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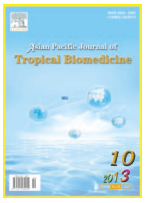

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Bioefficacy of crude extract of *Cyperus aromaticus* (Family: Cyperaceae) cultured cells, against *Aedes aegypti* and *Aedes albopictus* mosquitoes

Fatemeh Kamiabi[1,2*], Zairi Jaal[3], Chan Lai Keng[3]

[1]*School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia*

[2]*Faculty of Health, Kerman University of Medical Sciences, Kerman, Iran*

[3]*School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia*

PEER REVIEW

Peer reviewer

Dr. Abbas Aghaei Afshar, Assistant Professor,Department of Medical Parasitology Kerman university of Medical Sciences, Iran.
Tel: +98 0341 3221660
E-mail: afshara2@yahoo.com

Comments

It was an interesting study that had good functional results. I accept the present article because for the following reasons: 1. The finding is practical and could be used for planning feature research projects. 2. the results are innovative and open a new approaches for control of insects.
Details on Page 773

ABSTRACT

Objective: To evaluate the growth inhibition activity of the crude extract of *Cyperus aromaticus* (*C. aromaticus*) cultured cells against the 3rd instar larvae of *Aedes aegypti* (Linn.) and *Aedes albopictus* Skuse (*Ae. albopictus*) under laboratory conditions, and determine the sublethal effects (EI_{50}) of the crude extract of *C. aromaticus* cultured cells on some biological and morphological parameters of both *Aedes* mosquito species during two generations as well.

Methods: The cell suspension cultures of *C. aromaticus* were activated from five callus lines (P4, Pa, Z1, Z6 and Ml) derived from the root explants of *in vitro* plantlets. The cultured cells were extracted in chloroform and used as plant material for the present study. For detection of juvenile hormone III, the crude extracts were analyzed by HPLC. Then the crude extracts of the three *C. aromaticus* cultured cell lines which contained varied amounts of juvenile hormone III [high level (P4 cell line), medium level (Z1 cell line) and low level (Ml cell line)] were tested against *Aedes* mosquito species. Laboratory evaluation was performed against late third instar larvae of the Vector Control Research Unit strains of *Ae. aegypti* and *Ae. albopictus* using the standard WHO method. The effects of EI_{50} of the *C. aromaticus* cultured P4 cells on fecundity, fertility, growth period, sex ratio, adult size and longevity of *Aedes* mosquitoes were assessed.

Results: Bioassay tests presented the remarkable growth inhibition activity of the crude extracts of *C. aromaticus* cultured cells against the two *Aedes* mosquitoes. Between the two mosquito species, *Ae. albopictus* was more susceptible to the crude extracts with lower EI_{50} values. EI_{50} of the crude extract of *C. aromaticus* cultured cells (P4) increased the sterility indices in the parental generation females in both *Aedes* mosquito species. A significant delay in the pupal formation and adult emergence were observed in the parental generation of the both mosquito species. The sex ratio of the adult population either parental or F1 generation of the *Aedes* mosquito species was not significantly affected by the EI_{50} dosage of the crude extract of *C. aromaticus* cultured P4 cells. A significant decrease in the wing length of the treated adult (female and male) of *Aedes aegypti* as well as the treated female of *Ae. albopictus* were observed. Longevity of the adult female of the parental generation of both *Aedes* mosquitoes as well as females of F1 generation of *Ae. albopictus* were significantly decreased.

Conclusions: The present study revealed the potential of the crude extract of *C. aromaticus* cultured cells in controlling vector mosquito populations in the effort to reduce the transmission of vector borne diseases.

KEYWORDS

Aedes aegypti, *Aedes albopictus*, *Cyperus aromaticus*, Plant cell culture, Juvenile hormone III, Emergence inhibition

*Corresponding author: Fatemeh Kamiabi, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia, and Faculty of Health, Kerman University of Medical Sciences, Kerman, Iran.
Tel: 00983413205107
Fax: 00983413205105
E-mail: fkamiabi@yahoo.com

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1. Introduction

Presently mosquito borne diseases have become a global health problem with serious social and economic implications especially in tropical and subtropical countries[1,2]. *Aedes aegypti* (Linn.) (*Ae. aegypti*) and *Aedes albopictus* (*Ae. albopictus*) are considered as the two main vectors of dengue fever in Malaysia[3,4]. Dengue is endemic and found mostly in the urban and suburban areas in Malaysia[5]. The morbidity and mortality rates of dengue fever and dengue hemorrhagic fever (DF and DHF) in this country have been on the rise since 1999. In 2010, 46 171 dengue cases with 134 deaths were reported[6]. *Ae. aegypti* and *Ae. albopictus* larvae breed in natural and artificial containers with clean and organically rich water[3,7]. Different approaches have been used to decrease the prevalence of mosquito borne diseases in endemic zones of the world. The use of insecticides is a main strategy. Chemical control of mosquito vectors is an effective way of reducing vector population, but wide-scale application of synthetic insecticides has many drawbacks such as the development of insecticide resistance among vector species, even to bioinsecticides such as *Bacillus thuringiensis* (*B. thuringiensis*) and *Bacillus sphaericus* and also to insect growth regulators such as methoprene, and adverse effect on non-target organisms and environmental contamination[8-15]. Thus the search for newer insecticides, which are environmentally friendly as well as effective without any harmful effects on non-target organisms, is important[16-18]. Recently the search for such compounds has been focused on a wide range of plant species[19,20]. Many active components of phytochemicals have great potential for the natural control of insect pests[21]. Plants produce secondary metabolites that act as insecticides, larvicides, growth inhibitions, antifeedants and insect repellents[22,23]. Some phytochemicals contain compounds that interfere with the normal development of insects[24-26]. Natural resources from tropical rain forest are a great source of biologically active compounds. Malaysia is a tropical country, which is rich in natural resources. Numerous studies have shown the bioefficacy of local Malaysian plants against mosquito vector species[27-32].

C. 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. The identification of very high levels of juvenile hormone III (JH III) in *C. aromaticus* and the observation of biological effects on insects induced an attractive attention to this plant in the native habitat[33]. Clearly *C. aromaticus* represents a valuable source of (10R)-JH III and can be used for the production of bioinsecticides. Since the presence of JH III in 10-week-old *in vitro* plantlets of *C. aromaticus* was reported[34], cell suspension cultures of *C. aromaticus* were established[35]. The *in vitro* culture system could be an alternative method for the production of JH III from *C. aromaticus*. This system will be a fast and effective approach for mass production of *C. aromaticus* cells, JH III and its precursors[34].

The aim of this study was to investigate the bioactivity of crude extract of *C. aromaticus* cultured cells against *Ae. aegypti* and *Ae. albopictus* and its consequent sublethal effects on some biological parameters in the *Aedes* mosquito species during two generations.

2. Materials and methods

2.1. Production of cell biomass of C. aromaticus in vitro

The cell suspension cultures of *C. aromaticus* were activated from five callus lines (P4, Pa, Z1, Z6 and Ml) derived from the root explants of *in vitro* plantlets[35]. The cells were inoculated into 250, 500 and 1 000 mL Erlenmeyer flasks (0.01 g/mL) containing Murashige and Skoog culture medium supplemented with 4.5 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D; 20.4 μmol/L) and 5.5 mg/L 1-naphthalene acetic acid (29.5 μmol/L). The cultures were placed in an incubator shaker with a rotary gyration of 120 r/min, at (25.0±2.0) °C with continuous lighting of 2 600-3 000 lux. Cells were harvested and subcultured at 12 d interval. The cell culture procedure was repeated on a continues basis. Harvested cells were dried at room temperature and stored in 250 mL bottles and kept in the laboratory for further experiments.

2.2. Extraction of the crude extracts and determination of JH III

The crude extracts of different cultured cell lines of *C. aromaticus* were extracted[35]. Dried cultured cells of *C. aromaticus* were grounded and soaked in chloroform (System® ChemAR™) (0.1 g/mL) for 24 h, and then filtered through a Whatman No.1 filter paper by suction. This process was repeated twice. The extracts were evaporated using rotary evaporator (Eyela Rotary Vacuum Evaporator N-N Series) at 35 °C until the solvent was completely removed. The crude extracts were stored in 10 mL bottles, sealed and covered with aluminium foil, and kept in the freezer for HPLC analysis and bioassay tests on mosquitoes.

For high resolution separation and detection of JH III, the crude extracts were analyzed by HPLC[35]. For preparation of the samples, 0.02 g of each crude extract was dissolved in 2 mL acetonitrile (HPLC grade) and filtered using 0.25 μm micro membrane syringe filter (Whatman® Puradisc™ 25TF). The HPLC system consisted of a reverse phase liquid chromatography column measuring 250.0 mm long and 4.6 internal diameter. The column was packed with C18 (octadecylsilyl, or ODS) coated beads bonded to a silica core (5 μ Hypersil®), connected to a UV photodiode micro array detector (SPD-10AVvp). Samples with a volume of 25 μL were used as loading volume. Pure acetonitrile (HPLC grade) was

used as the mobile phase and was filtered and degassed by the HPLC automatically. The mobile phase was pumped into the column at a flow rate of 0.5 mL/min for 10 min. JH III was then detected using UV photodiode micro array (SPD-10AVvp) at 216 nm wavelength. The standard solutions of JH III (Sigma, St. Louis, MO) (5 to 400 mg/L) were prepared for the calibration of JH III content in all the samples.

2.3. 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 Vector Control Research Unit in University Sains Malaysia since the 1980s and have not been exposed to any insecticides. The eggs of the both mosquito species obtained from the VCRU were placed separately in different culture trays containing seasoned water and kept under laboratory condition at a temperature of (27±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 at the ratio of 1:1:1:2. The late third instar larvae of each species were picked and used for bioassays.

2.4. Emergence inhibition activity of the crude extracts of C. aromaticus cultured cells on Aedes mosquito species

The standard WHO method with slight modification was used to determine the effectiveness of the crude extracts of the three *C. aromaticus* cultured cell lines which contained varied amounts of JH III [high level (P4 cell line), medium level (Z1 cell line) and low level (Ml cell line)] against *Ae. aegypti* and *Ae. albopictus*[36]. Two percent stock solutions were prepared by dissolving 400 mg of each crude extract in 20 mL acetone. In all tests, 20 late third 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 each crude extract. Preliminary tests were conducted to determine the maximum and minimum doses which caused more than 0% and less than 100% growth inhibition. Then six concentrations of the P4 and Z1 extracts and seven concentrations of the Ml extract within the activity range were tested on both *Aedes* mosquitoes. Each concentration was replicated three times and three controls treated with 1 mL of acetone. All tests were repeated three times on different occasions. An appropriate amount of larval food (10 mg/larva) was added to each cup after the larvae were introduced in the water containing the crude extract in the test and control cups as well. All tests were conducted in a temperature controlled laboratory with (27±2) ℃ and relative humidity of (80±5)%. All the cups were covered with netting to prevent adults from escaping. Mortality at larval, pupal and adult stages was recorded. Moribund larvae, 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.

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

To evaluate the biological and morphological impacts of sublethal dosage (EI_{50}) of the *C. aromaticus* cultured cells on *Ae. aegypti* and *Ae. albopictus* mosquito species, the crude extract of cultured P4 cell line, which was the most effective against the study mosquito species, was selected. Initially a total of 300-500 late third instar larvae of *Ae. aegypti* and *Ae. albopictus* were treated with the crude extract of *C. aromaticus* (cultured P4 cells) at the concentration of EI_{50} doses (61.22 and 48.68 mg/L 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 with (27±2) ℃ and relative humidity of (80±5)%. Adults were allowed to emerge, and emerged adults were fed by giving access to cotton wick soaked with 10% sucrose, and kept for further experiments.

2.5.1. Fecundity and fertility assays

To study the effect of sublethal dose (EI_{50}) of the cultured cell extract on the fecundity of the two mosquito species, a cohort of 4-5 d old (mated) females from each control and treated of both mosquito species were separately placed in foldable experimental cages (four cages). A white mouse restrained within a piece of wire-netting was placed in each cage during the day as a source for blood meal. The number of blood-engorged females of the mosquito species was recorded a day after. Twenty blood fed mosquitoes of each the *Aedes* species (treated and control) 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 the 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 7 d.

To investigate the effect of sublethal dose of the crude extract on the hatching performance mosquito species, the filter papers containing eggs collected from the fecundity tests were used. The filter papers were kept wet for two more days and then allowed to be dried. About 10 d after egg laying, two to three filter papers (from each *Aedes* mosquitoes and control as well) were separately submerged in a culture tray containing one liter of seasoned water and held at standard rearing conditions for 48 h 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 8th day for the female mosquitoes in the experimental cages, and the data mentioned earlier were recorded for the second gonotrophic cycle.

2.5.2. Growth period and sex ratio assays

The effects of sublethal dose of the crude extract of *C. aromaticus* cultured cells on developmental period (time required to pupal formation and adult emergence) and also sex ratio of *Aedes* mosquito species were evaluated. A total of 300 late 3rd instar larvae of each *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 (61.22 and 48.68 mg/L respectively) and 200 larvae were treated with only 1 mL of acetone as the control (10 larvae per cup). More larvae were used for treatment with the crude extract at EI_{50} dose because half of the larvae treated with this dose would be died. 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 h 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 (cultured cell extract and control) were transferred to culture trays (50 larvae per tray) containing one 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 h and the emerged pupae were transferred into plastic cups containing 100 mL seasoned water (10 pupae per cup) and covered with a piece of mesh cloth. Adult emergence (male and female) was observed and recorded every 12 h.

2.5.3. Adult size

The length of the adult wing (an indicator of body size) was used to study the sublethal effect of the crude extract of *C. aromaticus* cultured cells on the adult size of the *Aedes* mosquito species[37]. After adult emergence, 30 males and 30 females of each treated mosquito species (cultured cell extract and control) were randomly aspirated out of the emergence cage and maintained in separate cages and fed only on seasoned water for 24 h. Then 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×.

2.5.4. Adult mosquito longevity

To determine the impact of a sublethal dose of the crude extract of *C. aromaticus* cultured cells on the longevity of the adult mosquito species, 40 males and 40 females of the emerged adults of each treated mosquito species at 3rd instar larva (cultured cell extract and control) were maintained in foldable cages separately (20 males and 20 females 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 plant material.

2.5.5. Statistical analysis

The comparison of the mean JH III content in the crude extracts of *C. aromaticus* cultured cells was made by one way ANOVA and Duncan test.

Abbott formula was used and the survival percentages were corrected if necessary. The emergence inhibition (EI) values obtained at each concentration were calculated using WHO guideline[36]. 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 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.

3. Results

In the present study according to the HPLC analysis, the production of JH III from the five cultured cell lines was significantly different ($P \leq 0.05$). Among the cultured cell lines, P4 cell line produced significantly the highest JH III content [(174.88±12.30) μg/g dried weight], followed by Z1, Pa, Z6 and Ml cell lines by (127.40±6.00), (86.88±5.85), (77.21±4.46) and (75.98±5.13) μg/g dried weight respectively (Figure 1).

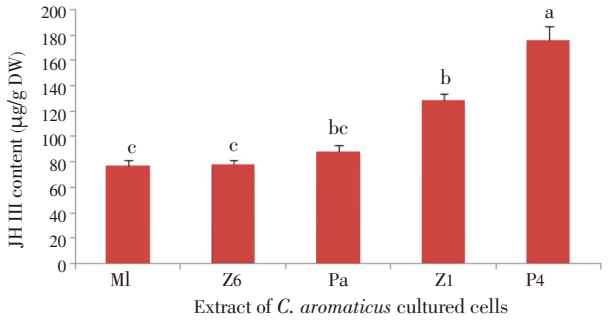

Figure 1. JH III content (Mean±SE) in the crude extracts of *C. aromaticus* cultured cell lines after seven subcultures. n=3, Mean values with different letters are significantly different at $P \leq 0.05$ (Duncan test).

Table 1

The adult emergence inhibition and slope values of the crude extracts of *C. aromaticus* cultured cells on 3rd instar larvae of *Aedes* mosquito species.

Crude extract of cultured cells	EI_{50} (mg/L) (95% *CI*)		EI_{90} (mg/L) (95% *CI*)		Slope±SE	
	Ae. aegypti	*Ae. albopictus*	*Ae. aegypti*	*Ae. albopictus*	*Ae. aegypti*	*Ae. albopictus*
P4	61.22 (45.32-76.94)	48.68 (39.73-57.19)	171.52 (123.10-349.54)	147.54 (117.77-209.61)	2.82±0.21	2.66±0.20
Z1	65.02 (44.54-90.48)	53.01 (39.78-65.88)	184.35 (121.67-590.86)	154.65 (114.60-274.75)	2.85±0.21	2.75±0.20
Ml	75.90 (59.23-90.46)	72.38 (60.36-83.22)	241.32 (187.95-397.97)	167.99 (137.15-239.86)	2.55±0.22	3.50±0.28

EI: Emergence inhibition, *CI*: Confidence interval, SE: Standard error.

Table 2

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* cultured cells and the F1 generation.

Mosquito Species	G	GC	Number of egg		ER (%)	Egg hatch (%)	
			Control	Treated		Control	Treated
Ae. aegypti	P	1st	118.40±3.30	110.29±2.90	6.9	96.36±0.70	72.33±10.70*
		2nd	117.80±3.60	115.45±3.70	2.0	95.20±0.40	81.87±7.40
	F1	1st	125.90±2.80	122.71±3.00	2.6	96.66±0.30	92.99±1.00
		2nd	123.30±2.60	123.07±4.80	0.2	97.52±0.00	95.94±0.90
Ae. albopictus	P	1st	114.10±4.00	93.52±4.10*	18.1	97.54±0.60	68.72±5.80*
		2nd	96.60±3.60	91.21±3.20	5.6	94.82±2.40	75.60±4.50*
	F1	1st	112.60±3.80	106.61±3.60	5.4	93.38±1.60	81.05±8.40
		2nd	114.70±4.20	122.09±4 90	0	91.04±2.30	91.50±2.00

*Mean values within a row are significantly different at $P\leqslant 0.05$.

G: Generation, GC: Gonotrophic cycle, P: Parental, ER: Effective reduction of producing eggs.

The adult emergence inhibition activity of the crude extracts of *C. aromaticus* cultured cells was evaluated against *Ae. aegypti* and *Ae. albopictus*. Mosquito larvae of the both *Aedes* species displayed different susceptibilities to the crude extracts. *Ae. albopictus* was significantly more susceptible to the crude extracts with the lower EI_{50} values. The EI_{50} values were 48.68, 53.01 and 72.38 mg/L for P4, Z1 and Ml cultured cell lines respectively, whereas these values for *Ae. aegypti* were 61.22, 65.02 and 75.90 mg/L respectively (Table 1).

Although the crude extract of *C. aromaticus* cultured cells reduced the mean number of eggs oviposited by treated female of *Ae. aegypti* mosquitoes, either parental or F1 generation during the first and second gonotrophic cycles relative to control, but the differences were not significant at $P\geqslant 0.05$ (Table 2). The percentages of the reduction effect were calculated[38]. The data indicated that these values were 6.9% and 2.0% for parental and 2.6% and 0.2% for F1 generation respectively. The mean number of eggs produced by the parental generation of *Ae. albopictus* over the first gonotrophic cycle was significantly decreased by 18.1% at $P\leqslant 0.05$ (Table 2), whereas the differences between the mean number of eggs produced by the parental generation (over the second gonotrophic cycle) and F1 generation of this mosquito (over the first two gonotrophic cycles) relative to controls were not significant at $P\geqslant 0.05$. The percentage of reduction effect of the crude extract of *C. aromaticus* cultured cells for parental generation was 5.6% during the second gonotrophic cycle and for the F1 generation were 5.4% and 0% during the first and second gonotrophic cycles respectively (Table 2).

There was a significant reduction in the hatching percentage of eggs produced by the parental generation of *Ae. aegypti* over the first gonotrophic cycle (24.03%) and *Ae. albopictus* over the first and second gonotrophic cycles (28.82% and 19.22% respectively) compared to the controls at $P\leqslant 0.05$ (Table 2). There were no significant effect of the sublethal dosage of the crude extract of *C. aromaticus* cultured cells on the hatching percentage of eggs produced by the F1 generation of both *Aedes* during the first two gonotrophic cycles at $P\geqslant 0.05$ (Table 2).

The sterility indices of the parental generation of mosquitoes were calculated using the obtained fecundity and fertility data[39]. The sublethal dose of the crude extract of the cultured cells induced the higher sterility index in *Ae. albopictus* during the first and second gonotrophic cycles (42.72% and 24.50% respectively) and the lower in *Ae. aegypti* (30.36% and 15.78% respectively) (Figure 2).

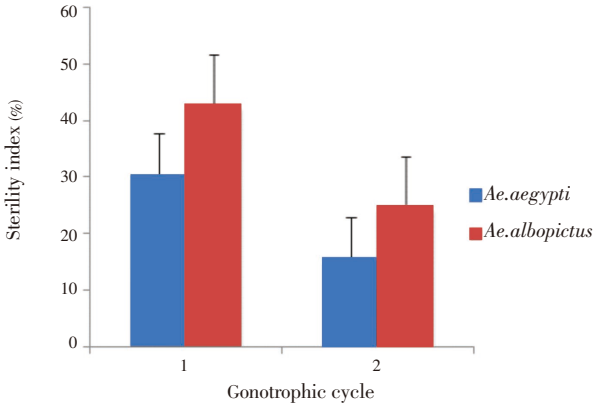

Figure 2. Sterility index (%) (mean±SE) of the mosquitoes obtained from the larvae treated with the EI_{50} of the extract of *C. aromaticus* cultured cells.

Table 3

Pupal formation and emergence time (mean±SE) for the parental of the *Aedes* mosquito species (treated with the crude extract of *C. aromaticus* cultured cells) and the F1 generation.

Mosquito Species	G	Pupation period (Day)		Emergence period (Day)			
		Control	Treated	Female		Male	
				Control	Treated	Control	Treated
Ae. aegypti	P[1]	2.49±0.01	3.99±0.04*	4.56±0.02	6.31±0.07*	4.03±0.01	5.56±0.08*
	F1	7.03±0.01	7.09±0.01	9.72±0.03	9.68±0.03	9.42±0.02	9.45±0.03
Ae. albopictus	P[1]	2.57±0.01	3.27±0.03*	5.02±0.02	6.07±0.04*	4.48±0.02	5.19±0.03*
	F1	7.45±0.02	7.52±0.03	10.91±0.03	10.95±0.03	10.30±0.03	10.39±0.04

*Mean values within a column are significantly different at $P \leq 0.05$.

G: Generation, P: parental generation, F1: F1 generation, 1: Days from day of treatment.

Larvae of two *Aedes* mosquito species exposed to the sublethal dose of the crude extract of *C. aromaticus* cultured cells since late 3rd instar showed a significant delay in pupal formation when compared with the controls ($P \leq 0.05$). The crude extract induced a significantly affect on pupal formation in the *Ae. aegypti* and *Ae. albopictus* with a delay of 1.50 and 0.70 d respectively (Table 3). The effects of the crude extract of *C. aromaticus* cultured cells on the pupal formation time for F1 generation of both *Aedes* mosquitoes were not significant at $P \geq 0.05$ (Table 3).

Adult emergence from the third instar larvae of the two *Aedes* mosquito species exposed to the sublethal dose of the crude extract of *C. aromaticus* cultured cells (parental generation) was significantly longer than the controls at $P \leq 0.05$. The adult emergence of *Ae. aegypti* and *Ae. albopictus* were delayed by 1.75 and 1.05 d in the females and 1.53 and 0.71 d in the males respectively (Table 3). The effect of the crude extract of *C. aromaticus* cultured cells on the adult emergence time for F1 generation of either females or males of both *Aedes* mosquitoes were not significant at $P \geq 0.05$ (Table 3). Analysis of obtained data indicated that the sex ratio of both *Aedes* mosquitoes either parental or F1 generation was not significantly affected at $P \geq 0.05$ by exposure of the 3rd instar larvae of each mosquito to the EI_{50} dose of the crude extract of *C. aromaticus* cultured cells (Table 4).

Table 4

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

Group	Sex Ratio (Male : Female)			
	Ae. aegypti		*Ae. albopictus*	
	P	F1	P	F1
Control	1.18±0.12	1.12±0.01	1.15±0.16	1.03±0.07
Treated	1.33±0.10	1.13±0.06	1.05±0.37	1.04±0.12

P: Parental generation, F1: F1 generation

Exposure of the 3rd instar larvae of the *Aedes* mosquitoes to sublethal dose of the extract of *C. aromaticus* cultured cells significantly reduced the wing length of emerged adults (males and females) of *Ae. aegypti* as well as females of *Ae. albopictus* compared to the untreated controls at $P \leq 0.05$. The reduction values were 0.09 and 0.06 mm for females and males of *Ae. aegypti* respectively and 0.06 mm for females of *Ae. albopictus* (Table 5). However, the reduction effects of the crude extract on wing length of males of the parental generation of *Ae. albopictus* as well as wing length of the adults (males and females) of both *Aedes* mosquitoes which emerged from non exposed larvae (F1 generation) were not significant compared to the controls at $P \geq 0.05$ (Table 5). The mean longevity of females of the both *Aedes* mosquitoes which emerged from the 3rd instar larvae exposed to the crude extract of *C. aromaticus* cultured cells (parental) as well as F1 generation was significantly shorter

Table 5

Adult wing length and adult longevity (mean±SE) for the parental mosquitoes treated with the crude extract of *C. aromaticus* cultured cells and the F1 generation of the *Aedes* mosquito species.

Mosquito species	Generation	Gender	Wing length (mm)		Time of adult survival (Day)	
			Control	Treated	Control	Treated
Ae. aegypti	Parental	Female	2.64±0.01	2.55±0.01*	49.82±1.45	44.00±1.28*
		Male	2.03±0.01	1.97±0.00*	24.57±0.87	22.24±0.82
	F1	Female	2.98±0.01	2.95±0.01	54.24±1.83	48.80±1.45*
		Male	2.20±0.01	2.22±0.01	21.40±0.99	19.20±0.69
Ae. albopictus	Parental	Female	2.50±0.01	2.44±0.01*	44.85±1.74	39.44±1.51*
		Male	2.10±0.00	2.06±0.00	25.43±1.07	23.78±0.95
	F1	Female	2.61±0.01	2.60±0.01	43.18±1.30	38.97±1.44*
		MMale	2.16±0.00	2.17±0.00	17.31±0.70	16.55±0.60

*: Mean values within a row are significantly different at $P \leq 0.05$.

relative to controls at $P \leqslant 0.05$. The mean longevity of females of *Ae. aegypti* during two generations was significantly more affected by reduction of 5.82 and 5.44 d respectively, whereas these values for *Ae. albopictus* was 5.41 and 4.21 d respectively (Table 5). Although the cultured cell extract reduced the longevity of the males of both *Aedes* mosquitoes either parental or F1 generation, but the differences were not significant at $P \geqslant 0.05$ (Table 5).

4. Discussion

The crude extracts of *C. aromaticus* cultured cells contained JH III. The JH III isolated from *C. aromaticus* collected from the field (mature mother plant) and that of *in vitro* plantlets have the same 10 R configuration as insect JH III[34]. Similarly JH III was found in the mother plant and cultured cell extracts of *Cyperus iria*[24,33].

The low EI_{50} values of the crude extracts of *C. aromaticus* cultured cells on the both *Aedes* mosquito species reported in the present study indicated their remarkable potential in reducing the population of vector mosquito species. A few studies have been found in the literature applying the extracts from *in vitro* cultures (tissue and cultured cells) of some other plants against several insect pests including mosquitoes. The bioefficacy of callus extract of *Indigofera tinctoria* containing rotenoid against the larvae of *Anopheles stephensi* and adults of pulse beetle (*Callosobruchus chinensis*) was reported[40]. They noted the higher effects of callus extract as compared to plant part extract against both the tested insects. In the other study, the hexane extract of hairy root callus culture of *Tagetes patula* L. which contains a high quantity of thiophenes showed larvicidal effect against *Culex quinquefasciatus* mosquitoes. The extract showed 50% mortality at 0.06 mg/L as compared to standard thiophenes, which showed 55% mortality at the same concentration[41]. Similarly, application of pyrethrins extracted from the callus tissue of *Tagetes erecta* plant (5 mg/mL) against *Tribolium* spp. showed immediate 'knock down' effect on the tested insects[42]. Using 50 mg/L of the root-derived callus of the *Balanites aegyptiaca* which contained a high quantity of saponin that induced 18% adult emergence inhibition of *Ae. aegypti* population relative to the control. The extracted saponin also showed dose-dependent larvicidal effect on *Aedes* mosquito larvae[43].

Results of this study indicated that a sublethal dosage of the crude extracts of *C. aromaticus* cultured cells can exert effect on the biological and morphological aspects of the treated mosquitoes and even on their F1 generation. The finding of the current study showed that phytochemicals with insect growth regulator activity 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[44,45]. Variability among the anti fertility effect of active compounds of various plant extracts could cause different effects on the reproduction potential[39]. The extract of *C. aromaticus* cultured cells might produce 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 juvenile hormone active IGRs in the female body or after the egg deposition[46]. Prolongation of 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[47].

This might be the first study that shows the possibility of using cell culture of *C. aromaticus* against vector mosquito species. 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. In this circumstance, *in vitro* culture system of *C. aromaticus* as an alternative approach for producing JH III as an IGR product would be advantageous. Further investigations are needed to evaluate the utility of extract of *C. aromaticus* cultured cells 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. Therefore, further scaling up and optimization study of large scale production of *C. aromaticus* cell culture containing high level of JH III is suggested. Further to that, a readily available plant culture may lead to a future development of botanical JH that could be integrated into integrated vector management programmes for the control of vector mosquito species in regions plagued by vector borne diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The crude extract of many plants have been already studied for their insecticidal and repellent properties against insect pests. Since the middle ages, plant extracts have been widely employed for bactericidal, virucidal, fungicidal, parasiticide, insecticidal, medicinal, and cosmetic applications. The Asian tiger mosquito, *Ae. albopictus* and *Ae. aegypti* (Diptera: Culicidae) are currently considered the most invasive mosquitoes species in the world.

Research frontiers

In this study the cell suspension cultures of *C. aromaticus* were activated from five callus lines (P4, Pa, Z1, Z6 and Ml) derived from the root explants of *in vitro* plantlets and then was carried out against late third instar larvae of the VCRU strains of *Ae. aegypti* and *Ae. albopictus* using the standard WHO method.

Related reports

The results of this study with other studies that have used extracts from other plants are consistent. For example, the results of the studied Giovanni Benelli showed that the plants of Apiaceae can have a lethal effect on insects.

Innovations and breakthroughs

The new findings in this study was the effect of the JH III in *C. aromaticus* on insects. Although other studies about the JH III have been conducted, but in this study the JH III had a high inhibitory properties.

Applications

It is important that we have a lot of knowledge about insect control and use safe methods to control pests and insects. In the present study identification of very high levels of JH III in *C. aromaticus* and the observation of biological effects on insects induced an attractive attention to this plant in the native habitat. Clearly *C. aromaticus* represents a valuable source of (10R)-JH III and can be used for the production of bioinsecticides.

Peer review

Given the importance of insects in disease transmission to humans, this study has been well performed. Since the product showed potent inhibitory effect, it could be used as a good candidate for substitution of chemical insecticide.

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