Original article

Evaluation of the effects of neem leaf (*Azadirachta indica*) extract on the morphology, development, biochemical composition and digestive enzyme activity in *Drosophila melanogaster*

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Abstract

The present study investigates the impact of neem (*Azadirachta indica*) leaf extract on *Drosophila melanogaster*. Flies were reared on 50 mL of food medium supplemented with varying concentrations of neem extract (1%, 5%, and 10%). Observations were made on changes in body size and external morphology, development and metamorphosis, and biochemical responses. Significant alterations were recorded in the levels of total proteins and amino acids, as well as in the activity of key digestive enzymes such as amylase, protease, and trehalase. Larval development was adversely affected, and severe morphological deformities were observed when the highest concentration of the extract was used. Neem extracts caused varying levels of stress responses depending on their concentrations. The study thus demonstrates that neem extract exerts dose-dependent effects on *Drosophila melanogaster*, potentially affecting digestion, metabolism, growth, larval development, and metamorphosis, and may serve as a natural and eco-friendly biopesticide alternative.

Keywords: Amylase, *Azadiracta indica*, biopesticide, *Drosophila melanogaster,* protease, trehalase,

Introduction

The increasing global demand for food security has led to intensive agricultural practices that heavily rely on synthetic pesticides for crop protection. These chemical insecticides have improved agricultural productivity; however, their widespread and indiscriminate use causes numerous environmental and health concerns. Synthetic pesticides persist in the environment, contaminate soil and water resources, disrupt ecological balance by affecting non-target organisms, and pose serious health risks to humans through bioaccumulation in the food chain. Furthermore, the development of pesticide resistance in target pest populations has necessitated the application of higher concentrations and more frequent treatments, further exacerbating these problems. In response to these concerns, there has been a shift towards developing sustainable and eco-friendly alternatives to conventional pest control methods. Natural plant-based insecticides have emerged as promising candidates due to their biodegradable nature, lower environmental persistence, and reduced toxicity to non-target organisms. Neem (*Azadirachta indica*) has gained considerable attention as a potential source of natural insecticides.[1] *Azadirachta indica*, commonly called 'Indian Lilac' belongs to the family

Meliaceae.[2] Neem exhibits antiallergenic, antidermatic, antifeedant, antifungal, anti-inflammatory, antipyorrhoeic, antiscabic, cardiac, diuretic, insecticidal, larvicidal, nematicidal, oviposition-deterrent, and spermicidal activities among others. These biological properties are attributed to the presence of bioactive compounds such as azadirachtin, nimbin, salannin, and other limonoids.[3] These compounds interfere with various physiological processes in insects, including hormone regulation, feeding behaviour, reproduction, and development, making neem extracts highly effective as natural pest control agents. A neem-based fertilizer has been effective against the pest, southern armyworm. Neem cake is often used as a fertilizer. *Azadirachta indica* has been found to be toxic to non-target organisms as well.[4]

Drosophila melanogaster, commonly known as the fruit fly can be used as an ideal model organism for investigating the biochemical effects of potential insecticides.[5] This species has been extensively used in biological research due to its short life cycle, well-characterized genetics, ease of culturing, low cost, short generation time, and physiological similarities to other insects.[6] The wealth of genetic and molecular tools available for *Drosophila melanogaster* makes it particularly suitable for understanding the mechanisms of action of bioactive compounds and their effects on insect physiology and behaviour.[7]

Understanding the biochemical mechanisms underlying the insecticidal activity of neem leaf extract is important for optimizing its application as a natural pesticide. It may contribute to the development of more effective formulations, appropriate dosage recommendations, and targeted application methods. The development of natural insecticides represents a critical step towards sustainable agricultural practices that minimize environmental impact while maintaining effective pest control.The present study aims to investigate the biochemical effects of neem leaf extract on development and metamorphosis and various physiological and biochemical parameters in *Drosophila melanogaster*, with a view to providing insights into the cautious and environmentally safe use of neem-based formulations as pesticides. The findings from this investigation are expected to contribute to the growing body of knowledge on the application of biopesticides in pest management.

Materials and Methods

Preparation of neem leaf extract

Fresh *Azadirachta indica* leaves were collected and washed with running tap water and rinsed with distilled water to remove dust and debris. The neem leaves were crushed using a mortar and pestle, adding distilled water (4 mL of water /g). The extract of the crushed leaves was filtered through Whatman filter paper and the filtrate obtained was used for further experiments.[8]

Preparation of *Drosophila* food medium

A base culture medium was prepared by adding corn flour (5g) and agar (1g) in 100 mL distilled water. The mixture was boiled and subsequently cooled. Before solidification, yeast and sucrose were added. Test food media containing 1%, 5%, and 10% neem leaf extracts were prepared by adding the respective extracts to the base medium, in the proportions required, maintaining the final volume at 50 mL. Each of these were then mixed thoroughly, and allowed to solidify. The base medium without the neem leaf extract was used as the negative control.

Collection of *Drosophila melanogaster*

Drosophila melanogaster specimens were collected using a beaker with curd as an attractant. The beaker was covered with cling film perforated with small pores, allowing the flies to enter while preventing their escape.

Rearing of *Drosophila*

Adult *Drosophila* (20–50 individuals per vial) were introduced into culture vials containing the test food medium treated with a specific concentration of neem extract. The flies were maintained in these vials for 2–3 days to allow females to lay eggs. Thereafter, the adults were removed, and the vials were plugged with cotton. The eggs were allowed to hatch, and the newly emerged larvae were maintained on the same treated food medium until the end of the experiment.

Biochemical estimations

Estimation of protein and amino acid

Protein and amino acid estimation was done using Lowry method.[9] *Drosophila* adults that were previously exposed to different concentrations of neem leaf extract were used for the estimation of protein and amino acid. For this, 50 mg of *Drosophila* adults were cold anaesthetised and homogenized in 1mL ice cold water. 250 µL of the homogenate was taken in an Eppendorf tube. 250 µL of 10 % trichloroacetic acid was added to it and shaken well and centrifuged at 3000 rpm for 10 min. The pellet obtained was used for protein estimation and the supernatant for amino acid estimation.

For protein estimation, the pellet was dissolved in 0.1N NaOH and made up to 1mL. One mL of distilled water was taken as the blank. To the sample, 3.5 mL of alkaline copper sulphate solution was added and mixed well and kept it for 10 min. To this, 0.5 mL of Follin's reagent was added. It was mixed well by vigorous shaking and kept at room temperature for 30 min. 2.5% Bovine serum albumin was used as the standard. Optical density (OD) was recorded at 660 nm.

Supernatant was used for amino acid estimation. To 0.5 mL of supernatant, 0.5 mL of NaOH was added. To this, 3.5 mL of alkaline copper sulphate solution was added and mixed well and kept it for 10 min followed after adding 0.5 mL of Follin's reagent. It was mixed well by vigorous shaking and kept it at room temperature for 30 min. One mL of distilled water similarly treated with reagents was taken as the blank. A 2.5 % solution of tyrosine was used as the standard. Optical density was measured at 540 nm. The concentration of protein / amino acid in the sample was calculated by the formula:

$$\frac{\text{(Concentration of standard) (OD of test)}}{\text{OD of standard}}$$

Digestive enzyme assay

Preparation of enzyme extract

Cold anaesthetised 50 mg *Drosophila* adults, after washing thoroughly with insect saline were transferred to 1 mL of ice-cold distilled water in an Eppendorf tube, homogenized and centrifuged at 5000 rpm for 10 min at 4ºC. The supernatant obtained was used for digestive enzyme assay.

Amylase

The method used by Sreekumar and Prabhu (1988)[10] was followed for determining amylase activity. To 0.2 mL of enzyme extract, 0.2 mL of Tris- HCl buffer (pH 8.2) and 0.4 mL of 1% starch solution were added and incubated at 37° C for 30 min. The reaction was stopped by adding 1.2 mL of 3, 5-dinitrosalicylic acid followed by heating at 50°C for 5 min. The absorbance of the solution was read at 550 nm and µg of maltose equivalents liberated was calculated using 0.01% of maltose solution as standard.

Protease

Protease activity was determined using the method of Birk *et al*. (1962).[11] To 0.2 mL of enzyme extract, 0.2 mL glycine NaOH buffer (pH 9) and 0.4 mL 1% casein solution were added. Enzyme activity was terminated after 30 min of incubation at 37°C by adding 1.5 mL of 5% trichloroacetic acid, and centrifuged at 13000 rpm for 15 min. The supernatant was read at 250 nm using 0.005% of tyrosine as standard.

Trehalase

The method of Friedman (1996)[12] was used for the estimation of trehalase activity based on the rate of glucose generation from trehalose. To 0.2 mL of enzyme extract, 0.2 mL of 60 mM citrate buffer and 0.2 mL 10 mM trehalose were added and incubated for 15 min at 32ºC. The reaction was stopped by the addition of 1 mL of $Ba(OH)_2$ and 1 mL of 0.5 M $ZnSO_4$. The volume was made up to 5 mL by adding distilled water. The reaction mixture was centrifuged at 6000 rpm for 5 min. The supernatant was used for the estimation of glucose. To 1 mL of supernatant, 4 mL of anthrone reagent was added, mixed well and kept in a water bath for 15 min. For the blank, 1 mL of distilled water was used instead of the enzyme extract and 0.1% glucose was used as standard. OD was measured at 620 nm.

Statistical analysis of data

Student's *t*-test was employed to evaluate the statistical significance of the data obtained from the experiments.

Observations and Results

Life cycle of *Drosophila*

The life cycle of *Drosophila melanogaster* includes four stages, *viz.*, egg, larvae comprising three instars, pupa and adult (Figure 1). The duration of various instars with their duration, and morphometric parameters are presented in Table 1.

Effect of neem leaf extract treatment on *Drosophila*

In *Drosophila* larvae reared on food media containing neem leaf extract, development and growth were found to be impaired depending on the concentration of the extract. The effects were less pronounced

Figure 1: The developmental stages of Drosophila melanogaster. Above, from left to right: egg, first instar larva, and second instar larva; below, from left to right: third instar larva, pupa and adult

when the concentration of the extract was low at 1%. During the early stages, the larvae appeared pale and slender, indicating a clear alimentary canal devoid of food. As development progressed, the body became darker, and the cuticle showed a wrinkled appearance. These effects were more pronounced at higher concentrations of the extract, adversely affecting pupation and adult emergence (Figure 2). The adults exhibited developmental deformities such

Table 1: Duration of developmental stages and related morphometric parameters in *Drosophila melanogaster*

Developmental stages*	Duration	Length (mm)	Width (mm)
Egg	24 h	0.51±0.02	0.21 ±0.03
First instar	24 h	1.02±0.03	0.24 ±0.03
Second instar	24 h	2.40±0.10	0.52 ±0.02
Third instar	48 h	4.50±0.20	1.02 ±0.03
Pupa	4 -5 days	3.27±0.26	1.21 ±0.04
Adult	40-50 days	2.96±0.50	1.00 ±0.20

*The values are presented as mean ± standard deviation of 8 observations.

as crumpled bodies or malformed wings, as shown in Figure 2. The duration of the life cycle revealed an increase in treated larvae, compared to the control.

Figure 2: Malformed larvae and adults of *Drosophila melanogaster*, treated with neem leaf extract

Effect of neem leaf extract treatment on protein and amino acid levels in *Drosophila*

Neem leaf extract caused a significant reduction in both protein and amino acid levels. The decrease was evident at all tested concentrations, with a pronounced decline in protein content observed at the highest concentration (10%) of the extract. (Table 2 and Figure 3).

Effect of neem leaf extract treatment on digestive enzyme levels in *Drosophila*

Protease activity increased with increasing concentrations of neem leaf extract from 1% to 10%. Amylase activity showed fluctuations at different concentrations of the extract while Trehalase showed a dose dependant decrease in activity when compared with controls (Table 3 and Figure 4).

Table 2: Effect of varying concentrations of neem leaf extract on protein and amino acid levels in *Drosophila melanogaster*

Concentration of extract (%)	Protein level (mg/ mL)	Amino acid level (mg/mL)
Control	8.07± 0.88	11.20 ± 0.64
1	4.63 ±0.31*	5.65 ± 1.39*
5	5.89 ±0.52*	8.44 ± 0.69*
10	3.81 ±0.34*	7.05 ± 0.30*

*Significant at 0.05 level with respect to control and the values are presented as mean ± standard deviation of 6 observations.

Table 3: Effect of varying concentrations of neem leaf extract on protease, amylase and trehalase levels in *Drosophila melanogaster*

Concentration of extract (%)	Protease activity in units [a]	Amylase activity in units [b]	Trehalase activity in units [c]
Control	80.00 ± 3.977	11.54 ± 1.15	43.67 ± 1.53
1	76.34 ± 3.69*	9.50 ± 1.095*	39.89 ± 1.39*
5	86.23 ± 2.17*	27.67 ± 2.52*	39.11 ± 1.02*
10	93.22 ±1.34*	14.89 ± 0.86*	34.74 ± 2.29*

[a]µg of tyrosine liberated / min /mL of extract; [b]µg of maltose equivalents liberated /min /mL of extract; [c]µg of glucose liberated / min /mL of extract.

*Indicates values that are significant with respect to control at 0.05 level and the values are presented as mean ± standard deviation of 6 observations.

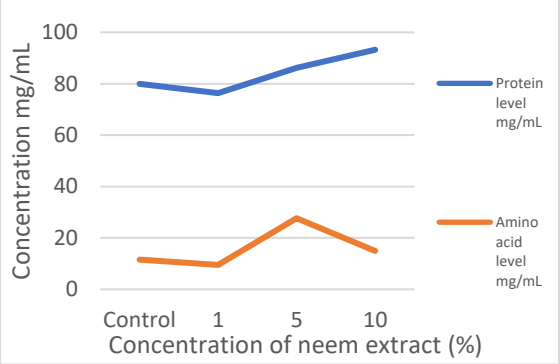

Figure 3: Effect of different concentrations of neem leaf extract on protein and amino acid concentration in *Drosophila melanogaster*

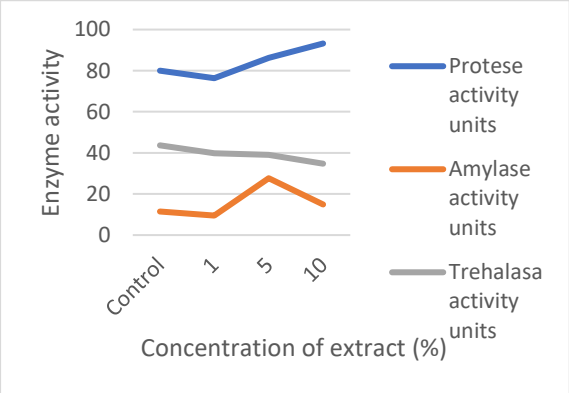

Figure 4: Effect of different concentration of neem leaf extract on digestive enzyme activity of *Drosophila melanogaster*

Discussion

Drosophila melanogaster being a holometabolous insect, the life cycle includes a larval stage comprising three instars, followed by pupal and adult stages. Neem contains a large number of biologically active compounds, and more than 140 compounds have been isolated from different parts of the neem. Neem leaf constituents are found to have immunomodulatory, anti-inflammatory, hyperglycemic and antibiotic properties.[13]

It was observed in this study that the presence of neem leaf extract in food media impaired the growth and development of *Drosophila* larvae; this effect was dependent on the concentration of the extract. Effects were minimal at a low concentration of 1%. Initially, larvae appeared pale and slender, suggesting an empty alimentary canal and reduced feeding. At the later stages, the body darkened, and the cuticle became wrinkled. These adverse effects intensified with higher extract concentrations, severely inhibiting pupation and adult emergence. The initiation of metamorphosis depends on several external and internal cues. In *Oryctes rhinoceros*, it has been observed that the final instar larvae must either attain a critical age[14] or reach a threshold body weight [15] to successfully pupate. Neem-treated larvae often fail to undergo metamorphosis, as they are unable to achieve these critical conditions. This results in a prolonged larval instar duration. The present study shows that surviving adults of *Drosophila* treated with neem extract, exhibit severe developmental deformities, such as crumpled bodies or malformed wings as may be seen in Figure 2. Such deformities are also observed in other insects. [16,17] The prolonged life cycle observed in larvae treated with neem extract, compared to the control, observed in the present study, can be attributed to the interference of certain neem compounds with the endocrine system. This interference may be mediated by certain compounds present in the neem extract that may mimic or block hormones such as ecdysone and juvenile hormone, thereby disrupting critical processes like moulting, metamorphosis, and overall growth. In addition to its direct toxicity, azadirachtin influences several physiological processes in insects, including growth regulation, protein synthesis, reproduction, and hormonal balance, by affecting both ecdysteroid and juvenile hormone titres.[18,19]

In the present study, a decrease in whole-body protein content was observed in neem-treated larvae. This reduction may be attributed to the neem compound azadirachtin, which can interfere with ribosomal function, enhance proteolysis, and trigger stress and energy demand, causing insect to break down proteins by proteolysis.[20] Neem compounds are also

known to induce oxidative stress, resulting in protein denaturation and degradation. An earlier study reports that treatment with neem extract causes a significant reduction in total protein, carbohydrate, and lipid levels in the lepidopteran larvae of *Pericallia ricini*.[21]

Insects maintain a rich pool of amino acids that act as anti-stress agents, protecting them from various environmental stressors. Under stress conditions, these amino acids are utilized for the synthesis of specific stress-related proteins, such as heat shock proteins, antimicrobial peptides[22] or other novel peptides, which may lead to an elevation in total protein content. Amino acids are essential for protein synthesis, and most insects obtain their amino acid requirements from dietary proteins. In the present study, the total free amino acid levels in adult *Drosophila melanogaster* cultured on food media containing different concentrations of neem extract were estimated. The results showed that treatment with neem extract significantly decreased the total free amino acid supporting the observations of the aforementioned report. Feeding inhibition, disruption of protein metabolism, or mild oxidative stress may also explain the decrease in amino acid concentration.

Among the most important benefits of neem application are the insecticidal and feeding deterrent characteristics of the products.[19,23] In insects, feeding activity strongly influences the secretion of digestive enzymes. The act of feeding itself, or the presence of food materials in the midgut—particularly proteins, which serve as major secretagogues—can stimulate enzyme release. While feeding is regulated by neural mechanisms, enzyme secretion by secretagogues is mediated through neuroendocrine pathways, aided by midgut hormones.[24] Azadirachtin, in neem extracts, is known to disrupt insect physiology and digestion. In *Drosophila* larvae, neem treatment may induce a state of starvation by reducing feeding activity, thereby adversely affecting enzyme secretion.[25]

Amylase is an enzyme that degrades starch, first into oligosaccharides and then into maltose and glucose, by hydrolyzing α-1,4-glycosidic bonds.[26] Khosravi and Sendi (2013) reported that treatment of fifth-instar *Glyphodes pyloalis* larvae with azadirachtin resulted in a reduction in α-amylase activity, with the decrease becoming more pronounced at higher concentrations of the plant extract.[27] Similarly, sublethal concentrations of pyrethroids were found to decrease α-amylase activity in the larval gut of the beetle *Tribolium castaneum*.[28] Treatment with *Artemisia annua* extract also caused a reduction in α-amylase activity in *Eurygaster integriceps*, and this reduction increased with higher concentrations of the extract.[29] . In the elm leaf beetle treated with *Artemisia annua* extract, α-amylase activity decreased after 24 hours but sharply increased after 48 hours, indicating a possible loss of potency of the extract over time. [30] In the present study, treatment of *Drosophila melanogaster* with a low concentration (1%) of neem leaf extract caused a reduction in amylase activity. The reduction in α-amylase activity caused by plant extracts could be attributed to plant defence compounds that act on insect gut enzymes or to the cytotoxic effects of the extract on amylase-secreting cells of the midgut.[31] At lower concentrations, neem's active constituents (azadirachtin) might slightly inhibit enzyme synthesis or activity. However, when the concentration of neem extract was increased to 5%, amylase activity showed an increase, followed by a decrease at the highest concentration tested. These results suggest that both the concentration of neem extract and the duration of exposure may influence amylase secretion through multiple physiological mechanisms.

The present study revealed a dose-dependent decrease in trehalase activity at different concentrations of neem extract.

Trehalase plays a vital role in maintaining haemolymph sugar levels, which are primarily composed of trehalose, a disaccharide. An increase in trehalose concentration raises the osmotic pressure of the haemolymph, which can inhibit gut emptying and indirectly suppress feeding.[32] The decrease in trehalase activity, which remained relatively constant across all concentrations of the extract, observed in the present study, may represent a response to physiological stress, for maintaining homeostasis.

Azadirachtin inhibits peristalsis, reduces enzyme production as food passes through the gut, interferes with midgut cell renewal, and suppresses feeding.[33] It is reported that botanical insecticides can interfere with the production of certain proteases, impairing the digestion of ingested proteins.[34] Azadirachtin A inhibits the growth and development of *Bactrocera dorsalis* larvae by releasing cathepsin in the midgut.[35] Similarly, azadirachtin causes a reduction of the protease activity in *Glyphodes pyloalis*.[27,36] It has also been reported that digestive protease of *Cnaphalocrocis medinalis* was suppressed by extracts of *Vitex negundo* and *Azadirachta indica*.[37] Similar to their effects on α-amylase activity, plant defence compounds can also influence the secretion of proteinases.[38,39] *Heliothis virescens* larvae reared on an artificial diet containing azadirachtin exhibited increased digestibility,[37] presumably due to a reduced rate of food passage through the gut.[40] In the present study, *Drosophila melanogaster* larvae showed a significant decrease in protease activity when treated with a low concentration of neem extract. However, protease activity increased as the concentration of neem extract was raised. This increase could represent a physiological response to stress induced by azadirachtin or other bioactive compounds present in the extract. Digestive physiology plays a critical role in insect adaptation to stress factors. Although insects exhibit remarkable plasticity in their digestive systems, a comprehensive understanding of how digestive physiology mediates adaptation to various stressors remains limited.[41]

Phytophagous insects are known to regulate their digestive enzyme activity in response to plant defence proteins such as chitinases, lectins, and enzyme inhibitors.[42-44] Such responses may not always follow the usual pattern of enzyme regulation. For example, the midgut trypsin activity of *Ephestia kuehniella* larvae decreased following treatment with *Inga vera* trypsin inhibitor, whereas chymotrypsin-like activity increased.[45] *Tenebrio molitor* compensates for trypsin inhibition by enhancing cathepsin activity, thereby maintaining digestive efficiency.[46]. Similarly, *Hyphantria cunea* larvae exposed to α-amylase inhibitors showed decreased midgut α-amylase activity but increased trypsin activity.[41] Some insects also employ homeostatic strategies to modulate digestive enzyme activity when exposed to plant secondary metabolites. Exposure to azadirachtin significantly inhibited the growth of *Bactrocera dorsalis* larvae but was accompanied by a notable increase in cathepsin activity.[35] The elevated cathepsin activity in *Bactrocera dorsalis* may represent a compensatory digestive response, enabling larvae to meet the heightened energy demands associated with metamorphosis under azadirachtin-induced stress. The results of the present study indicate that digestive enzyme secretion in *Drosophila* larvae exhibits variable responses to neem extract depending on both the duration of exposure and the concentration of the extract. It is possible that at low concentrations, the insects did not initiate a compensatory stress response. However, at higher concentrations (around 5% or above), enzyme activity may increase, as moderate or elevated stress levels could induce adaptive metabolic adjustments, including the upregulation of digestive enzymes to counteract the physiological challenge. The effects of neem application are not

always consistent, as they depend on several factors such as insect species, timing and method of application, and the concentration used. [47] Excessive concentrations can be toxic, highlighting the importance of dose optimization in potential pest control applications. For this reason, it is necessary to have more information on the gut enzymatic activity of insects to devise a rational strategy for insect pest control utilizing plant extracts.[27]

Conclusion

This study characterizes the bioactive effects of neem leaf extract on *Drosophila melanogaster* larvae. When incorporated into the culture medium, the extract acts as a potent antifeedant. It appears to induce physiological stress, leading to adverse alterations in key biochemical parameters, including haemolymph protein and amino acid concentrations, as well as digestive enzyme secretion. These disruptions result in arrested larval development and metamorphosis, confirming the extract's strong potential as a natural insect growth regulator.

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Conflicts of interest

There are no conflicts of interest.

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