Journal of Chemical Health Risks

www.jchr.org

JCHR (2026) 16(2), 621-625 | ISSN:2251-6727

Entomotoxic Effects of *Clerodendrum Infortunatum*: Analysis of Larval Midgut Histology and Digestive Enzymes of *Culex Quinquefasciatus* Say (Diptera: Culicidae)

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(Received: 05 January 2026 Revised: 15 February 2026 Accepted: 05 March 2026)

KEYWORDS

Culex quinquefasciatus, *Clerodendrum infortunatum, histology*, digestive enzymes

ABSTRACT:

Introduction: Mosquitoes are medically important vectors globally transmitting diseases such as dengue, malaria and filariasis. Among mosquito species, *Culex quinquefasciatus* is a major vector responsible for lymphatic filariasis. For the control of mosquitoes, natural and man-made insecticides are used. Increased use of chemical insecticides causes insecticidal resistance and adverse effects on the environment. Therefore, safer alternatives are necessary, which are eco-friendly and cost-effective. Phytochemicals are a promising source of botanical insecticides. *Clerodendrum infortunatum* belongs to the family Lamiaceae.

Objectives: The present study investigates the insecticidal potential of *Clerodendrum infortunatum* against fourth instar larvae of *Culex quinquefasciatus,* emphasising its effect on histology and the activity of digestive enzymes α – amylase and protease in the treated larvae compared to control ones.

Methods: The histopathological effect of the acetone extract of *Clerodendrum infortunatum* against fourth instar larvae of *Culex quinquefasciatus* was examined using Hematoxylin-eosin staining. The activity of α- amylase and protease was estimated to understand the effect of plant extract on the larval digestive system.

Results: Fourth instar larvae of *Culex quinquefasciatus* were monitored after 24 h treatment with the acetone extract of *Clerodendrum infortunatum*. Several histological aberrations were observed in treated larvae. The midgut showed damage, causing cell wall protrusion and vacuolisation. The digestive enzymes α-Amylase and Protease were decreased compared to the control. The observed histo-enzymological disruptions collectively contribute to impaired larval development and subsequent mortality.

Conclusions: The study provides insight into the effect of the acetone extract of *Clerodendrum infortunatum* against fourth instar larvae of *Culex quinquefasciatus*. *Clerodendrum infortunatum* can be used as a promising botanical larvicide with different modes of action. Further studies are necessary for identifying the active components of the plant extract.

1. Introduction

Mosquitoes are a concern for public health due to their role as vectors of pathogenic microorganisms. Their role in disease transmission, such as Malaria, Dengue, Lymphatic Filariasis etc make them a vector of major importance. They can thrive in various habitats and transmit diseases among individuals. Control of mosquitoes is now gaining public attention because of the health risks and economic loss. When a female mosquito feeds on infected blood, the pathogens are passed to the subsequent human host. Transmission of pathogens depends on the abundance of mosquito vectors.[1]

Culex quinquefasciatus, commonly known as the southern house mosquito, is the major vector of Lymphatic filariasis. By adjusting to sailing ships built for lengthy journeys, the larvae, found in natural and anthropogenic habitats, spread to far-off locations.[2] Eradication of Culex species gains importance as it causes economic losses due to the spread of diseases. A large number of chemical and biological insecticides are used to control larvae as well as adult mosquitoes.

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JCHR (2026) 16(2), 621-625 | ISSN:2251-6727

Among the different life stages of mosquitoes, the larval stage is easier to manage as they reside in one location and exhibit limited mobility. The larval stage constitutes the main phase of feeding and growth, making it the primary target for administering larvicides to evaluate their biological activity because a clear understanding of the mode of action and target sites of larvicides are crucial.[3]

Understanding the digestive system of insects is crucial for elucidating the utilisation of assimilated nutrients and for identifying the biochemical mechanisms targeted by insecticidal agents.[4] The insect alimentary canal is anatomically divided into the foregut, midgut and hindgut, each with distinct functions. Among these regions, the midgut is the principal site of digestion and absorption due to the presence of major digestive enzymes. The enzymes are regulated according to the nature of the ingested diet.

Insects rely on digestive enzymes such as proteases, amylases, lipases, and esterases to break down complex dietary compounds into simple, absorbable nutrients essential for survival, growth and reproduction. Disruption of enzyme activity limits nutrient uptake and discourages feeding, leading to mortality, thus making digestive enzymes key biochemical targets.[5]

Continued application of organophosphates and insect growth regulators is used worldwide for the control of mosquitoes[1]. When chemical pesticides are used for an extended period of time, resistance develops, and the species becomes accustomed to the insecticide, making eradication extremely challenging and causing mosquitoes to resurge. The biochemical and physiological characteristics of the midgut determine the resistance of insects to plant metabolites.[6]

The rising demand for alternative mosquito control strategies highlights plant-based metabolites as a promising and eco-friendly source. *Clerodendrum infortunatum,* belonging to the family Lamiaceae, distributed in tropical and subtropical regions of the world, has many ethnobotanical uses. Clerodendrum species have antimicrobial, anticancer, antimalarial, antioxidant, antidiabetic and antidiarrheal activities.[7]

2. Objectives

The objective of the study was to evaluate the effect of the acetone extract of *Clerodendrum infortunatum* against fourth instar larvae of *Culex quinquefasciatus*, emphasising the actions induced in the midgut histology and digestive enzymes α- amylase and protease.

3. Methods

Collection and culture of mosquito

The fourth instar larvae of *Culex quinquefasciatus* were reared in the laboratory by mixing dechlorinated tap water and two beaten eggs, and kept in the laboratory for a week.[8] Pungent smell created by the solution attracted mosquitoes for oviposition. The egg rafts were collected and maintained in the laboratory. Emerged larvae were identified using standard taxonomic keys.[9] Larvae were fed crushed dog biscuits in water. Fourth instar larvae were used for further analysis.

Larvicidal bioassay

Fourth instar larvae of *Culex quinquefasciatus* were exposed to 300 ppm acetone extract of *Clerodendrum infortunatum* for 24 hours. A sublethal concentration of 300 ppm was selected based on preliminary larvicidal bioassays (unpublished data). Experiments were conducted in six replicates with 20 larvae each. Control groups were maintained in distilled water without plant extract. After exposure, surviving larvae were collected for histological and enzymatic analyses.

Collection and preparation of plant extract

Fresh leaves of *Clerodendrum infortunatum* were collected from Thiruvananthapuram district, Kerala and washed thoroughly with dechlorinated tap water. The fresh leaves were cut into small pieces and shade-dried for 2 weeks. Dried leaves were pulverised in a domestic grinder. The plant powder was extracted in a Soxhlet apparatus using acetone as solvent and concentrated using a rotary evaporator. It was tested for various biological activities against *Culex quinquefasciatus*.

Preparation of stock solution

The dried plant extracts were made into 10% stock solution. A concentration of 300 ppm was used for further analysis.

Histopathological observation

The whole body of control and treated larvae were fixed in 10% formaldehyde for 48 h, followed by dehydration and cleared in xylene. Processed larvae were embedded

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in paraffin wax, sectioned and stained with Hematoxylin-eosin staining and photographed using a microscopic mounted camera (LABOMED).

Estimation of α- amylase

In a test tube 1mL of starch solution and 1 mL of tissue homogenate were mixed and incubated for 27^{0}C for 15 minutes. 2 mL of dinitrosalicylic acid was added, followed by heating in a boiling water bath for 5 minutes. 1 mL of potassium sodium tartrate solution was added to the warm tubes and cooled in running tap water. The reaction mixture was made up to 10 mL by adding distilled water. Using a spectrophotometer, the absorbance was read at 560 nm.[10]

Estimation of protease

The tissue homogenised with phosphate buffer, pH 7.4, was combined with 5 ml of casein reagent and mixed thoroughly and incubated for 10 minutes at 37^{0}C. After 10 minutes, 5 ml of Trichloroacetic acid (TCA) reagent was added and centrifuged at 3000 rpm for 2 minutes. 5 ml sodium carbonate and 1ml of Folin's reagent were added, mixed well and incubated for 30 minutes at 37^{0}C. The absorbance was measured at 600 nm. Blank served with no tissue homogenate. Tyrosine was used as a standard.[11]

Statistical analysis

Statistical analysis was done by Student's t-test using SPSS 20. The difference was considered significant at $p < 0.05$.

4. Results

Histological examination of stained tissue sections showed the effect of a sublethal concentration of 300 ppm in treated larvae compared to control. Post treatment, larvae exhibited significant histological aberrations. The control larvae showed normal and intact midgut (Fig.1). The observations under the light microscope revealed that acetone extract treatment at sublethal concentration affected gastric caecae and normal midgut architecture of the fourth instar larvae. Well-developed pharyngeal pump and gastric caecae were observed in the control group. The pharyngeal pump was enlarged and disintegrated in treated larvae. The epithelial cells of the midgut protruded, disordered with vacuole formation, cells were swollen and exhibited destruction of the basement membrane (Fig.2).

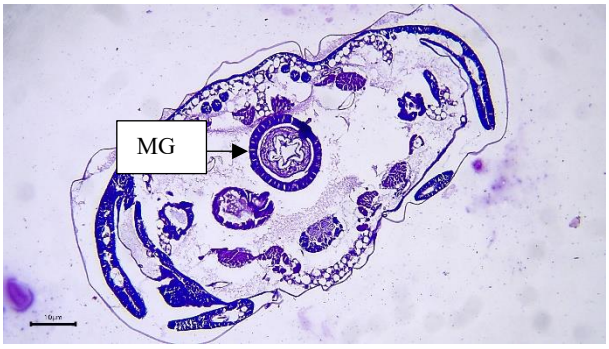

Figure 1. Photomicrograph of the cross-section of midgut (MG) of *Culex quinquefasciatus Say* (control). H&E 10x; scale bar = 10 µm

Alpha-amylase activity was determined using the dinitrosalicylic acid (DNS) method and expressed as µmol of maltose released per minute. The present study revealed that the α – amylase enzyme activity of the fourth instar larvae of *Culex quinquefasciatus* in control was 0.017 ± 0.00008 µmol maltose min^{-1}, while in the case of the larvae treated with the acetone extract of *Clerodendrum infortunatum* for 24h was 0.013 ± 0.00006 µmol maltose min^{-1}. The result showed a decrease in amylase activity with an inhibition of 23% in treated larvae compared to the control.

Figure 2. Photomicrograph of the cross-section of disrupted midgut (DMG) of *Culex quinquefasciatus Say* (treated).H&E 10x; scale bar = 10 µm

Protease activity was determined using casein as substrate and expressed as µmol of tyrosine equivalents released per minute. The results revealed a significant decrease in the protease activity with an inhibition of 81% in treated larvae compared to the control. The protease enzyme activity of the fourth instar larvae of *Culex quinquefasciatus* in control was 0.54 ± 0.004 µmol tyrosine min^{-1}, while in the case of the larvae treated with the acetone extract of *Clerodendrum infortunatum* for

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24hrs was 0.10 ± 0.003 µmol tyrosine min^{-1}. Each datum represents the mean ± SE of 6 replicates ($p < 0.05$).

5. Discussion

Most effective strategy for lowering target populations of dipterans presently seems to be the biological control of immature stages using plant-derived larvicides, as they are more promising, cost-effective and eco-friendly.

The present study reveals the histopathological changes in the fourth instar larvae of *Culex quinquefasciatus* treated with the acetone extract of *Clerodendrum infortunatum*. Sublethal concentration of the acetone extract of *Clerodendrum infortunatum* exhibited adverse effects on the midgut physiology of fourth instar larvae of *Culex quinquefasciatus*. Alterations in the structure of the midgut were found in the fourth instar larvae of *Culex quinquefasciatus*. Observation under a light microscope revealed that the treated larval midgut found to be severely damaged compared to the control post 24h treatment.

Normal nuclei and intact tall epithelial cells with intercellular contact and plasma membrane with well-developed brush border and basement membrane were observed in the control. In post-treated larvae, the midgut epithelial tissues protruded with vacuole formation, which shows cellular disruption. Vacuole formation might have resulted from the plant extract-induced cellular stress. Vacuoles sequester plant-derived compounds and trigger cellular responses. Similar observations were noticed when the leaf extract of *Schinus terebinthifolius* was treated against *Aedes aegypti,* where the midgut epithelium of the larvae disorganised with the formation of vacuoles. The elimination of the gut content enclosed in the peritrophic matrix indicated that the leaf extract interfered with the food passage along the digestive tract, and suggests that the presence of the extract in the larval environment disturbed the structural organisation of the midgut.[12]

Different plant-derived insecticides show subtle effects on the larval midgut. *Culex pipiens* larvae, when treated with *Lepidium sativum* seed extract, showed blebbing and protruding of epithelial cells, degraded microvilli with damaged basal membrane and vacuolized cells without proper nuclei.[13] Natural compound Pellitorine caused devastated effect on the midgut of *Aedes aegypti* larvae, where midgut epithelial cells were completely damaged, and fat bodies were demolished.[14] In the present study, the treated larvae also exhibited damaged fat bodies.

Acetone extract-treated larvae showed aberrations due to the reaction of the extract with the midgut and release of gut contents, with the decreased activity of proteases and amylases. The larval midgut assimilates plant-derived compounds from the gut lumen, like nutrients, through the midgut epithelium.[15] Disruption of the midgut causes loss of nutrients and damage to digestive cells and disruption of microvilli. Enteroendocrine cell damage causes deregulation of secretion and absorption of substances. Partial or complete disorganisation of the midgut and peritrophic membrane (PM) results in an imbalance of homeostasis.[13]

Amylases are hydrolytic enzymes that catalyse the hydrolysis of the α-D-(1,4)-glucan linkage in glycogen and other related carbohydrates.[16] Proteases are crucial enzymes in insects as they break down peptide bonds in dietary proteins to release amino acids essential for growth, survival and reproduction.[17] The current study revealed the inhibition of amylase and protease when treated with plant extract. Since the insect midgut is the primary source of digestive enzymes, a disrupted midgut leads to a decline in digestive enzymes. Disorganised midgut together with a decline in enzymatic activity might be the reason for larval mortality after treatment.

6. Conclusions

The work reports the effect of the acetone extract of *Clerodendrum infortunatum* on the midgut and digestive enzymes of fourth instar larvae of *Culex quinquefasciatus*. Major digestion of dietary products takes place in the midgut. Plant extracts are directly assimilated by the cells of the midgut, leading to deleterious effects on midgut histology. Extract of *Clerodendrum infortunatum* inhibited the digestive enzymes α-amylase and protease of fourth instar larvae. The midgut was completely damaged, exhibiting blebbing and vacuolisation. Further studies are necessary for the isolation of active components in the plant extracts which are responsible for the insecticidal activity.

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