tennyson, 2011a) and *Abutilon indicum* (Arivoli and Tennyson, 2011b) against the early 3rd instar larvae of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*, significantly prolonged the development of the larvae and pupae stages in all of them. This pattern was also reported using different concentration of n-hexane fraction of acetone extract from the leaves of *Catharanthus roseus* against *An. stephensi* (Kuppusamy *et al*., 2009). Likewise, treatment of the 3rd instar larvae of this *Anopheles* with 12.5, 25, 50 and 100 µg/ml of methanol extract of *Azadirachta indica* caused the developmental period of 10 days in the control to be extended to 11, 12, 14 and 15 days, respectively (Sharma *et al*., 2006). An extension in the larval developmental period of *Ae. aegypti* after exposure to 10 µg/ml of the acetone fraction of the petroleum ether extract from the seeds of the *Argemone mexicana* plant also has been reported (Sakthivadivel and Thilagavathy, 2003). Also, sublethal dosage of the methanolic extracts from *Nerium indicum* and *Euphorbia royleana* induced a significant delay in pupation in treated larvae of *Cx. quinquefasciatus* (Srivastava *et al*., 2003). Similar findings were reported in this mosquito treated with 400 µg/ml of *Momordica tuberosa* leaf extract (Sethuraman *et al*., 2010). Such prolonged larval and pupal periods of insects following exposure to phytoextracts indicates the interference by the bio-active compounds on the normal hormonal activity coordination of the metabolic processes of the developing stages probably due to interference in the endocrine mechanism (Sakthivadivel and Thilagavathy, 2003).

The sex ratio of all mosquito species investigated in the present study, was not significantly affected when the 3rd instar larvae were treated with a sublethal dose of the crude extract of *C. aromaticus*. There were no available information on the sublethal effects of phytoextracts on mosquito sex ratio, but several researchers reported the sublethal effects of phytoextracts on the sex ratio of other insect pests. Changes in the sex ratios of emerged adults tended to favour males following treatment of *Musca domestica* larvae with LC_{25}, LC_{50} and LC_{75} of *Cupressus macrocarpa*, *Lantana camara*, *Pelargonium zonale*, *Cyperus rotundus* and *Acacia nilotica* have been reported (Elkattan *et al*., 2011). Similarly the number of male *Drosophila melanogaster* was greater than females when the flies were reared on a medium with extract from the plant, *Chenopodium ambrosioides* (Wohlenberg and Lopes-Da-Silva, 2009). Likewise, treated eggs of the coffee leaf miner (*Leucoptera coffeella*) with *Momordica charantia* extract produced a sex ratio which was skewed towards females (Alves *et al*., 2011). These findings were in contrast with the results of the present study, probably due to the different plant extracts with various bioactive compounds and the different test insect species. In the present study, the wing length of only *Ae. aegypti* (females) was significantly reduced following treatment of the 3rd instar larvae with an IE_{50} dose of crude extract of *C. aromaticus*,. This result was in agreement with the findings of the study on *Ae. aegypti* using two microalgal chlorophytes, (*Scenedesmus quadricauda* and *Chlorococcum sp*) (Rohani *et al*., 2001). Whereas, in another study, the body size of *Liriomyza huidobrensis* was not affected when the first and third instar larvae were treated with different extract solutions of the *Melia azedarach* fruit (Banchio *et al*., 2003).

The effect of the plant extracts on the longevity of mosquito species in the present study was similar to findings of several studies using sublethal dosage of phytochemicals against various mosquito species. For instance, methanol extracts of the leaves of *Dysoxylum malabaricum* (Nathan *et al*., 2006a), extracts from *Azadirachta indica* (Nathan *et al*., 2005) and the ethanol and methanol extracts of *Andrographis paniculata* (Kuppusamy and Murugan, 2010) induced noticeable reduction in the adult longevity of *An. stephensi*. Reduction in adult longevity was also observed when the larvae of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* were treated with several phytoextracts including the crude leaf extracts of *Murraya koenigii* (Arivoli and Tennyson, 2011c) crude extract of *Citrullus colocynthis* (Arivoli and Tennyson, 2011a) and the leaf extracts of *Abutilon indicum* (Arivoli and Tennyson, 2011b). Also, a reduction in the adult longevity of *An. stephensi* after the larvae were treated with the n-hexane fraction of acetone extract of the leaves of *Catharanthus roseus* was observed (Kuppusamy *et al*., 2009). In summary, the results of the present work suggest a strong and potent effect of crude extract of *C. aromaticus* against dengue vector mosquitoes. In addition, the application of a sublethal dosage of the extract of *C. aromaticus* significantly affect the morphological and biological characteristics of mosquitoes which in turn could result in a reduction of the population of vector mosquitoes.

CONCLUSION

In conclusion the use of plant-derived natural products as biopesticides to reduce the immature aquatic stages can provide many associated benefits in vector control and as major alternatives to synthetic products for the control of important vector borne diseases. Further investigations are needed to evaluate the utility of phytoextracts of *C. aromaticus* under small and large field condition against vector mosquito species. Also the active ingredient(s) of the extract responsible for the adult emergence inhibition activity against vector mosquitoes should be identified and utilized, in preparing a commercial product/formulation to be used as an IGR agent.

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