Original Article

EVALUATION OF AQUEOUS EXTRACTS DERIVED FROM *CLERODENDRUM INERME* (L.) GAERTN. PLANT LEAVES FOR LARVICIDAL ACTIVITY AGAINST *AEDES AEGYPTI* L.

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ABSTRACT

Background: *Aedes aegypti* mosquito is a well-known vector of dengue, chikungunya, Zika, yellow fever etc. In the present study

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aqueous leaf extracts of *Clerodendrum inerme* plant were tested for insecticidal activities against third instar larvae of *Aedes aegypti.*

Methods: Aqueous extracts of fresh leaves and leaf powder of *C. inerme* were subjected to vacuum flash rotary evaporator and the residue obtained was re-dissolved in aqueous phase to prepare 10% stock solution. Third instar larvae of *Ae. aegypti* were exposed to graded concentrations of the extracts and observations on the developmental stages were recorded till adult emergence. Bioassay against *Gambusia affinis* fish was conducted to test the toxic effect of the leaf powder extract.

Results: Adult emergence inhibition (EI_{50}) were 864.28ppm and 148.95ppm for fresh leaf and dried leaf powder extracts respectively. Observation of the larvae and pupae dead showed developmental abnormalities *viz.,* visual eye pigment reduction/loss in dead larvae and incomplete ecdysis during moulting from larval to pupal stage with exuviae attached at the head capsule. The dried leaf powder extract tested for toxicity against *Gambusia affinis* fish had no effect on its survivability even at 4000ppm concentration.

Conclusion: The aqueous extracts revealed growth disruptive properties leading to mortality during developmental stages of *Ae. aegypti.* Extract of dried leaf powder was found to be more effective compared to extraction of fresh leaves. The present study indicated that the aqueous extracts of *C. inerme* could be used as a cost-effective and eco-friendly natural product in the integrated mosquito control program especially against *Ae. aegypti*.

Keywords : *Clerodendrum inerme*; *Aedes aegypti*; dengue; Gambusia affinis

INTRODUCTION

Mosquitoes are well known for their medical importance as they transmit parasites/pathogens causing diseases such as, for example, dengue, chikungunya, Zika, malaria, yellow fever, Japanese encephalitis and West Nile fever etc. to humans around the globe especially in the tropical and subtropical regions. Dengue

is one of the most important mosquito-borne diseases transmitted by the *Aedes aegypti* mosquito, the primary vector for dengue worldwide.[1] During the last few decades incidence of dengue fever has increased many fold and is estimated to be 3.9 billion people at risk in 128 countries, of which 390 million infections and 96 million clinically manifest the disease.[2-4] As prevention is better than cure, controlling of mosquito population is the prime approach in management of mosquito borne diseases. In past various chemical insecticides were used to reduce the mosquito populations, however the relief and benefits were short lived due to development of resistance in vectors, in addition to adverse effects on the non-target organisms and environment. Therefore, an eco-friendly, target-specific, biodegradable, effective, long-term alternative vector-borne disease management is the need of the hour to control vector mosquitoes.[5-8]

Biologically active natural chemical constituents have larvicidal properties and could be one of the effective alternative techniques for mosquito population control, without the fear of resistance development in vector and adverse effects on humans and environment.[9-10] Plant derived products are considered to be promising sources mainly due to their combined/synergistic mode of action eventually restraining the possibility of resistance development in insects[6]. Use of plant/plant products have been well documented not only for their insecticidal activity against several medically important insects including mosquitoes but also as growth disruptor, anti-juvenile hormone activity and repellants. *Azadirachta indica* (neem tree) shows excellent insecticidal properties against several medically important insects and vector mosquito species.[11-13]. Modern technology may be used to explore the insecticidal properties of natural products available from plants and their implementation in vector borne disease management. As a step towards this direction, we attempted to investigate the larvicidal property of *Clerodendrum inerme* against *Ae. aegypti* mosquito. Larvicidal property of petroleum ether extracts of *C. inerme* has already been reported earlier against *Ae. aegypti*, *Culex quinquefasciatus* and *Anopheles stephensi*.[14] Volatile oil of *C. inerme* showed toxicological deteriorations effect against *Musca domestica* by inducing serious effects on the biology and biotic potential.[15] The insecticidal properties of *C. inerme* plant has been studied against several insects including *Achaea janata*, *Spodoptera litura* and mosquito species.[16-21] In the present study the aqueous extracts of *C. inerme* derived from fresh leaves and dried leaf powder were tested by exposing third instar larval stages of *Ae. aegypti* to graded concentrations of extracts.

MATERIALS AND METHODS

Clerodendrum inerme Gaertn. Plant:

Clerodendrum inerme is commonly known as glorybower, bagflower and bleeding-heart, and Kundali in Hindi, and is grown as a hedge plant. The plant has attractive evergreen foliage and has white fragrant flowers in clusters and are accented by delicate red protruding stamens. Leaves of *C. inerme* collected in and around the Karnatak University, Dharwad, Karnataka State, India were used for deriving aqueous extracts.

Fresh leaf extract:

For preparation of fresh leaf extract 500 gms of fresh leaves collected were cleaned with tap water, pulverized in a grinder and squeezed using cheesecloth and filtered through coarse filter paper. The filtrate was subjected for water evaporation under reduced vacuum pressure in a rotary flash evaporator. The concentrated semi-liquid residue was diluted with double distilled water to prepare a 10% stock solution and stored at 4°C for further experiments.

Dried Leaf powder extract:

The extract hereafter is referred to as leaf powder extract. For preparation of leaf powder extract, fresh leaves of *C. inerme* were exposed to sunlight for one week for drying and pulverized to get fine powder. Fifty grams of leaf powder prepared was soaked overnight in one liter of distilled water and the mixture was then stirred for a period of two hours on a magnetic stirrer and filtered using coarse filter paper. The filtrate obtained was subjected for solvent evaporation under reduced pressure in the rotary evaporator and the residue was diluted in double distilled water to prepare 10% stock solution and stored in the refrigerator for further experiments.

Culture of Aedes aegypti:

Cyclic colony of *Aedes aegypti* was maintained under optimum laboratory conditions (28±2 °C temperature and 70-75% RH). Eggs were hatched in tap water and the newly hatched larvae were fed with a mixture of ground dog biscuit and yeast at 2:1 ratio. The larval rearing water was renewed every alternate day to avoid scum formation on the water surface. Pupae formed were transferred to adult

rearing cages ($45cm^3$) and emerging adults were provided with 5 percent honey soaked in cotton pads. Albino rats (Western strain) were used as a source of blood meal for feeding female adults twice a week. A cotton pad soaked in distilled water and surface covered with whatman filter paper in a petri dish was provided in the rearing cages for oviposition, and water was added frequently to maintain the surface moist for egg laying.

Experiments and statistical analysis:

Experiments were conducted under laboratory conditions by exposing freshly moulted third instar *Ae. aegypti* larvae to plant derived extracts as per guidelines of World Health Organization.[22] Initially experiments were set up by exposing the test insect at various concentrations of fresh leaves and leaf powder extracts to determine the final concentrations of extracts for graded-mortalities. Concentrations between 600 to 1400ppm for fresh leaf extract and between 40 to 280ppm for leaf powder extract were finally considered for bioassay. Tap water was used to prepare test concentrations with a final volume of 100 ml placed in 250ml polythene cups. In each test concentrations and control, 25 third instar larvae were introduced and control groups with water were maintained. Experiments were performed in four replicates for each tested concentration with concurrent control groups. Food was provided *ad libitum* and observations were recorded at an interval of 24 hour till the end of the experiment or emergence of adults, if any.

Statistical analysis was carried out for multiple comparison of dose-response between the concentrations following Tukey's-b test and probit analysis to determine concentrations of emergence inhibition (EI_{50} and EI_{90}) for adults using SPSS statistical software.

Toxicity evaluation against Gambusia affinis fish (a bio-control agent):

Gambusia affinis fish for testing toxicity of leaf powder extract were collected from permanent water bodies around the region of Dharwad, Karnataka State, India. Bioassay was set up in a glass jar of 1000ml capacity by exposing five fishes to each test concentrations prepared in 250ml of tap water. Initially the fish (Size ranging 40-45 were exposed to concentrations ranging between 100 to 280ppm with an increment of 20ppm subsequently up to 500ppm and then to 4000ppm with an increment of 500ppm. All the fish were exposed to test concentrations for a period of 6 days equivalent to the number of experimental days observed for leaf powder

extract tested against *Ae. aegypti* larvae. The aqueous test concentrations and water in the control groups were renewed daily and observations were recorded at 24 hours intervals for mortality if any.

RESULTS

Experimental results on the stage-specific mortality treated with aqueous extract of fresh and dried leaves are summarized in Table 1 and Fig. 1, 2 and 3. Adult emergence inhibition values (EI_{50} and EI_{90}) for fresh leaf extract were 864.28 and 1201.83 ppm and for leaf powder extract were 148.95 and 257.59ppm respectively (Table 1). It was evident from the results that the extract obtained from leaf powder demonstrated higher insecticidal activity compared to that derived from fresh leaves. Leaf powder extract revealed 100% inhibition of adult emergence at 280ppm, while 100% inhibition with fresh leaf extract was observed at 1400ppm (Fig. 1 and 2). The larvae treated with fresh leaf extract exhibited mortality during the fourth instar stage with maximum of 80% at 1400ppm, while leaf powder extract exhibited mortality during pupal stage with 93 percent at 280ppm. Microscopic observations showed reduction / loss of visual eye pigmentation in the dead larvae and incomplete ecdysis during larval-pupal moult with exuviae attached at the head capsule in the dead pupae (Fig. 4 and 5). Mortality observed during different stages for both the extracts was during the normal developmental period of 4-5 days (i.e. third instar to pupal stage) which was comparable to the duration of the stages in the control groups.

Third instar larvae treated with the aqueous extracts successfully moulted to fourth instar and no mortality was observed. Effect of the extracts tested was evident in later stages exhibiting mortality during fourth instar larval and pupal stages. Mortality during fourth instar larvae treated with fresh leaf extract varied between 2 to 80% (Fig. 1), while in larvae treated with leaf powder extract, mortality observed during fourth instar stage was below 8% for all the test concentrations (Fig. 2). Pupal mortality observed for fresh leaf extract showed non-linear relationship against the concentrations tested exhibiting mortality as low as 10% at 800ppm and maximum 33% mortality at 1000 ppm, whereas with leaf powder extract, pupal mortality was significantly evident exhibiting 28% at 80ppm and 93% at 280ppm concentration. Total larval/pupal mortality for fresh leaf extract and leaf powder extract was found to be 1400ppm and 280ppm, respectively.

Regression analysis of the dose-response for total mortality observed was highly significant for both the extracts tested indicating the dependency of mortality on test concentrations (Table 1).

Table 1. Analysis of dose-response of fresh leaf and leaf powder extracts of *C. inerme* tested against third instar *Ae. aegypti* larvae

	EI_{50} ppm	EI_{90} ppm	Intercept ± SE	Regression coefficient	Pearson X^2 for goodness-of-fit test (*df*)	*P value*
	Fiducial limits with 95% confidence (Lower limit – Upper limit)					
Fresh Leaf extract	864.28 (806.75 – 914.63)	1201.83 (1132.74 – 1301.90)	-3.28 ± 0.43	0.00380	5.47 (8)	NS
Sundried leaf powder extract	148.95 (120.97 – 178.31)	257.59 (219.59 – 328.98)	-1.75 ± 0.23	0.01180	8.75 (6)	NS

EI – Emergence inhibition; df - Degree of freedom; NS – Non-significant; $p < 0.05$

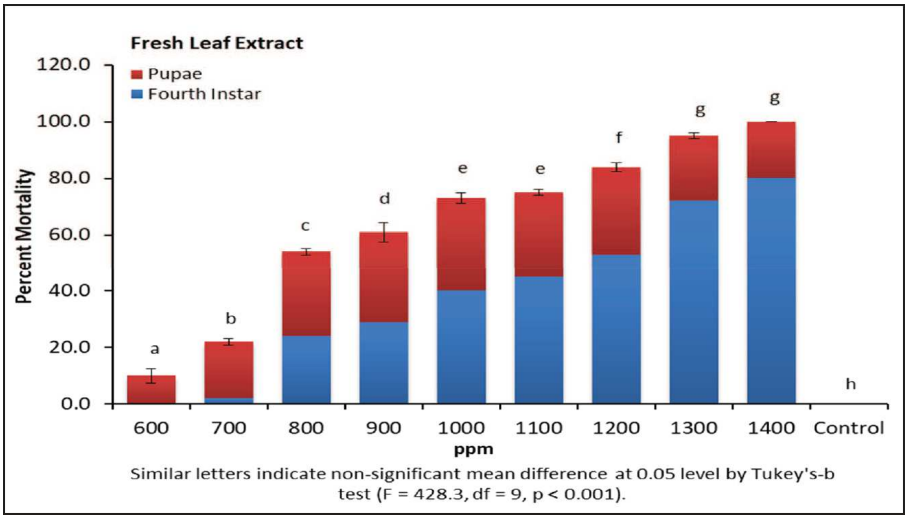

Fig. 1. Mortality during larval and pupal stages following treatment with *C. inerme* fresh leaf extract against third instar *Ae. aegypti* larvae

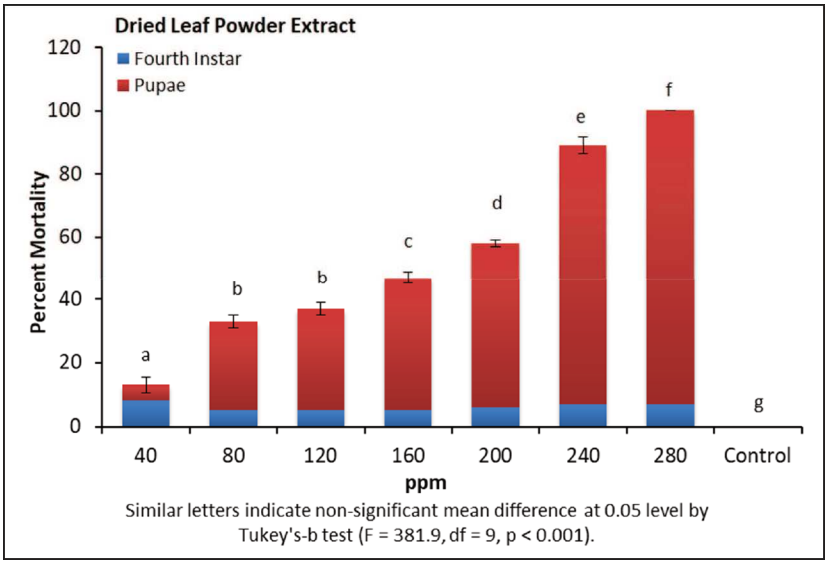

Fig. 2. Mortality during larval and pupal stages following treatment with *C. inerme* leaf powder extract against third instar *Ae. aegypti* larvae

In another set of experiments the aqueous extract of leaf powder was tested at higher concentrations ranging between 600 ppm to 2000 ppm to observe the effect during larval stages. No significant effect was observed during the third instar larval stage and they moulted normally to the fourth instar similar to the observations visible in the control groups. However, in the fourth instar stage, progressive mortality was observed at grading concentrations of leaf powder extract (Fig. 3), and the mortality was found to be significant varying between 32% at 600 ppm and 95% at 2000 ppm ($F=180.01$, $df= 8$, $P< 0.001$). The fourth instar larvae, those moulted successfully to pupae died during the normal developmental period of pupal stage and there was no emergence of adults observed in any of the tested concentrations ($F= 64.23$, $df= 8$, $P< 0.001$).

Extract of leaf powder was tested against a non-target organism *i.e., G. affinis* fish to evaluate toxicity level. Initially the fish were exposed to concentrations ranging between 100 ppm to 280 ppm with an increment of 20 ppm and thereafter to higher concentrations up to 4000 ppm. There was neither any evidence of visible effect on survivability nor mortality at the concentrations tested against the *G. affinins*.

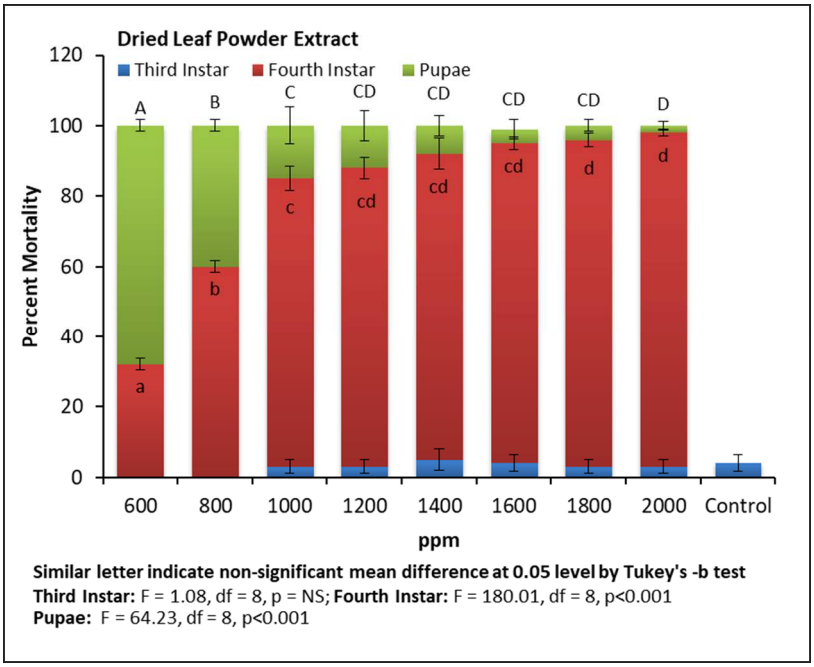

Fig. 3. Impact of *C. inerme* leaf powder extract at concentrations above 600ppm treated against third instar *Ae. aegypti* larvae

Fig. 4. Effect of *C. inerme* extracts on *Ae. aegypti* larval eye pigmentation, arrow indicates the affected eye pigment in the treated group

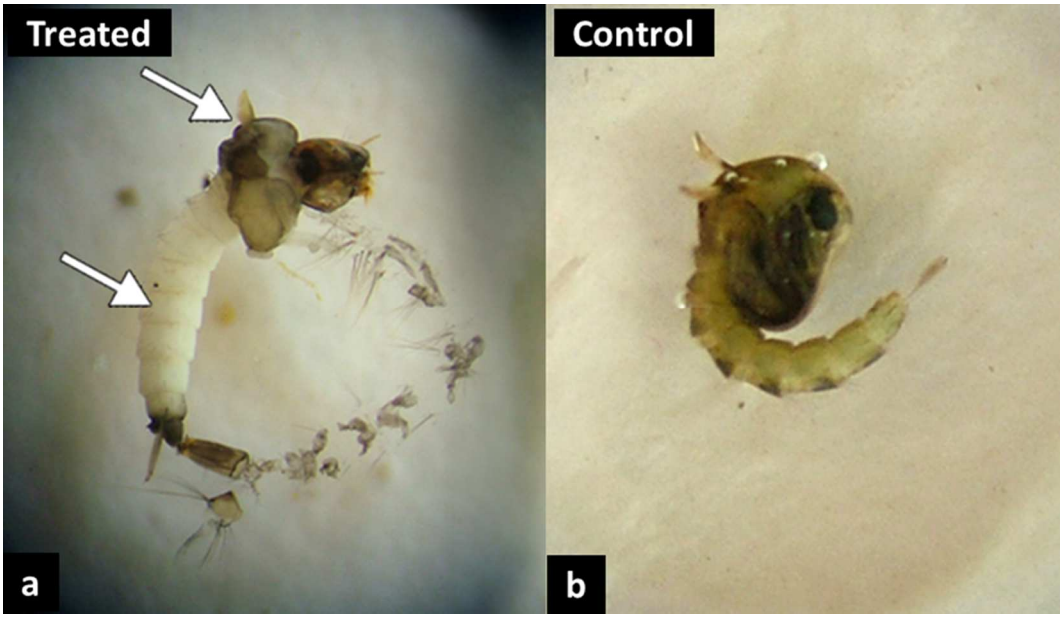

Fig. 5. Effect of *C. inerme* extracts on ecdysis during larval-pupal moulting phase, arrow indicates exuviae attached at the head capsule

DISCUSSION

Research on plant based insecticides has been on the focus since the beginning of 19th century to explore their use in pest insect control including disease transmitting vector mosquitoes.[23] However, a major part of control measures implemented against the vector mosquitoes consists of applying synthetic insecticides. DDT, an agent known to possess broad spectrum insecticidal properties, is deployed against mosquito-borne diseases, mainly malaria. Indiscriminate use of the synthetic insecticides for a longer duration has led to several problems *viz.,* environmental contamination, insecticide resistance development and toxic effects to non-target organisms.[24] The risks following the use of these synthetic insecticides has compelled to explore for alternative insecticides, which are environmentally safe, target-specific and biodegradable.[25] In the present study aqueous extracts of *C. inerme* derived by two different methods were tested for insecticidal properties against *Ae. aegypti* larvae. The study findings

and observations on mode of mortality suggest that the *C. inerme* extracts possess promising growth disruptive activity against the larval and pupal stages of *Ae. aegypti*. Observations on the EI_{50} values suggest that aqueous extract of leaf powder was more than 5 times effective in suppressing the emergence of adults compared to extract derived from fresh leaves. The effect of leaf powder extract against the larvae at concentrations between 40 to 280 ppm was visible during pupal stages, while the larvae exposed to higher concentrations ranging between 600 to 2000ppm, the dose-response effect was reduced to greater extent leading to mortality during the fourth instar stage. It is noted that the quality of extract is influenced and is dependent on several basic parameters including plant part, method of extraction and type of solvent used for extraction.[26]

There are several reports to suggest that plant extracts possessing insecticidal properties induce developmental abnormalities in insects including mosquitoes.[20,27-29] Earlier Saxena and Yadav[30] have reported that acetone extract of *Oligochaeta ramosa* (Roxb) tested against *Ae. aegypti* larvae led to incomplete development leading to larval-pupal intermediate and abortive ecdysis. Petroleum ether extract of *Artemisia annua* when applied to eggs of *Anopheles stephensi* led to significant damage to hemolymphatic tissue, alimentary canal, fat bodies and tracheal network during the larval development (Sharma et al.[31]). Our observations of the dead larvae demonstrated loss of visual eye pigmentation, while pupae dead were found with incomplete sclerotization and exuviae found attached at the head capsule. Earlier investigations have shown that juvenoid applications against *Musca domestica* larvae caused similar morphological effects during the development stages like reduction in the cuticular sclerotization, reduced pigmentation in the eyes, and genital defects.[32] Several workers have reported similar findings of inhibition of proliferations of epidermal imaginal disks affecting genital disk, eye-antennal disk and reduced size of the eyes in other insects as well.[33-34] Studies have shown that *Ae. aegypti* larvae when reared aseptically on an artificial diet for one generation devoid of vitamin A or its precursor - β carotene led to a reduction of visual eye pigmentation leading to impairment in electrical response to light.[35] In our earlier study with crude fresh leaf extract of *C. inerme,* it was observed that there was an interference in the developmental process of treated fourth instar larvae of *Ae. aegypti*.[19] The reason for the development defects observed in the present study cannot be conclusively established, whether it is due to specific block during the changes in the developing stages or due to unspecified nutritional disturbances in

the treated larvae as the extracts used were crude. However, the latter explanation appears more plausible as it is supported by our earlier study showing *C. inerme* leaf powder treatment against fourth instar larvae of *Ae. aegypti* disturbed the midgut region leading to extrusion of the peritrophic membrane indicating that the leaf powder may be impairing the digestive process leading to deficiency of nutrition.[18] It may be argued based on our present findings that the extracts might have interfered in the digestive process leading to deficiency in nutrition and impairing development and causing morphological aberrations in the eye pigmentation and developmental process. Mortality observed during pupal stages may be theoretically conceivable because of inhibition of cell proliferation &/or evertion of the imaginal disk.[32] It is argued from our visual findings that incomplete moulting leading to pupae with attached exuviae might have caused suffocation leading to the death of the pupae.

The leaf powder extract was considered for toxicity evaluation against *G. affinis* fish on the basis of bioassay experiments demonstrating higher efficacy of leaf powder extract against *Ae. aegypti* larvae. Exposure of *G. affinis* to leaf powder extract at a concentration up to 4000 ppm, 26 times greater than EI_{50} value observed for leaf powder, had no visible effect and the fishes survived successfully at all the concentrations tested. Present findings on insecticidal properties and toxicity study against *G. affinis* revealed the aqueous extracts of *C. inerme* leaves to be safe. Our study suggests that further refinement and isolation of the active compound(s) from the plant extract derived might lead to a potential insect growth disrupter especially against dengue vector and other vector mosquito species. However it may be also noted that active compounds in its combined form with other compound(s) in the extract have synergistic effect than compared to the isolated compounds.[36]

It is concluded that the leaf powder extract of *C. inerme* plant leaves could be useful in the integrated mosquito control program as the extract were prepared in aqueous medium from a widely available plant and safer to non-target organisms.

Conflict of interest: The authors declare that they have no conflict of interest.

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