

Evaluation of larvicidal efficacy of *Clerodendrum infortunatum* Linn extracts against *Culex quinquefasciatus* Say (Diptera, Culicidae)

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ABSTRACT: *Clerodendrum infortunatum* Linn (Family, Lamiaceae), extracts of acetone, methanol and aqueous evaluated against larvae of *Culex quinquefasciatus* Say, showed promising results. Acetone leaf extract recorded an LC_{50} value of 345.41ppm, and was found effective for the management of *Cu. quinquefasciatus*. In vitro cytotoxic analysis of L929 mouse cell lines using the plant extract proved safe for non-target organisms, higher vertebrates. Phytochemical screening of the acetone extract revealed the presence of secondary metabolites that may have contributed to its insecticidal activity.

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KEY WORDS: Acetone leaf extract, LC_{50} , cytotoxicity, MTT assay, secondary metabolites

INTRODUCTION

The application of synthetic pesticides and chemicals is detrimental to all ecosystems, and nowadays they are being replaced by green or bio-pesticide methods to manage pollution hazards. Phytochemicals and other secondary metabolites produced in plant parts protect themselves by defensive mechanisms and serve humanity as larvicides, insecticidal agents, weedicides, medicines, nutraceuticals, anticancer agents, anti-feedants, etc., since they are safe for non-target organisms, readily available and environmentally benign (Ghosh *et al.*, 2012; Kekuda *et al.*, 2019). The spread of mosquito-borne diseases such as fevers and filariasis in both humans and animals is caused by *Culex quinquefasciatus* (Diptera, Culicidae). Additionally, it may spread the West Nile

virus and St. Louis encephalitis (Ragavendran *et al.*, 2017). It breeds widely in unclean water sources, such as burrow pits, cesspools, septic tanks with leaks, sluggish drains, and practically all organically damaged water sources (Suleiman *et al.*, 2014). Common weed plant in tropical plains, *Clerodendrum infortunatum* Linn (Family, Lamiaceae), locally referred to as Indian Bhand tree or peruvellam in Kerala, is a terrestrial shrub with an unpleasant stench that has several therapeutic and insecticidal uses (Abbaszadeh *et al.*, 2014; Kekuda *et al.*, 2019). Because of its accessibility and alleged health benefits, different components of *C. infortunatum* are employed for different purposes (Uddin *et al.*, 2021). The tribal community in Kokrajhar (Assam) uses *C. infortunatum* as a mosquito-repellent plant. It is a customary practice to use the leaves of this plant to keep flies and

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mosquitoes away from food (Patil *et al.*, 2014; Swargiary *et al.*, 2021). The present investigation was carried out to analyse the bioefficacy of different extracts of *C. infortunatum* leaves against fourth-instar larvae of *Cu. quinquefasciatus*. The study also evaluates cytotoxicity against the L929 cell line.

MATERIALS AND METHODS

The experiment was conducted in the Research Laboratory of the Zoology Department, University College, Thiruvananthapuram, Kerala. Five replicas of toxicity bioassay and different tests for different solvents of plant material were conducted with fourth-instar mosquito larvae under standard laboratory conditions. *Culex* egg rafts were collected by placing a plastic bucket (10L) filled three-fourths with water and two crushed domestic fowl eggs to attract oviposition (Ayana Gayathri and Evans, 2018). After 3 to 4 days, a foul smell was produced from the bucket, and the mosquito began to lay eggs. The eggs were laid in rafts and collected from the bucket with a soft brush. The rafts were transferred into small bottles containing de-chlorinated tap water and a small amount of egg solution from the bucket. After 24 hours, small, slender first-instar larvae emerged from the eggs, exhibiting wriggling movements. Larvae that emerged were pooled and identified using standard manual (Barraud, 1934) and maintained in distilled water for further experiments. During this time, crushed dog biscuits were provided to the medium to nourish the larvae.

Fresh leaves of *C. infortunatum* were washed thoroughly with de-chlorinated tap water and shade-dried for two weeks. The dried leaves were pulverised in a domestic grinder. According to the polarity, from low to high, different solvents such as acetone, methanol and distilled water were subjected to Soxhlet extraction (Madhu *et al.*, 2011). The extracts obtained were dried and used for further analysis. Ten per cent stock solution was prepared using the dried extract of acetone, methanol, and distilled water, with distilled water as the solvent. From the stock solution, concentrations ranging from 100 to 1000ppm were prepared.

Evaluation of larvicidal activity: Larvicidal activity was evaluated on fourth-instar larvae of uniform size, randomly selected from the stock culture in accordance with WHO guidelines (WHO, 2005). Larvae (20 Nos.) were exposed for a 24-hour bioassay to different concentrations of *C. infortunatum* extracts, ranging from 100 to 1000ppm, prepared from the stock, along with distilled water as a control. Larvae were considered dead when they showed no movement or when they were touched with a needle on their siphon. The lethal concentration (LC_{50}) and its 95 per cent upper and lower confidence limits were determined using probit analysis (Finney, 1971).

Ovicidal and pupicidal activity: Ovicidal and pupicidal activity were studied using the acetone extract of *C. infortunatum*. Eggs were separated carefully from the egg raft for ovicidal activity, and pupae were maintained for pupicidal activity in paper cups containing acetone leaf extracts of sub-lethal concentration 300 ppm and kept for 24 hours. Five replicates, including a control, were maintained and observed for hatchability and pupal emergence, respectively.

Hatchability (%) =

$$\frac{\text{Number of hatched larvae}}{\text{Total number of eggs}} \times 100$$

Pupicidal activity (%)

$$= \frac{\text{Number of dead pupae}}{\text{Total number of pupae}} \times 100$$

Cytotoxic analysis of plant extract on non-target cells: The L929 mouse fibroblast cell line cells were procured from National Centre for Cell Sciences (NCCS), Pune, India and cultured in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10 percent heat inactivated Fetal Bovine Serum (FBS) and one percent antibiotic cocktail containing penicillin (100 U/mL), streptomycin (0.1 mg/mL) and amphotericin B (0.25 µg/mL).

Cell viability assay: The assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was performed to determine the IC_{50} (Mosmann, 1983; Joseph *et al.*, 2012). The L929 cells were seeded on 96-well plates and

allowed to acclimate to the culture conditions. The samples were prepared in DMEM and filter-sterilised. The samples were further diluted in DMEM and added to the wells containing cultured cells at a concentration of $300\mu\text{g mL}^{-1}$. Control wells were kept untreated. After treatment, the plates were incubated for 24 hours. After the incubation period, the media were discarded. $100\mu\text{L}$ MTT solution in PBS (Phosphate-Buffered Saline) was added to the wells. The plates were incubated for an additional two hours to develop formazan crystals. After incubation, the supernatant was removed, and $100\mu\text{L}$ of DMSO (Dimethyl Sulfoxide) was added to each well. Absorbance was measured at 570nm . Three wells per plate without cells served as a blank. The formula calculated the cell viability:

$$\text{Cell viability (\%)} = \frac{\text{Average of absorbance of test}}{\text{Average of absorbance of control}} \times 100$$

Estimation of phytochemical content in *C. infortunatum*: The phytochemical screening of the acetone leaf extract of *C. infortunatum* was done for phenol, tannin, flavonoids, saponins, terpenoids, alkaloids, glycosides, quinones, fatty acids and steroids using standard protocols (Ejikeme *et al.*, 2014; Kumar *et al.*, 2014). Statistical analysis of the data was performed to determine the LC_{50} using probit analysis in R (R Core Team, 2025).

RESULTS AND DISCUSSION

The acetone, methanol and aqueous extracts of *C. infortunatum* induced mortality in fourth-instar larvae of *Cu. quinquefasciatus* after 24-hour exposure (Table 1). The acetone extract of *C. infortunatum* showed greater activity against the fourth-instar larvae compared to methanol and aqueous extracts. Low and moderate larvicidal activities were shown by aqueous and methanol extracts when treated with 100 to 1000ppm concentrations against fourth-instar larvae. No larval mortality was observed in the solvent-treated controls or in the control groups maintained in distilled water. The acetone extract caused higher mortality at higher concentrations, at 1000ppm, 100 per cent larval mortality, and the mortality decreased at lower concentrations. At 100ppm, no significant mortality was observed. With increasing concentration, larval mortality increased significantly. Mortality was also increased with exposure duration across all concentrations tested. In the methanol and aqueous extracts 100 per cent larval mortality was not observed after 24 hours of exposure. The acetone extract exhibited the highest larvicidal activity, with an LC_{50} of 345.41ppm, compared to the methanol extract ($\text{LC}_{50} = 2820.96\text{ppm}$) and the aqueous extract ($\text{LC}_{50} = 1637.65\text{ppm}$) (Table 2). The acetone extract was more effective against the fourth instar larvae in

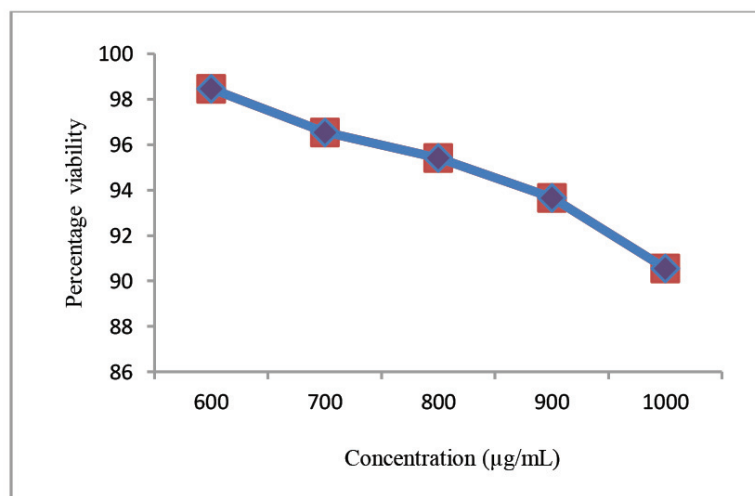


Fig. 1 Cell viability of the L929 cell line treated with a higher concentration of the acetone extract of *C. infortunatum*

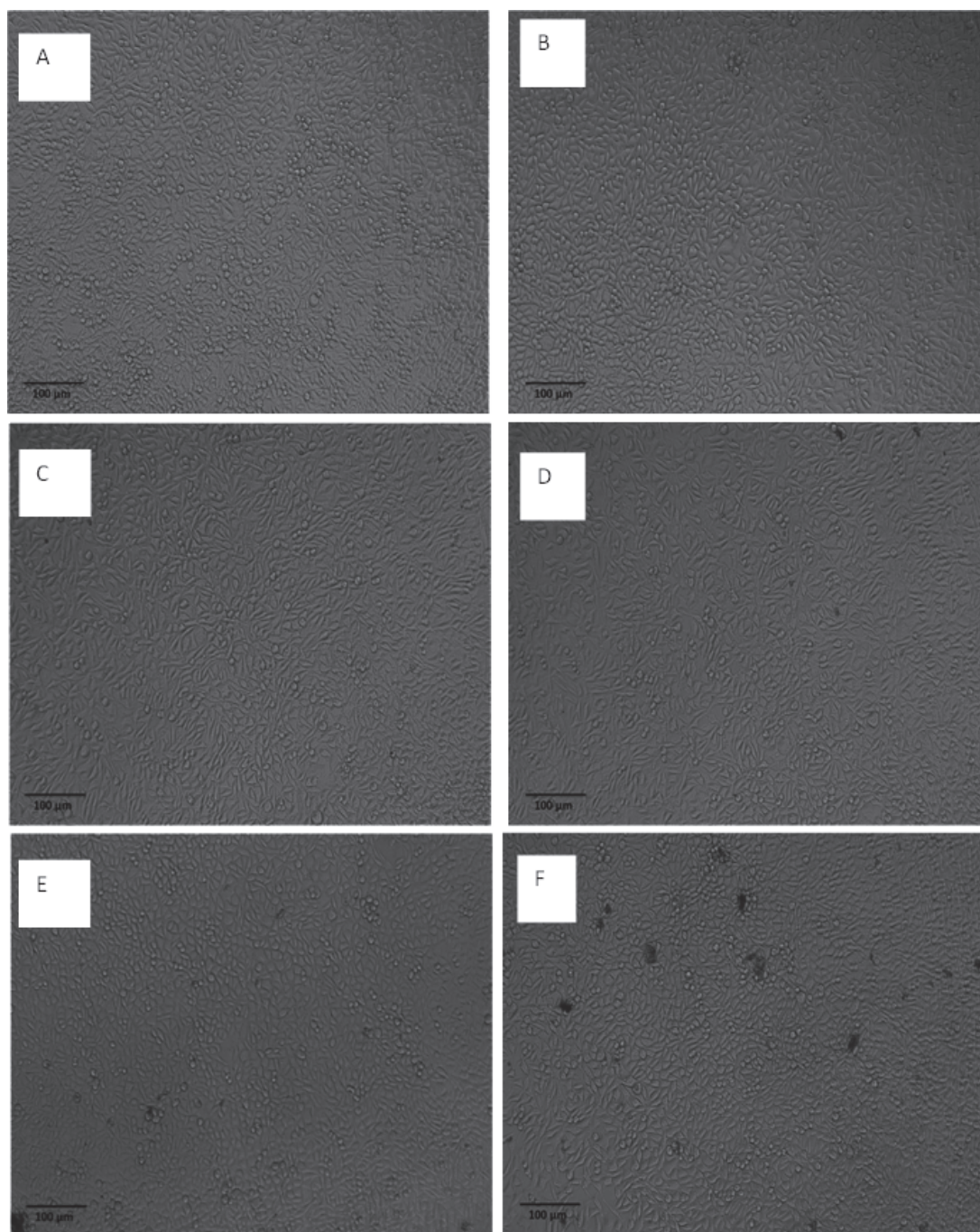


Plate 1. Photomicrographs of cultured L929 cells; A-Control; B, C, D, E, F represent cells exposed to 600, 700, 800, 900 and 1000 $\mu\text{g ml}^{-1}$ acetone extract. Images were taken using a light microscope at 40X magnification

Table 1. Mortality of *C. infortunatum* extracts against fourth-instar larvae of *Cu. quinquefasciatus*

Treatment concentration	Mean mortality after 24-h exposure %		
	Acetone	Methanol	Aqueous
Untreated	0	0	0
Solvent-treated	0	0	0
100ppm	16.66 ±2.58	5.83±2.04	3.33±5.16
200ppm	28.33±4.08	6.66±2.58	15.0±3.16
300ppm	39.16±5.84	9.16±3.76	16.66±4.08
400ppm	51.66±5.16	12.5±2.73	21.66±4.08
500ppm	54.16±2.04	15.83±4.91	25.0±3.16
600ppm	73.33±6.05	18.33±4.08	28.33±2.58
700ppm	75.83±5.84	20.0±3.16	30.0±3.16
800ppm	82.5±2.73	25.0±4.47	30.83±3.76
900ppm	91.66±6.83	32.5±2.73	32.50±5.24
1000ppm	100±0	34.16±2.04	41.66±4.08

terms of mortality than the methanol and aqueous extracts. Adewoyin *et al.* (2021) reported the insecticidal activity of the methanol leaf extract of *C. polycephalum* against *Aedes*, *Anopheles*, and *Culex* mosquito species at concentrations of 250 to 2000 ppm, and found it effective. Similarly, Nambiar and Ranjini (2018) reported that *C. infortunatum* affects sixth-instar larvae of the *Orthaga* species.

Ovicidal and pupicidal activity analysed using a sub-lethal concentration at 300ppm, showed 89.16per cent mortality in pupae and 57.5per cent mortality in the egg stage. Pupae were unable to emerge as adults and exhibited structural deformities in their wings. Larvae placed in distilled water as a control did not exhibit ovicidal and pupicidal effects. Remia and Lagaswamy (2009) reported the larvicidal efficacy of leaf extracts from two botanicals against the mosquito vector *Aedes aegypti*. Bioactivity of plant extracts on the larval and pupal stages of *A. aegypti* was also reported (Candido *et al.*, 2013). Target-specific larvicidal activity of the *Typhonium trilobatum* leaf extract against *Culex* mosquitoes

was reported by Haldar *et al.* (2011). The insecticidal properties of leaf extracts of *C. infortunatum* have been reported by various authors (Pavela, 2007; Yankanchi *et al.*, 2015; Jadhav *et al.*, 2016; Kekuda *et al.*, 2019; Swargiary *et al.*, 2021).

The toxicity of phytochemicals must be investigated *in vitro* because such data enable us to choose plant products with the least environmental impact. An alternate technique for examining toxicity is to use cell lines. To evaluate the potential hazards and unintended effects of insecticidal substances on the environment and human health, toxicity tests on non-target cells are essential. It is essential to determine the safety of a substance when used in the environment. The MTT assay is a widely used colorimetric method for assessing cellular metabolism as an indicator of cell viability, proliferation, and cytotoxicity. Cell lines are a relevant model system for studying the toxic effects of plant extracts. L929 mouse fibroblasts are rapidly proliferating, spindle-shaped, fibroblast-like cells derived from mouse connective tissue.

The cytotoxic activity study of the acetone extract of *C. infortunatum* was assessed using the MTT assay in the L929 cell line at concentrations ranging from 600 to 1000µg mL⁻¹. The cell viability studies indicated that the acetone extract of *C. infortunatum* was non-toxic to the L929 mouse fibroblast cell line at the concentrations tested (Fig. 1). Cell survival analysis showed no significant effect on viability or change in cell morphology (Plate 1). The viability was 98.46 per cent at 600µg mL⁻¹, 96.55 per cent at 700µg mL⁻¹, 95.42 per cent at 800µg mL⁻¹, 93.67 per cent at 900µg mL⁻¹, and 90.7 per cent at 1000µg mL⁻¹. The results showed that the plant extract was least toxic to non-target cells

Table 2. LC₅₀ value of different extracts of *C. infortunatum* against *Cu. quinquefasciatus* larvae

Solvent	LC ₅₀ (ppm)	LCL – UCL (95%)
Acetone	345.41±28.12	299.629 - 391.076
Methanol	2820.96±191.65	1945.370 - 5260.213
Distilled water	1637.66±143.54	1274.735 - 2406.159

with an IC_{50} value above $100\mu\text{g mL}^{-1}$. In most toxicological studies, an extract is considered safe or biocompatible if the cell viability remains above 70 or 80 per cent compared to the control group.

The qualitative phytochemical analysis of the acetone extract of *C. infortunatum* revealed the presence of phenol, tannins, flavonoids, saponins, terpenoids, alkaloids, glycosides, quinones, fatty acids and steroids. The phytochemical content and larvicidal properties of *C. infortunatum* were extensively explored (Swargiary *et al.*, 2021). Hepatoprotective potential of the methanol extract of *C. infortunatum* was proved by Sannigrahi *et al.* (2009). The bioactivity of the plant's secondary metabolites was reported by Helen *et al.* (2021). The cytotoxicity of *C. viscosum* was extensively reviewed by Srivastava *et al.* (2021), and the results were comparable to those reported here. Numerous studies have evaluated the insecticidal potential of the *Clerodendrum* family (Das *et al.*, 2014). Research showed that three diterpenoids isolated from *C. infortunatum* clerodin, 15-methoxy-14,15-dihydroclerodin and 15-hydroxy-14,15-dihydroclerodin showed insecticidal activity against *Helicoverpa armigera* (Abbaszadeh *et al.*, 2014). Hexane extract of *C. infortunatum* showed strong insecticidal activity against the sand fly *Phlebotomus argentipes* (Dinesh *et al.*, 2015). Ethyl acetate extract of *C. capitatum* showed strong repellent activity to adults of three major stored grain insects, *Sitophilus oryzae*, *Rhyzopertha dominica* and *Tribolium castaneum* (Adesina *et al.*, 2019). Studies revealed that the aqueous extract of *C. infortunatum* killed third-instar larvae of *Hyposidra talaca* (Geometridae), a new and serious tea pest, using the leaf-dip method (Roy *et al.*, 2015).

This study clearly demonstrates the larvicidal potential of *C. infortunatum* against *Cu. quinquefasciatus*, offering a sustainable, eco-friendly alternative to toxic synthetic insecticides. The high larval mortality rates observed, coupled with the plant's natural abundance, suggest its viability as a primary tool in Integrated Mosquito Management (IMM) strategies.

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