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Impact of *Calotropis procera* Leaf Extracts on the Survival, Morphology and Behaviour of Dengue Vector, *Aedes aegypti* L.

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ABSTRACT

The impact of a common weed, *Calotropis procera* was explored against dengue vector, *Aedes aegypti* in terms of larvicidal, behavioral and morphological response. The leaves of *C. procera* were extracted in hexane and screened for their larvicidal activity against dengue vector. Larvicidal bioassay conducted with the hexane leaf extract of *C. procera* established its efficacy revealing the LC_{50} and LC_{90} values as 78.39 and 100.60 ppm, respectively. The toxicity potential of the extract increased after prolonged periods of exposure of the larvae to the extracts, the LC_{50} values decreasing by 2.3%. The larvae assayed with *C. procera* extract exhibited increased wriggling speed with violent vertical movements. Most exciting observation was the insistent self-biting of anal gills with their own mouth parts resulting in the formation of ring-shaped larval structures suggesting probable impact of the extract on the neuromuscular system. Morphological studies of these larvae also revealed the loss of cuticular pigmentation and shrinkage of internal cuticle of anal gills of *Ae. aegypti* larvae indicating them as the probable action sites of the *C. procera* leaf extract. The mosquito management potential of *C. procera* as new larvicides is recommended for further exploration. The assessment of mosquito control efficiency of *C. procera* may provide constructive research with an objective of the formulation of new anti-mosquito agent above and beyond the weed management.

Keywords: Anal biting, anal papillae, behavioural, *Calotropis*, larvicidal

INTRODUCTION

Aedes aegypti, a primary carrier of Dengue Virus, has attracted considerable attention worldwide being a vector of many alarming diseases. The vector has been reported to be prevalent over large tropical and subtropical areas and due to its high potential to exploit even adverse environmental conditions, mosquitoes can rapidly increase their population. Since past few years, dengue is on alarming rise becoming a prime public-health issue accounting for huge mortality and morbidity in the prevalent areas. Since last 50 years, the alarming 30-fold rise in the global prevalence of dengue indicates it as the major and quickly spreading mosquito-borne disease (1). In 2012, World Health Organization reported that more than 40% of the world's population, approximately 2.5 billion, is at the dengue threat (2). Almost 50 to 100 million dengue infections have been predicted each year. According to the WHO reports, at present more or less 75% of the worldwide populace exposed to dengue resides in the Asia-Pacific region further leading to a continued rise in the number of countries affecting with the disease (2). In India, the Union health ministry records clearly reveal the immense augmentation in the dengue prevalence within the country. The corresponding figure till end December, 2013, stood at 75,808 cases and 196 fatalities in contrast to 50,222 cases and 242 deaths in 2012 (3).

Since early times, synthetic chemical insecticides have been in use against mosquitoes because of their immediate action and high efficacy. Nevertheless, the unsystematic and recurrent application of these synthetic chemicals has caused diverse problems including resistance to insecticides, toxicity to non-target organisms, lethal impacts on the individuals and residual effects on the environment and its destabilization (4, 5). Keeping this in view, the focus of research has been diverted towards plant extracts and isolated phytochemicals from them as probable sources of mosquito control agents to prevent the predictable toxicity and resistance problems caused by chemical insecticides (6).

Several reports are available that have proved phytochemicals as green, eco-safe, recyclable and affordable innate toxicants with target specificity. Researchers have shown the cidal and repellent efficacy of a huge number of plant extracts against mosquito vectors (7, 8), though the insecticidal effects of phytochemicals are based on the diverse factors. Their efficacy depends on not only the plant species, but also the mosquito species, plant parts and the extraction methodology. Phytochemicals have been reported safe for the environment possessing manifold effects against vector mosquitoes which include cidal effects, hormono-mimetic effects, reproductive disadvantage, fecundity suppression, sterility and oviposition deterrent activity (9, 10, 11). However, currently only a handful reports are available concerning the anti-mosquito potential of weeds in terms of the larvicidal, ovicidal and oviposition deterrent potential (10, 12, 13).

Calotropis procera (Aiton) W.T. Aiton is a common blossoming plant belonging to the Family, Apocynaceae. It is indigenous to North and Tropical Africa, Western and South Asia, and Indochina. The plant, popularly known as Osher, Mudar, Apple of Sodom, Sodom apple or Stabragh, has been found to exhibit purgative, antipyretic, alexipharmic, anticonvulsant, anthelmintic, analgesic, anxiolytic, and sedative effects (14, 15). It has also been used for the treatment of various diseases, including ulcers, leprosy, leucoderma, lumps, piles and splenic, hepatic and abdominal diseases (16). They have been shown to contain a noxious bitter and opaque sap because of which they are also commonly known as *milkweeds*. Variety of components has been isolated from the milky sap, such as cardenolides, cardiac glycosides and flavonoids (17). The possible efficacy of the extracts prepared from the leaves of *C. procera* has been explored against the larvae of dengue vector *Ae. aegypti*, malarial vector *Anopheles stephensi* and filarial mosquito *Culex quinquefasciatus* (18).

The available literature, however, reveals that this plant hasn't been investigated extensively as mosquito larvicide or affecting their biological characteristics. Hence, this study was undertaken to evaluate the probable use of leaf extracts of *C. procera* against dengue vector larvae causing mortality, behavioural changes or morphological aberrations. The assessment of mosquito control efficiency of *C. procera* may provide constructive research with an objective of the formulation of new anti-mosquito agent above and beyond the weed management.

METHODOLOGY

Rearing of Dengue Vector

The dengue vector *Ae. aegypti* used in the present studies were collected from fields of New Delhi and nearby areas. The culture of the dengue vector was maintained in an insectary at controlled conditions maintained at 28 ± 1°C, 14h light and 10h dark, and 80 ± 5% relative humidity (8). A small swab of moist cotton was placed on the top portion of each mosquito cage as a source of water for the adults. Moist split raisins were kept in the cage primarily as a food source for the adult males whereas intermittent blood meals were offered to the females required for the maturation of their eggs. Small Whatman filter paper-lined enamel bowl (4" diameter), filled half with the de-chlorinated water, was placed in each cage for oviposition. The egg strips were transferred to the trays containing de-chlorinated water for hatching. Upon hatching, the larvae were fed upon a mixture of finely grounded yeast and dog biscuits (1:3 by weight). Water was changed every alternate day so as to prevent formation of any froth on the surface. The pupae formed were immediately collected in a bowl that was kept in the screened cages to allow emergence of the adults.

Collection of *C. procera* leaves

Leaves of the *C. procera* plant were collected from the premises of Acharya Narendra Dev College, New Delhi (Figure-I). The leaves were scrupulously washed with tap water and disease/infection-free leaves were selected for the investigations. The selected leaves were dried indoors at temperature of 28 ± 1 °C for about 20 days and carefully scrutinized each day for any fungal or other infections. When completely dried, the leaves were converted into powdered form and sieved meticulously to obtain fine powder.

Figure-I: *Calotropis procera*

Source: https://upload.wikimedia.org/wikipedia/commons/4/45/Starr_070207-4333_Calotropis_procera.jpg

Preparation of the leaf extract

The powdered plant leaves (30 g) were extracted in 250 mL of hexane in a soxhlet extraction apparatus. The extraction was carried out for 3 days, 8 h per day, at a temperature less than the boiling point of hexane (68 °C). The crude extract formed was concentrated at 45 °C using a Buchi's vacuum evaporator till the solvent evaporated completely. The concentrated extract formed was collected in a volumetric flask and stored at 4ºC as the stock solution (Figure-II). Desired concentrations of the extract were prepared from the stock solution, whenever needed, for investigating the cidal, behavioural and morphological effects against larvae of *Ae. aegypti*.

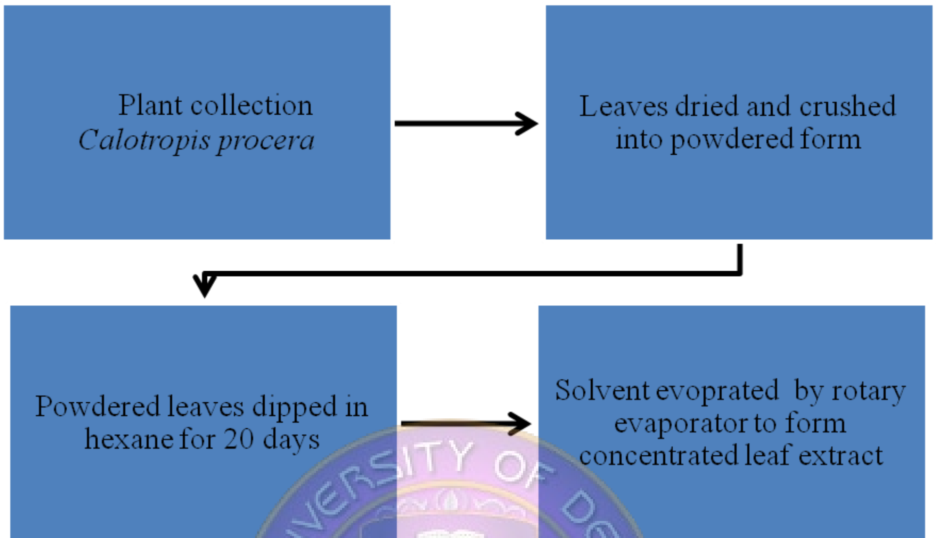

Figure II: Steps followed in Preparation of Extract

Larvicidal bioassay against *Ae. Aegypti*

The larvicidal bioassay with *C. procera* hexane leaf extract was performed at controlled conditions of temperature (28 ± 1^{o}C). The early fourth instars of *Ae. aegypti* were exposed to the extracts as per the procedure of WHO, with minor variations (19). The treatments were carried out in a 250 mL beaker containing 100 mL of distilled water to which 1 mL of a particular concentration of the leaf extract was added. Two solutions were mixed vigorously with the help of a glass rod to ensure a consistent treatment solution. Active early fourth instars of *Ae. aegypti*, taken in batches of 20, were kept separately in bowls filled with 99 mL of distilled water. These larvae were transferred carefully to the glass jars with the test solutions and stirred lightly. Keeping in view the probable heterogeneity in the mosquito population, each test concentration had four replicates. The larvae were exposed to only ethanol in control treatments. The jars were kept undisturbed in the insectary and the dead/moribund larvae were scored after 24 as well as 48 h of exposure.

Statistical investigation of the data

The larvicidal bioassays exhibiting more than 20% larval death in control treatments or formation of more than 20% pupae were rejected and repeated. The data with mortality ranging from 5 to 20% in the control treatments was corrected as per the following formula (20).

$$\%\text{Corrected Mortality} = \frac{\%\text{ Mortality (Experimental treatments} - \text{Control treatments)} \times 100}{(100 - \%\text{Mortality in Control treatments})}$$

The data was analyzed for probit regression using SPSS 22.0 Programme software. The LC_{50} and LC_{90} values with 95% confidence limits were calculated to measure difference between the test samples.

Behavioural studies in *Ae. aegypti* larvae treated with leaf extract of *C. procera*

During the larvicidal bioassay, the larvae of *Ae. aegypti* were monitored carefully for behavioural alterations, if any. The observations included wriggling speed, movements in different directions, aggregation behaviour, etc. The larval behaviour was recorded and photographed with Canon Power Shot SX50HS. Similar observations were made in controls for comparison with the treated larvae.

Morphological studies in *Ae. aegypti* larvae treated with leaf extract of *C. procera*

After the larvicidal bioassay, the dead larvae were separated and probed under light microscopy for any aberrations in their morphology. Each body segment of the larvae, eyes, antennae, mouth brushes, setae and saddle; and the anal gills were observed carefully for structural changes and variation in the pigmentation pattern. Any aberrations observed was recorded and compared with the features observed in the controls.

RESULTS

The potential of hexane leaf extract of *C. procera* was studied against *Ae. aegypti* larvae for assessing the beneficial use of potentially harmful weed as eco-friendly substitute for synthetic insecticides. The data obtained with the larvicidal bioassay performed against early fourth instars of *Ae. aegypti* is presented in Table-I which clearly establishes the usefulness

Table-I: Larvicidal bioassay with leaf extracts of *Calotropis procera* against early fourth instars of *Aedes aegypti*

Concentration of *C. procera* Extract (ppm)	**% Larval Mortality**		**% Mortality**	
	After 24 h	**After 48 h**	**After 24 h**	**After 48 h**
Control	Nil	Nil	Nil	Nil
40	Nil	Nil	Nil	Nil
60	3.5	4.0	17.5	20.0
80	6.5	7.5	32.5	37.5
90	16.5	16.5	82.5	82.5
100	19.0	19.0	95.0	95.0
200	20.0	20.0	100.0	100.0

*For each test; n=80 (20 larvae in 4 replicates) of hexane leaf extract of *C. procera* against the larvae. It was also observed that the treatments with leaf extracts did not result in any pupal or adult emergence indicated the adequate selection of early fourth instars. Moreover, the control treatments did not cause any mortality within 48 h indicated the optimum conditions of treatments.

When the early fourth instars of *Ae. aegypti* were assayed with the hexane leaf extract of *C. procera*, the data analysis revealed the LC_{50} and LC_{90} values as 78.39 and 100.60 ppm, respectively. It is also worthy to note that the toxicity potential increased after prolonged periods of exposure of the larvae to the extracts, the LC_{50} values decreasing after 48 h of exposure (Table-II). The results revealed that the LC_{50} value of the hexane leaf extract of *C. procera* decreased by 2.3% after 48 h of exposure of the extract to the early fourth instars of *Ae. aegypti* resulting in an LC_{50} value of 76.57 ppm (Table-II).

Table-II: Larvicidal efficacy of the leaf extracts of *Calotropis procera a*gainst early fourth instars of *Aedes aegypti*

Time of exposure	**Larvicidal activity**					
	LC_{50}	**LC_{90}**	**SE**	**χ^2**	**df**	**Regression coefficient**
24 h	78.39	100.60	2.2	5.98	4	11.827
48 h	76.57	100.94	1.9	5.84	4	10.682

*For each test; n=80 (20 larvae in 4 replicates)

Behavioural observations of the larvae on treatment with the hexane leaf extract of *C. procera* revealed fascinating behavioural symptoms. The immediate exposure to the extract resulted in a normal feeding pattern, vertical movements and wriggling motion in all the larvae. It continued for 5-10 min of exposure after which the larvae exhibited radically increased wriggling speed with violent vertical movements. Most exciting observation was the insistent self-biting of anal gills with their own mouth parts resulting in the formation of ring-shaped larval structures (Figure-III). The pattern continued for 2-3 h followed by the immobility in the larvae and failure to move upwards for breathing leading to heavy larval mortality.

Figure-III: Digital photomicrographs of early IV instars of *Aedes aegypti* treated with hexane leaf extract of *Calotropis procera* showing the formation of ring-shaped structure

When the dead larvae of *Ae. aegypti* were observed after the larvicidal bioassay, the larvae showed morphological aberrations and structural alterations in the anal gills on comparison to the control larvae. The observations clearly established the damaged anal papillae possessing a shrunken and folded cuticle of the internal membrane. On the other hand, the external membrane of anal papillae was completely intact with no structural changes (Figure-IV). Another noteworthy observation was the change in pigmentation pattern with loss of pigment in the cuticle as compared to that in the control larvae.

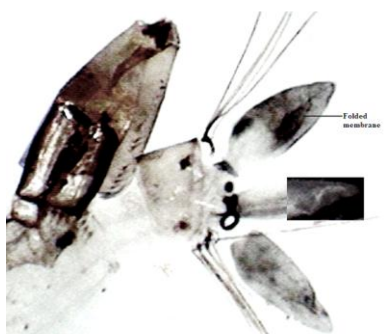

Figure IV: Micrographs of anal gills of *Aedes aegypti* larvae treated with leaf extract of *Calotropis procera* showing four anal gills with externally normal appearance but internally shrunken structure and folded membrane

DISCUSSION

Extensive and indiscriminate application of synthetic insecticides for mosquito control has led to irreparable damage to the environment and the increased resistance in mosquitoes causing their control difficult. The continued rise in mosquito-borne diseases since past few years has necessitated the formulation of novel strategies for mosquito control. Keeping in view the problems and health hazards occurrence due to the use of insecticides, the researchers have shown inclination towards natural and innocuous phytochemicals as probable and safe alternatives to synthetic chemical insecticides. In fact, the crude plants extracts from various species have been used against insect pests for many centuries. Several researchers have recently documented the efficacy of plant extracts against mosquito larvae (6, 13).

C. procera is a common plant (Family: Apocynaceae) reported to exhibit diverse medicinal effects (14, 15) and thus, used for treatment for various diseases (16). It has been shown to contain a toxic, bitter milky sap from which variety of components have been isolated. The larvicidal potential of the leaf extract of *C. procera* has been explored against larvae of all the three mosquito species; *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* (18). Most of the studies however have been carried out with the latex of the plant. For the first time, Giridhar *et al*. (21) had reported compounds with larvicidal activity in the latex of *C. procera*. Keeping in view the limited research work with the plant, present studies were performed to evaluate the probable use of *C. procera* in dengue vector management program.

Our investigations established the efficacy and usefulness of hexane leaf extract of *C. procera* against early fourth instars of *Ae. aegypti* revealing LC_{50} and LC_{90} values as 78.39 and 100.60 ppm, respectively. These results are in conformity with that reported by Kumar *et al.* (22) who obtained LC_{50} values of 137.9 ppm against *Cx. gelidus* and 110.05 ppm against *Cx. tritaeniorhynchus* when assayed with aqueous extracts of *C. gigantea.* Ramos *et al.* (23) revealed the larvicidal compounds in *C. procera* latex which could inflict 100% mortality in III instars of *Ae. aegypti* just after exposure of 5 min. Likewise, Markouk *et al.* (24) demonstrated 50% mortality caused by 28 ppm *C. procera* latex against *An. labranchiae*. Larvicidal bioassays conducted by Warikoo and Kumar (8) with hexane root extracts of *Argemone mexicana* has shown the LC_{50} and LC_{90} values as 91.331 ppm and 156.684 ppm, respectively against early fourth instars of *Ae. aegypti* when exposed for 24 h. They also reported 1.1-fold rise in the cidal potential of the extract when the larvae were exposed for 48 h.

Raj Mohan and Ramaswamy (25) had reported the moderate efficacy of the *Aegeratina adenophora* leaf extracts against IV instars of *Ae. aegypti* and *Cx. quinquefasciatus*. They reported the LC_{50} value of 256.70 and 227.20 ppm, respectively after 24 h of exposure. They recommended that the weed can be successfully used in stagnant water bodies against mosquito larvae. Likewise, the leaf extracts of *Lantana camara* has also shown to possess larvicidal potential against *Ae. aegypti* and *Cx. quinquefasciatus* larvae (12). The investigations with the hexane extracts prepared from the dried fruits of three species of peppercorns; Long pepper, *Piper longum* L., Black pepper, *P. nigrum,* and White pepper, *P. nigrum* revealed their cidal efficacy against larvae of *Ae. aegypti* (9). They revealed the

Black *P. nigrum* extract as the most effectual larvicidal followed by that of *P. longum*, whereas White pepper was found as the least effective. On the contrary, the hexane leaf extracts prepared from citrus plant, *Citrus sinensis* has been shown to possess reasonable larvicidal potential against dengue vector, the bioassay resulting in very high LC_{50} and LC_{90} values of 446.84 and 1370.96 ppm, respectively (26).

The behavioural observations of the larvae treated with hexane leaf extract of *C. procera* revealed interesting modifications leading to excitation, violent vertical and horizontal movements, and haphazard motility. These symptoms are quite similar to those caused by nerve poisons suggesting that the extract could possibly affect the neuromuscular synchronization leading to changes in chemical synapses. These findings are in conformity to those of a few earlier studies (8, 9, 27) which indicate that plant extracts could act as neuro-toxicants. Our investigations also showed a noticeable behaviour of aggressive anal biting in treated larvae leading to the formation of ring-shaped structure. Similar kinds of observations have been reported by Warikoo and Kumar (8) when the larvae of *Ae. aegypti* were assayed with extracts prepared from *A. mexicana.* The anal papillae of mosquitoes have been reported to have some role in regulating electrolyte balance required for the life sustainability (28) suggesting the cytotoxic effects of *C. procera* extract leading to electrolyte imbalance in the anal region causing violent anal biting.

Present investigations also showed structural alteration of anal papillae in the treated early IV instars of *Ae. aegypti*, the internal structures of the anal papillae exhibiting remarkable shrinkage and foldings. These results are in accordance with that of Warikoo and Kumar (8) who also obtained the similar internally shrinked anal papillae of *Ae. aegypti* larvae when assayed with variable extracts of *A. mexicana*. Comparable destructed anal papillae exhibiting shrunken border of the larval cuticle has been observed by Insun *et al.* (29) in *Cx. quinquefasciatus* larvae on treatment with ethanolic extract of *Kaempferia galangal.* It has been indicated that the structural deformity in the larval anal papillae perhaps led to abnormal functions probably leading to disruption of the osmotic and ionic regulation (27). They suggested that the disruptive regulatory functions may have caused the mortality of the larvae.

CONCLUSION

Our investigations have indicated the potential of *C. procera* leaves as the possible and effective control agent against *Ae. aegypti* larvae. The studies recommend that this plant could form a safe and eco-friendly alternative to eco-enemy synthetic pesticides. However, the active ingredient present in the extract and mechanism involved causing larval mortality needs to be explored further. The isolation and identification of the bio-active ingredient from the extract could help to devise line of attack for mosquito management. Further studies regarding enhancing effectiveness, constancy, noxious effects and impacts on non-target population and the environment, along with field trials are required to recommend *C. procera* as an anti-mosquito agent.

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