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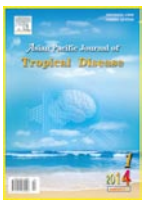

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Clerodendron inerme Gaertn. plant as an effective natural product against dengue and filarial vector mosquitoes

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PEER REVIEW

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Comments

Mosquito borne diseases have gained considerable attention due to frequent outbreaks of diseases around the world. Alternative and eco-friendly control methods are most salutation approaches in the integrated approach for mosquito control. The present investigation on *C. inerme* extracts has significant results and could be a valuable resource for utilization of plant based on natural insecticides and need to be analysis under field conditions.

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ABSTRACT

Objective: To investigate insecticidal properties of organic solvent extracts of *Clerodendron inerme* (*C. inerme*) leaves against larval stages of *Aedes aegypti* (*Ae. aegypti*) and *Culex quinquefasciatus* (*Cx. quinquefasciatus*) mosquito species.

Methods: The sundried leaf powder of *C. inerme* was subjected for extraction using organic solvents *viz.* methanol, chloroform, petroleum ether and hexane extract, and were tested against third/fourth instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus* species in accordance with WHO standard methods. Experiments were conducted in four replicates with control group containing water alone and positive control group containing respective solvent (dimethylsulfoxide/acetone) used for dissolving the extracts.

Results: Among the four solvent extracts, hexane extract has effective growth disruptive activity against *Ae. aegypti*, and showed positive tests for presence of four groups of phytochemical constituents *viz.* tanin, phytosteriod, terpenoid and cardiac glycoside. The hexane extract was tested against field collected filarial vector *Cx. quinquefasciatus* larvae for growth disruptive activity. Adult emergence inhibition values for 50 percent suppression (EI_{50}) of the tested population for methanol, chloroform, petroleum ether and hexane extracts treated against third instar larvae of *Ae. aegypti* were found to be 37.45, 14.79, 2.56 and 1.96 mg/L respectively, while hexane extract treated against *Cx. quinquefasciatus* was found to be equally effective with EI_{50} value of 3.74 mg/L. Hexane extract treated against fourth instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus* showed EI_{50} values of 8.07 and 19.55 mg/L respectively in comparison with that of standard insect growth regulator methoprene demonstrating EI_{50} value of 0.05 mg/L. Besides, the hexane extract was also found to possess toxic effect against non-target organism *Gambusia affinis* (a bio-control agent), however, the lethal concentration (LC_{50}=172.7 mg/L for 24 h) against *Gambusia affinis* was much higher in comparison with that of tested concentrations against *Ae. aegypti* and *Cx. quinquefasciatus*.

Conclusions: We conclude from the present investigation that the hexane extract of *C. inerme* has positive implications for its consideration in integrated control of dengue/filarial vectors due to their ecological distinctness in comparison with non-target organism and further due to ecofriendly nature of the extract in terms of easy biodegradability.

KEYWORDS

Clerodendron inerme, *Aedes aegypti*, *Culex quinquefasciatus*, Phytochemicals, Methoprene, *Gambusia affinis*

1. Introduction

Plant based natural products have been of tremendous importance due to their ecofriendly nature, easily biodegradability, cost effectiveness and their benefits to the mankind without any harmful effects, and is the only

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immediate and easily available rich natural resource (*i.e.* secondary metabolites) around our surrounding environment. Efforts to explore these plants for their prospective in various aspects have led to the invention of their broad spectrum applicability in various fields especially in health care (medicine) and agriculture insect pest control/vector control (Biopesticides). These plant derived products have been well known for their insecticidal properties (repellants, antifeedants, phagostimulants and toxins) to combat against various pest insects including the vector mosquitoes[1-3], which are well known for their potentiality in transmitting deadly diseases like malaria, dengue, chikungunya, elephantiasis *etc*. causing heavy economic burden to the nations and human grievances[4-5]. Whilst several plants have been broadly investigated and reported for possessing insecticidal properties including the well known commonly available tree *Azadirachta indica* (neem tree), which has been utilized effectively to certain extent in pest insect control[6]. However, development of modern technology led to understanding and over exploitation of these plant derivatives to gain maximum benefit to mankind ultimately discovering modern synthetic insecticides, which eventually led to serious repercussions like insecticidal resistance, toxicity, bioaccumulation, biomagnification *etc*[7]. These repercussions during the years have compelled to setback and relook for alternatives *i.e.* plant based products, for the betterment of our environment. Nature has provided a diverse range of plants as rich resource in our immediate environment which can be utilized effectively without any hazardous effect, but need to be explored in effective way for their implementation against pest insects/vector insects. In the present study, a widely available hedge plant *Clerodendron inerme* (*C. inerme*) was investigated for bioefficacy against vector mosquitoes *Aedes aegypti* (*Ae. aegypti*) and *Culex quinquefasciatus* (*Cx. quinquefasciatus*). This *C. inerme* plant has been studied for its insecticidal properties against *Spodoptera litura*, *Achaea janata* and *Ae. aegypti* mosquito[8-10]. Our earlier report on leaf powder of *C. inerme* has shown effectiveness on the development stages of the *Ae. aegypti*[11]. The present study was carried out with following objectives: a) efficacy of the *C. inerme* leaf extracts against fresh water breeding mosquito *Ae. aegypti* and *Cx. quinquefasciatus*, which usually breeds in polluted water, b) toxic effect against non-target organism *Gambussia affinis* (*G. affinis*) fish (a bio-control agent), c) qualitative analysis of phytochemical constituents of the extracts, d) separation of the active fraction by thin layer chromatography (TLC) method.

2. Materials and methods

2.1. C. inerme Gaertn. (Family: Verbenaceae)

C. inerme is commonly called as seaside clerodendrum, embrert, Indian privet, Glorybower, Kashmir bouquet *etc*., and is valued in landscaping as a groundcover or hedge plant. It has attractive evergreen foliage with fragrant white flowers that form in clusters and are accented by delicate red protruding stamens and is evergreen sprawling shrub 1.0-1.8 m tall (Figure 1). Medicinal properties: *C. inerme* plant is traditionally known for its use as an abortifacient and to treat constipation, oedema, bacterial infections, cancer and diabetes. The plant has been reported for its use in treating rheumatism, common fever, skin diseases, suppuration, elephantiasis, asthma, topical burns, venereal diseases and has been known for increasing appetite as well as substitute for quinine in the treatment of malarial fever[12].

Figure 1. *C. inerme* Gaertn.

2.2. Plant extraction

Fresh leaves of *C. inerme* collected from campus of Karnatak University, Dharwad, Karnataka State, India were dried under sunlight for 6 d before pulverization to fine powder using grinder for subsequent extraction. Soxhlet extraction was carried out using 50 g of finely ground leaf powder for each organic solvent *viz.* methanol, chloroform, petroleum ether and hexane, for 36 h. The extracts obtained were subjected to vacuum flash evaporator for solvent evaporation and subsequent preparation of 10% stock solution in acetone except for methanol extract which was dissolved in dimethylsulfoxide (DMSO) as the residue was insoluble in acetone and stored in refrigerator for further evaluation.

2.3. Ae. aegypti L. culture

Experimental cohorts for *Ae. aegypti* larvae were used from the cyclic colony maintained under laboratory conditions at (28±2) °C temperature, relative humidity 70% to 75% and photo period of 14:10 (light:dark). Paper strips holding eggs were submerged in tap water in enamel trays (16×21 cm) overnight for hatching and the larvae were provided *ad libitum* with diet containing mixture of finely ground dog's biscuit and yeast (2:1). Water was renewed every alternate day to avoid

formation of scum on the water surface due to presence of yeast in the diet. Pupae formed were separated in paper boats and were transferred to adult rearing cage (45 cm^3) with 2:1 ratio (female:male). Emerged adults are provided with 5 percent honey in distilled water soaked in cotton in a petridish. In addition to honey feeding, female adults were also allowed to blood feed on albino rat (Wester strain) twice a week for development of eggs. Oviposition cups containing cotton soaked in distilled water and covered with Whatman filter paper were placed inside for egg laying. Eggs laid were stored under laboratory conditions for further use in experimental setup/maintaining colony when required.

2.4. Cx. quinquefasciatus Say

Cx. quinquefasciatus larvae were collected around the Karnatak University campus and were grown till third/fourth instar stage in the water medium collected from the source and used for further bioassay.

2.5. Experimental setup

Experiments were carried out under laboratory conditions against freshly moulted third instar/fourth instar *Ae. aegypti*/*Cx. quinquefasciatus* larvae as per the guidelines of WHO[13]. Initially experiments were carried out at different concentrations for both extracts to determine the final concentrations of the extracts for graded-mortalities against the third instar larvae. All the test concentrations were prepared in 100 mL tap water in polythene cups of 250 mL capacity. Twenty five freshly moulted third instar larvae were introduced into each test concentrations with four replicates along with control group containing water alone and positive control group containing respective solvent (DMSO/acetone) used for dissolving the extracts. Food was provided *ad libatum* and observations were recorded at 24 h interval until the emergence of adults if any.

2.6. Toxicity study against G. affinis

G. affinis fish were collected from permanent water bodies around region of Dharwad, Karnataka state, India and were used for experiments. Hexane extract of *C. inerme* was evaluated for toxicity study against *G. affinis*. Experiments were conducted in triplicates at concentrations between 50 to 275 mg/L with an increment of 25 mg/L in 500 mL of fresh water along with a control group by introducing 10 fish in each test concentrations for 48 h.

2.7. Phytochemical screening

The phytochemical screening of extracts for presence of alkaloids, tannins, saponins, steroids, terpenoids, flavanoids and cardiac glycosides were carried out by standard methods reported earlier[14-19].

2.7.1. Alkaloid

The alcoholic extract was evaporated to dryness and the residue (around 0.1 g) was heated on a boiling water bath with 10% HCl (5 mL). After cooling, the mixture was filtered and divided into two equal portions. One portion was treated with a few drops of Mayer's reagent. Turbidity or precipitation indicated presence of alkaloids.

2.7.2. Tanin

About 0.1 g of the extract was boiled in 10 mL of distilled water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green, blue, blue-black, green or green black coloration or precipitate indicated the presence of tannins.

2.7.3. Saponin

About 0.1 g of the extract was boiled in 2 mL of distilled water and cooled. The solution was later vigorously shaken and observed for froth formation. The presence of froth was taken as an indication of the presence of saponin in the extract.

2.7.4. Flavanoid

Three methods were followed for screening of flavanoid as follows: i. About 0.1 g of the extract was mixed with 5 mL of ethanol followed by shaking and filtering. To 1 mL of the filtrate was added a few drops of 0.5 mmol/L alcoholic KOH. Yellowish suspension or precipitate indicated the presence of flavanoid. ii. Around 0.1 g of the extract was suspended in 5 mL of ethyl acetate followed by shaking vigorously and filtering. To 1 mL of the filtrate was added few drops of dilute ammonia solution. The alkaloid layer was observed for turning light or deep brown. iii. About 5 mL of dilute ammonia solution was added to a portion of the extract, followed by addition of concentrated H_2SO_4. A yellow coloration observed indicated the presence of flavanoids. The yellow colour disappears on standing.

2.7.5. Cardiac glycoside

About 0.1 g of the extract was mixed with 5 mL of chloroform followed by filtration. Concentrated sulphuric acid was carefully layered at the bottom of the test tube without disturbing the solution and observed for the formation of a sharp brown ring at the chloroform/sulphuric acid interface. This indicates the presence of cardiac glycosides.

2.7.6. Steriod (Lieberman's Burchard test)

About 1 mL of the extract was dissolved in 0.5 mL of acetic anhydride and cooled well in ice. This was mixed with 0.5

mL of chloroform and 1 mL of concentrated H_2SO_4 was then carefully added by means of a pipette. At the separating level of the two liquids, a reddish-brown ring was formed, as an indication of the presence of steroids.

2.7.7. Terpenoid (Salkowski test)

About 0.5 g of the extract was mixed in 1 mL of chloroform and concentrated H_2SO_4 (3 mL) was carefully added to form a layer. A reddish brown coloration of the interface indicated positive result for the presence of terpenoids.

2.8. TLC

2.8.1. Readymade TLC plates

Polygram® Silica G/UV_{254} Fertigfolien pre-coated plastic sheets (Macherey-Nagel MN) of 5×20 cm of 0.25 mm thick silica gel with fluorescent indicator were used initially for separation and illumination of fractions.

2.8.2. Preparation, loading and illumination of TLC plates

TLC plates of 1 mm thickness were prepared by coating silica gel-G on glass (5×20 cm) using TLC applicator. To perform TLC, the sample extract was loaded (5-10 μL) using thin capillary tube at 1.5 cm from bottom edge of the plate. Solvent system of 30 mL containing combinations of hexane, benzene, methanol and distilled water in the ratio 2:6.5:1:0.5 respectively was used. The solvent was allowed to move up the TLC upto 90% of the plate and the distance traveled by the solvent was marked and the plates were illuminated in iodine chamber. Retention factor (R_f) of fractions were calculated using the formula: R_f=Distance travelled by the fraction/Distance travelled by the solvent front. The silica gel containing the fractions was extracted in acetone to confirm the bioactivity of the fractions against *Ae. aegypti* larvae.

2.9. Methoprene (Insect growth regulator)

Altosid grade (methoprene) was used as reference standard compound for comparative analysis. Stock solution of 0.01 percent methoprene was prepared in alcohol and bioassay was carried out against fourth instar *Ae. aegypti*.

2.10. Statistical analysis and calculations

The experimental data was subjected to multiple comparison analysis among the treatments by one-way anova following Tukey's test method and probit analysis to determine emergence inhibition (EI_{50} and EI_{90}) values for adults using statistical analysis software SPSS of 10 version. Growth index (GI) was calculated following the method by Saxena *et al.*[20] [GI=Percent Survival/Average Developmental Period (ADP)]. Mortality if any in the control groups was corrected using Abbott's formula[21].

3. Results

3.1. Organic solvent extracts against third instar Ae. aegypti larvae

In the present study, third instar *Ae. aegypti* larvae in all the experimental cohorts exposed to organic solvent extracts exhibited significant mortality during fourth instar larval and pupal stages, and the total mortality for all the extracts was found to be dose-dependent (Figures 2-5 and Table 1). Emergence values (EI_{50}) for organic solvent extracts were found to be values of 37.45 mg/L, 14.79 mg/L, 2.56 mg/L, 1.96 mg/L and EI_{90} values were 58.85 mg/L, 24.79 mg/L, 4.37 mg/L, 3.39 mg/L for methanol, chloroform, petroleum ether and hexane extracts respectively (Table 2). Mortality during third instar stage observed for chloroform and petroleum ether extracts was found to be low but significant compared to control groups with a maximum of 6% mortality at 6 mg/L petroleum ether extract. Adult mortality observed among the experimental cohorts was 12% for chloroform, 8% for petroleum ether and 11% for hexane extract at 10 mg/L, 5 mg/L and 3 mg/L respectively. Fourth instar mortality was 69% mortality for methanol, 76% for chloroform, 68% for petroleum ether and 42% for hexane extracts at 70, 30, 6 and 4 mg/L respectively. Pupal mortality was maximum at 50 mg/

Table 1
Multiple comparison analysis by one-way Anova using Tukey's method of observed mortality against extract concentrations treated against third instar stage.

Stages	*Ae. aegypti*								*Cx. quinquefasciatus*	
	Methanol extract		Chloroform extract		Petroleum ether extract		Hexane extract		Hexane extract	
	F value (*df*)	*P* value	*F* value (*df*)	*P* value	*F* value (*df*)	*P* value	*F* value (*df*)	*P* value	*F* value (*df*)	*P* value
Third instar mortality	--	--	1.4	NS	1.5	NS	--	--	--	--
Fourth instar mortality	1059.9 (7)	<0.001	61.9 (7)	<0.001	61.9 (7)	<0.001	71.3 (8)	<0.001	362.2 (9)	<0.001
Pupal mortality	138.3 (7)	<0.001	13.1 (7)	<0.001	13.1 (7)	<0.001	125.0 (8)	<0.001	45.1 (9)	<0.001
Adult mortality	--	--	12.6 (7)	<0.001	12.6 (7)	<0.001	8.4 (8)	<0.001	--	--
Total mortality	902.9 (7)	<0.001	119.5 (7)	<0.001	119.5 (7)	<0.001	629.8 (8)	<0.001	479.6 (9)	<0.001

df: Degree of freedom; NS: Non-significant.

Table 2

Probit analysis of emergence inhibition (EI) of *Ae. aegypti* and *Cx. quinquefasciatus* following the treatment with *C. inerme* solvent extracts.

Species	Extracts	EI_{50} (mg/L) Fiducial limits with 95% confidence (Lower limit–Upper limit)	EI_{90} (mg/L) Fiducial limits with 95% confidence (Lower limit–Upper limit)	Intercept±SE	Regression coefficient	Pearson X^2 for goodness-of-fit test (*df*)	*P* value
Ae. aegypti	Methanol	37.45 (33.86–40.85)	58.85 (53.18–67.55)	−2.21±0.29	0.0576	11.00 (5)	NS
	Chloroform	14.79 (12.74–16.84)	24.79 (22.06–28.95)	−1.89±0.26	0.1280	3.33 (5)	NS
	Petroleum ether	2.56 (2.20–2.91)	4.37 (3.92–5.04)	−1.81±0.27	0.7060	2.77 (5)	NS
	Hexane	1.96 (1.66–2.22)	3.39 (3.04–3.95)	−1.74±0.26	0.8870	4.69 (6)	NS
Cx. quinquefasciatus	Hexane	3.74 (2.52–4.78)	7.64 (6.40–9.77)	−1.23±0.22	0.3291	7.17 (6)	NS

df: Degree of freedom; NS: Non-significant.

L for methanol, 20 mg/L for chloroform, 4 mg/L for petroleum ether and hexane extracts exhibiting 40%, 35%, 44% and 58% mortality respectively. ADP was found to be effected with an evidence of significant increase for methanol extract between 20 to 60 mg/L, chloroform extract between 5 to 30 mg/L, petroleum ether extract between 1 to 2 mg/L and hexane extract between 1.5 to 4.0 mg/L, and the growth index dropped significantly with increasing concentrations in all the experimental cohorts (Figures 2–5).

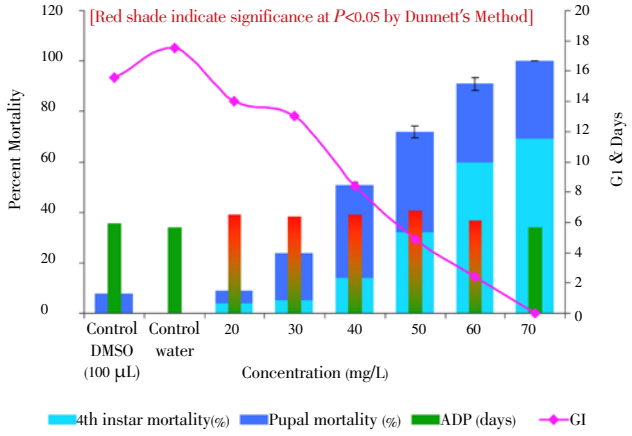

Figure 2. Percent mortality, developmental period and growth index of *Ae. aegypti* following the treatment with methanol extract against third instar.

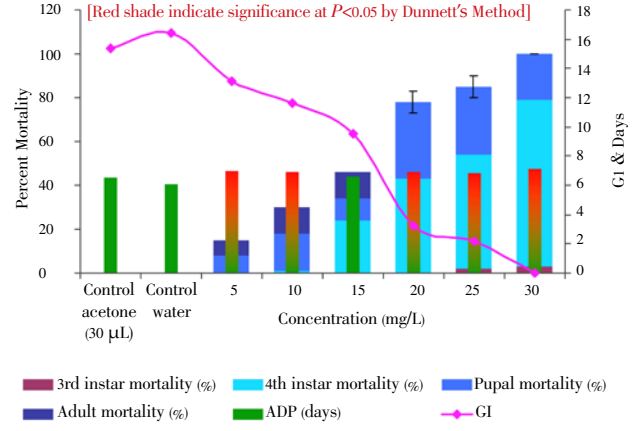

Figure 3. Percent mortality, developmental period and growth index of *Ae. aegypti* following the treatment with chloroform extract against third instar.

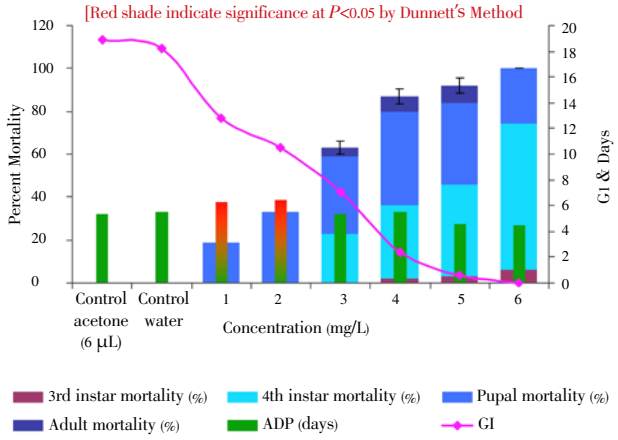

Figure 4. Regression of total mortality, developmental period and growth index of *Ae. aegypti* following the treatment with petroleum ether extract against third instar.

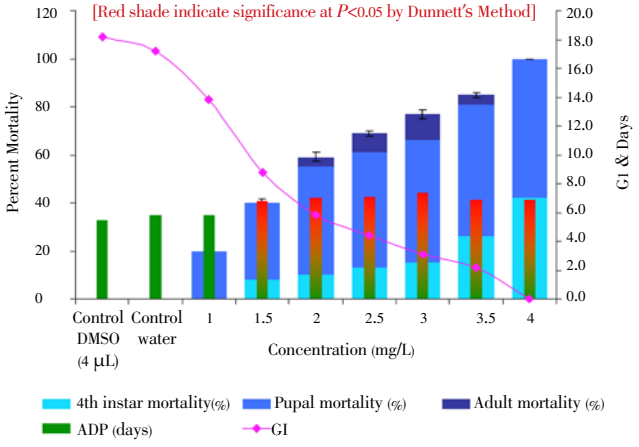

Figure 5. Percent mortality, developmental period and growth index of *Ae. aegypti* following the treatment with hexane extract against third instar.

3.2. Hexane extract against third/fourth instar Ae. aegypti and Cx. quinquefasciatus

Based on the bioactivity of the organic solvent extracts against third instar larvae of *Ae. aegypti* larvae, hexane extract was evaluated against *Cx. quinquefasciatus* (third instar and forth instar) and against fourth instar larvae of *Ae. aegypti* (Figures 6–8 and Table 3). Hexane extract treated

against third instar larvae of *Cx. quinquefasciatus* was found to be effective causing significant mortality during fourth instar and pupal stage; however, mortality during third instar and adult stage was not evident. ADP was found to be significantly effected in all the tested concentration except for 10, 12 and 16 mg/L. Growth index dropped down to 0 above 14 mg/L. The emergence inhibition values for 50% and 90% (EI_{50} and EI_{90}) suppression of *Cx. quinquefasciatus* adults were found to be 3.74 and 7.64 mg/L respectively (Table 2). Hexane extract tested against fourth instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus* showed mortality during fourth instar and pupal stages, and mortality was not evident adult stages for the both the tested insect species. Total mortality was found to be 100% above 12 and 32 mg/L for *Ae. aegypti* and *Cx. quinquefasciatus* respectively. Emergence inhibition values for 50% and 90% suppression of the population were found to be 8.07 and 10.76 mg/L for *Ae. aegypti* and 19.55 and 30.11 mg/L for *Cx. quinquefasciatus* respectively (Table 4).

Table 3

Multiple comparison analysis by one-way Anova using Tukey's method of observed mortality against extract concentrations treated against fourth instar stage.

Stages	Hexane extract			
	Ae. aegypti		*Cx. quinquefasciatus*	
	F value (*df*)	*P* value	F value (*df*)	*P* value
Fourth instar mortality	2.9 (8)	NS	20.6 (9)	<0.001
Pupal mortality	556.4 (8)	<0.001	190.8 (9)	<0.001
Adult mortality	--	--	--	--
Total mortality	499.8 (8)	<0.001	277.2(9)	<0.001

NS: Non-significant.

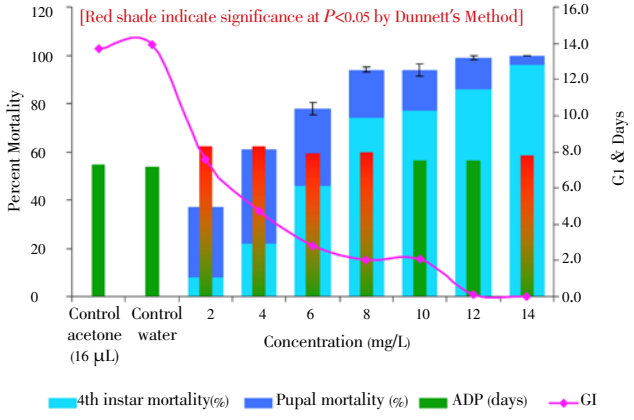

Figure 6. Percent mortality, developmental period and growth index of *Cx. quinquefasciatus* following the treatment with hexane extract against third instar.

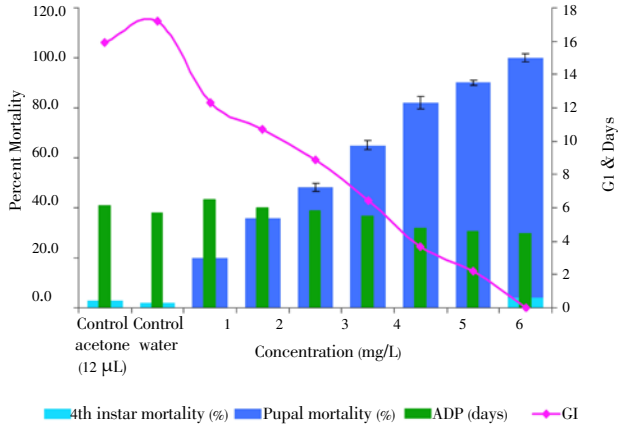

Figure 7. Dose-response relationship between hexane extract and mortality during developmental stages of *Ae. aegypti* following the treatment against fourth instar larvae.

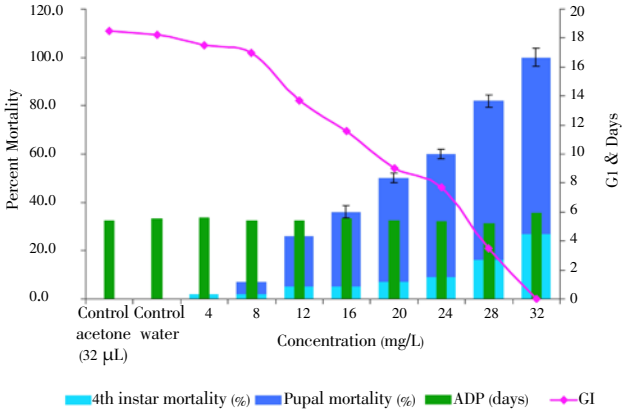

Figure 8. Dose-response relationship between hexane extract and mortality during developmental stages of *Cx. quinquefasciatus* following the treatment against fourth instar larvae.

Methoprene a standard insect growth regulator was evaluated for emergence inhibition activity against freshly moulted fourth instar larvae of *Ae. aegypti*. Methoprene was tested at concentrations between 0.02 to 0.12 mg/L and mortality was evident during pupal stage in all the tested concentrations exhibiting as low as 20 percent at 0.02 mg/L and 100 percent at 0.12 mg/L. EI_{50} and EI_{90} values were found to be 0.05 mg/L and 0.09 mg/L respectively (Table 4) and the *Chi*-square test for goodness of fit indicated good fit of the dose-response relationship.

Table 4

Probit analysis of emergence inhibition (EI) of *Ae. aegypti* and *Cx. quinquefasciatus* following the treatment with hexane extract in comparison with Methoprene [Technical Grade - Insect Growth Regulator] during fourth instar stage.

Species	EI_{50} (mg/L)	EI_{90} (mg/L)	Intercept±SE	Regression coefficient	Pearson X^2 for goodness-of-fit test (*df*)	*P* value
	Fiducial limits with 95% confidence (Lower limit-Upper limit)					
Ae. aegypti	8.07 (7.77-8.34)	10.76 (10.41-11.21)	-3.83±0.64	0.4753	1.22 (5)	NS
Cx. quinquefasciatus	19.55 (17.78-21.42)	30.11 (27.43-34.04)	-2.37±0.27	0.1213	5.02 (7)	NS
Methoprene	0.05 (0.04-0.06)	0.09 (0.08-0.10)	-1.67±0.25	33.2200	2.67 (5)	NS

df: Degree of freedom; NS: Non-significant.

3.3. Toxicity study against *G. affinis*

Lethal concentrations (LC_{50} and LC_{90}) for hexane extract against *G. affinis* were found to be 172.70 and 237.11 mg/L for 24 h and 101.66 and 165.31 mg/L for 48 h respectively (Table 5).

Table 5

Probit analysis of lethal concentration of *G. affinis* following the treatment with hexane extract.

G. affinis	LC_{50} (mg/L) Fiducial limits with 95% confidence (Lower limit-Upper limit)	LC_{90} (mg/L) Fiducial limits with 95% confidence (Lower limit-Upper limit)	Intercept±SE	Pearson X^2 for goodness-of-fit test (*df*)	*P* value
24 h	172.7 (161.96-183.92)	237.11 (222.95-260.27)	0.019±0.001	19.43 (7)	<0.01
48 h	105.66 (99.51-111.24)	165.31 (157.90-174.19)	0.021±0.001	2.95 (6)	NS

df: Degree of freedom; NS: Non-significant.

3.4. Phytochemical constituents of extracts

All the solvent extracts were subjected for analysis of phytochemical constituents *viz*. alkaloid, tanin, saponin, phytosteriod, terpenoid, flavnoid and cardiac glycoside (Table 6). Analysis for presence of tanin, phytosteriod, terpenoid and cardiac glycoside were found positive for all the extracts except for methanol extract where tanin was not evident, whereas alkaloid, saponin and flavnoid compounds were not evident in the analysis for all the extracts.

Table 6

Phytochemical constituents of organic solvent extracts of *C. inerme* leaf powder.

Solvent Extracts	Alkaloids	Tanin	Saponin	phytosteriod	Terpenoid	Flavanoid	Cardiac glycoside
Methanol	-	-	-	+	+	-	+
Chloroform	-	+	-	+	+	-	+
Petroleum ether	-	+	-	+	+	-	+
Hexane	-	+	-	+	+	-	+

+/- indicates presence/absence of the compound.

3.5. Active fraction of hexane extract

The extracts subjected for TLC separation resolved into 10 to 12 fractions including the origin fraction for chloroform, petroleum ether and hexane extract and methanol extract remained intact without any fraction resolution at the tested TLC solvent mixture. Hexane extract was found resolved into with four major fractions and initial testing of these fractions for bioactivity against third instar larvae of *Ae. aegypti* revealed bioactivity for fraction with R_f value of 0.393 (Figure 9), and other fractions were found to be least effective.

The identified active fraction of hexane extract was isolated using preparative TLC method to a required quantity for evaluation against *Ae. aegypti* larvae. Third instar larvae of *Ae. aegypti* were exposed to isolated active fraction at concentrations between 20 to 120 mg/L. Mortality was observed during third and fourth instar exhibiting 100% percent total mortality in all the tested concentrations (Figure 10). The ADP of third instar was significantly prolonged with the increasing concentrations of the active fraction to as high as 6.5 d compared to the larvae in the control groups with an average moulting period of 2 d. Those larvae moulted to fourth instar eventually died during the normal fourth instar larval developmental period.

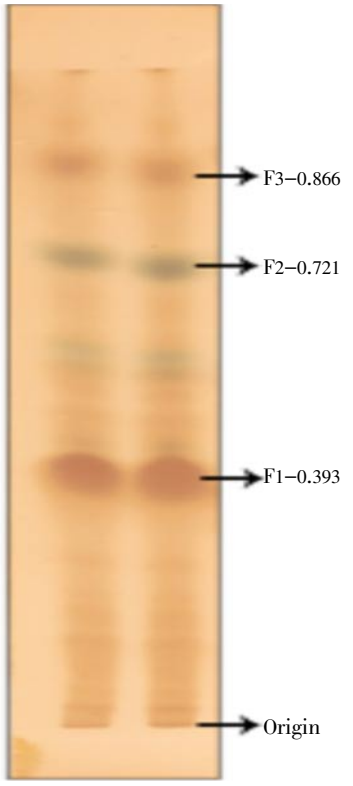

Figure 9. Fractionation of *C. inerme* hexane extract by TLC.

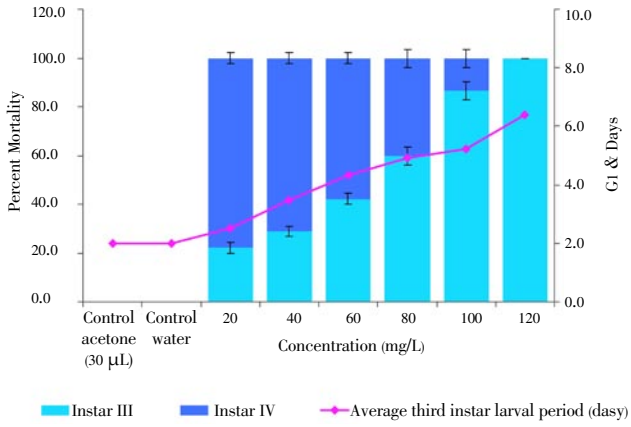

Figure 10. Bioactivity and dose-response relationship of the active fraction against *Ae. aegypti* following the treatment during third instar larvae.

3.6. Morphological observations

Dead larvae, pupae and adult stages from the treated groups were observed for morphological abnormalities. Pupae dead were found with incomplete development, incomplete sclerotization, exuvae attached at the head capsule and in some ecdysis was incomplete (Figure 11). Dead adults were found with incomplete emergence from the pupal case (Figure 11d).

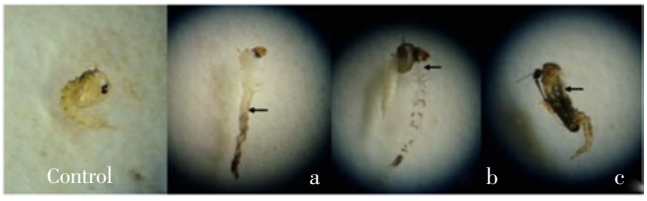

Figure 11. Effect of *C. inerme* extracts on development of *Ae. aegypti* larvae.
Arrows indicate incomplete ecdysis (a), incomplete sclerotization (b), incomplete adult emergence (c).

4. Discussion

Evidences and scientific reports on several plants possessing bioactivity suggest that plants are of worth consideration among all the control strategies involved in vector control in terms of feasibility, cost effectiveness and easy availability of plant resources[22], whilst plant based natural products are least focused in terms of its use in the integrated mosquito control program in the present situation. Inclusion and implementation of effective natural products in a systematic and appropriate way will definitely contribute to curb the vector population in the field to below disease transmitting threshold level. In the present investigation, *C. inerme* plant was studied for bioactivity against fresh water breeding mosquito species *Ae. aegypti* and *Cx. quinquefasciatus* species which breeds and survive in hostile aquatic conditions. The solvent used to extract the plant material were chosen based on their decreasing polarity index of the organic solvents, due to their varied affinity towards the active factor(s) present in the plant material *viz.* methanol (5.1)>chloroform (4.1)>petroleum ether (0.2)>hexane (0). Experimental results showed that all the solvent extracts were effective against *Ae. aegypti* larvae and the emergence inhibition activity observed for hexane extract was 19, 7 and 1 times greater than chloroform, petroleum ether, and methanol extract respectively with emergence inhibition (EI_{50} and EI_{90}) value of 1.96 mg/L and 3.39 mg/L respectively.

It is worth emphasizing here the fact that the factor (*i.e.* tap water used in the experiment), definitely has minimal interference on the active factors in the extracts, unlikely in the environment where the number of factors influencing the active factors positively/negatively are several (microbial load/chemical load/temperature/sunlight/others). Moreover, the extract tested was against laboratory reared fresh water *Ae. aegypti* mosquito, which has less genetic adaptation to hostile conditions as it breeds in fresh water in the environment compared to other mosquito species breeding in polluted water like *Cx. quinquefasciatus* which has a strong genetic makeup. In the above view, hexane extract was evaluated against field collected *Cx. quinquefasciatus* larvae under laboratory conditions using water from the source of field collection. Hexane extract against third instar *Cx. quinquefasciatus* was found to be active with an EI_{50} of 3.74 mg/L approximately double the concentration required for 50% inhibition of adult emergence of *Ae. aegypti*. Similarly hexane extract against fourth instar of *Cx. quinquefasciatus* for 50% inhibition of adult emergence was effective at concentration greater than 2 times observed against fourth instar *Ae. aegypti*.

Our basic understanding on most of the plant products against insects from earlier reports suggest that the extracts interfere in the physiological processes leading to growth inhibition / disruption of development in insects. One of the compounds well known for possessing such activity is methoprene[23], a synthetic insect growth regulator was tested against freshly moulted *Ae. aegypti* larvae to understand the efficacy and mode of action in comparison with hexane extract. Emergence inhibition activity (EI_{50}) was found at concentration 161 times lower than compared to that of hexane extract against fourth instar *Ae. aegypti*. Mortality for methoprene was maximum during pupal stage with most of the dead pupae attached with exuvae at the head capsule. Several plant extracts have been reported for possessing insecticidal activities and inducing abnormalities against mosquitoes. Shallan *et al.*[24] has reported that extract of *Callitris glaucophylla* extracts induced a wide range of sub-lethal effects on larval mortality, larval duration, pupicidal activity, pupal duration, adult emergence, sex ratio, adult mortality and malformation in *Ae. aegypti*. In another study, seaweed extract of *Enteromorpha intestinalis*, *Dictyota dichotoma* and *Acanthopora spicifera* were reported to possess enormous potentiality as a mosquito larvicide against *Ae. aegypti*[25]. Rodrigues and coworkers[26] has screened one hundred and ninty hexanic and ethanolic extracts from 27 plant species from the Cerrado biome of Brazil against third stage of *Ae. aegypti*. Out of which 14 extracts from 7 species were reported for activity with >65% mortality.

One of the important considerations in implementation of any plant extracts in vector control program is its specificity against target organism when released into the environment. Moreover, it is true that plant extracts may possess broad spectrum toxic effects against organisms including the co-existing non-target organisms. However, this should not halt from using plant extracts in the vector control program, nevertheless, it is of paramount importance to understand threshold level of plant extract concentration causing least effect against non-target organisms in the environment. Additionally plant extracts having affinity to easily biodegrade in the environment is one of the major advantages over other stable synthetic insecticides which lead to bioaccumulation and biomagnification in the ecosystem. In the view of these, hexane extract was tested against non-target organism *G. affinis*, and the extract was found to be toxic at 172.7 mg/L concentration causing 50% mortality within 24 h. Although it is not possible to calculate the SI/PSF/FSF (Suitability Index/Predator Safety Factor/Fish Safety Factor) based on the present results nonetheless the

experiment was intended to understand the level of potential concentration causing fatal effects against non-target organism.

Phytochemical analysis showed presence of compounds representing to four categories of secondary metabolites *viz.* tanin, phytosteriods, terpenoids and cardiac glycosides except for methanol extract where analysis for presence for tanin compounds was negative. Although it is not possible to deduce any conclusion on the analysis of phyto compounds present in the solvent extracts, it is worth consideration here that analysis of phytochemicals will give a broad understanding on the synergism effect of phytochemicals based on the bioassay results of the extracts against *Ae. aegypti*. It is well known that crude extracts derived from plant material contain mixture of phytochemicals producing combined effect against target pest insect, and is also the basis for reduced risk of resistance development in insects[27]. Our observations on bioactivity of the isolated active fractions of hexane extract against third instar larvae revealed the growth inhibitory activity leading to prolongation of the third instar larval period with maximum developmental period of 6.5 d at 120 mg/L compared to control groups (2 d). It is more appropriate to deduce from the present observations that the hexane extract in its crude form could prove potential candidate in the integrated mosquito control program, and further isolation/purification of the active compound(s) from the hexane may yield a potent insecticidal compound especially against dengue and filarial vectors. Further it is noted that the present study was intended to hold necessary investigations under laboratory conditions on the mosquitocidal activity, and further studies under field conditions are necessary and of paramount importance to prove its potentiality for its use against vector mosquitoes.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Mosquito species belonging to genus *Anopheles*, *Aedes* and *Culex* are the most commonly found in rural and urban area. These tiny creatures are medically important vectors and are responsible for spreading diseases like malaria, dengue, elephantiasis *etc*. Among these, *Ae. aegypti* is a fresh water breeding mosquito and is well known as a primary vector world wide for transmitting dengue disease other than chikungunya and yellow fever. Attempts to curb *Ae. aegypti* vector has been of prime focus world wide for the 21st century, as estimate by World Health Organization suggests that more than 2.5 billion people are at risk of dengue infection. Moreover developments of effective vaccines are still a long way. Most of the control strategies of these mosquito borne diseases relies on controlling the vector mosquito population, mainly dependent on application of synthetic insecticides. In the past, use of these synthetic insecticides has resulted in development of insecticide resistance indicating the need for alternative control strategies. Plant based natural products have been well known for their insecticidal properties against several insect pests including vector mosquitoes and have been considered as the safe insecticides due to their ecofriendly nature and easy biodegradability. Several plants have been investigated and reported for possessing repellent, larvicidal, growth disruptor and ovicidal properties against vector mosquito species.

Research frontiers

The present research on insecticidal properties of *C. inerme* plant against *Ae. aegypti* and *Cx. quinquefasciatus* provides significant information on the efficacy of plant extracts as well as on the importance of organic solvents in the extraction of bioactive compounds. The comparison of efficacy between the *Ae. aegypti* and *Cx. quinquefasciatus* is of major significance due to their difference in breeding habitats in natural environmental conditions. *Ae. aegypti* is a fresh water breeding species and prefers to breed in small water containers, whereas *C. quinquefaciatus* breeds in polluted water and is able to survive under hostile environmental conditions. The insecticidal properties of *C. inerme* has been explored to large extent with an attempt identify the active fractions and phytochemical constituents. The present investigation revealed significant information for further exploring the practical implications of the *C. inerme* hexane extract in the field for mosquito control.

Related reports

Reports on insecticidal properties of *C. inerme* are of inadequate. Earlier report by the same authors on sun dried leaf powder of *C. inerme* plant against fourth instar larvae of *Ae. aegypti* have shown growth disruptive properties and interference in the development leading to abnormalities. Review of literature suggests several organic solvent extracts of plant species have been reported for bioactivity against mosquito species. Present investigation on bioefficacy of organic solvent extracts against *Aedes* and *Culex* species shows the importance of organic solvents in the extraction of bioactive compounds with varied physical and chemical properties present in the plant species

Innovations & breakthroughs

C. inerme plant is a commonly available hedge plant

reported to possess medicinal properties, however insecticidal properties of the plant has been not explored to large extent against insects especially against vector mosquitoes. In the present investigation, a detailed study has been conducted for possible utilization of the plant extracts in future in the integrated mosquito control. The hexane extract of *C. inerme* leaves has been showed to be highly effective against developmental stages of *Ae. aegypti* and *Cx. quinquefasciatus* species.

Applications

C. inerme plant is a easily growing and commonly available. The present investigation has revealed promising results for use of *C. inerme* extract in the integrated approach for mosquito control in future, however yet need to be explored for efficacy under field conditions

Peer review

Mosquito borne diseases have gained considerable attention due to frequent outbreaks of diseases around the world. Alternative and eco-friendly control methods are most salutation approaches in the integrated approach for mosquito control. The present investigation on *C. inerme* extracts has significant results and could be a valuable resource for utilization of plant based on natural insecticides and need to be analysis under field conditions.

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