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Effect of Papaya Seed on Mortality and *Midgut* Histopathology in *Aedes aegypti* Larvaes

Cynthia Agustini[1], Hasmiwati[2*], Afriwardi[3]

[1]Department of Parasitology, Faculty of Medicine,Universitas Negeri Padang

[2]Department of Parasitology, Faculty of Medicine, Universitas Andalas

[3]Department of Physiology, Faculty of Medicine, Universitas Andalas

* Corresponding author.
E-mail address: hasmiwati65@med.unand.ac.id

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Abstract

Background: Indonesia is an endemic region for Dengue infection. The World Health Organization (WHO) reported Dengue as one of the top ten global health threats in 2020. Dengue is a viral infection transmitted primarily by the Aedes aegypti mosquito, causing Dengue Fever and Dengue Hemorrhagic Fever. Temephos 1.25% has been the WHO recommended standard larvicide. However, prolonged use and continuously increasing doses may result in resistance and toxicity, posing risks to the environment and human health. This necessitates the discovery of alternative larvicides that are effective in killing larvae with minimal side effects, particularly those derived from natural materials.
Methods: This research intends to illustrate the impact of papaya seed extract on the death rate of larvae and the histopathological changes in the midgut of Ae. aegypti larvae. A genuine experimental framework featuring a control group with only post-testing was utilized for this investigation. The sample comprised 750 Ae. aegypti larvae, which were categorized into six groups: one negative control group (aquades), one positive control group (temephos 1. 25%), and four treatment groups using papaya seed extract at varying concentrations of 2 mg/ml (P1), 2. 5 mg/ml (P2), 3 mg/ml (P3), and 3. 5 mg/ml (P4).
Results: The results showed that all larvaes died within 24 hours in the positive control, P1, P2, P3, and P4 groups. Probit analysis of papaya seed extract revealed LC50 at 2.599 mg/ml and LC90 at 4.776 mg/ml after 8 hours of exposure.
Conclusion: This study concludes that papaya seed extract affects larval mortality and midgut histopathology alterations in Ae.aegypti larvae. These findings indicate that papaya seed extract holds great potential as a natural and environmentally safe as alternative larvicide to disrupt the development of dengue fever vectors.

Keywords: *Aedes aegypti, Carica papaya, Dengue, Larvacide, Midgut, Temephos*

Introduction

Dengue is an illness brought on by the Dengue virus (DENV), which leads to Dengue Fever (DF) and Dengue Hemorrhagic Fever (DHF). Mosquitoes are the carriers of this disease, with Aedes aegypti being the main carrier and Aedes albopictus as a secondary carrier. This infection frequently occurs in tropical and subtropical areas, resulting in about 100 to 400 million incidents worldwide annually.[1] In early 2020, the World Health Organization (WHO) reported dengue as one of 10 diseases threatening global health. If this disease is not treated quickly and appropriately, it can trigger outbreaks event in the community, dengue shock syndrome (DSS) and even death.[2] Indonesia is one of the endemic countries to dengue. DHF cases in Indonesia in 2022 increased compared to previous years, with 143,266 cases reported and 1,237 deaths.[3]

The government has implemented various efforts to prevent DHF, such as eradicating vectors to break the transmission chain in disease control. The controls that have been carried out by the government are like

implementing fogging to kill adult mosquitoes and applying toxic substances to eliminate mosquito larvae such as sprinkling temephos 1.25% (abate) powder into water reservoirs in households. Temephos 1.25% is an organophosphate insecticide recommended by WHO as a standard. However, its use has been found to have side effects that can be dangerous for public health and environment. Several places have reported incidents of temephos resistance, such as Brazil, Bolivia, Argentina, Cuba, the Caribbean and Thailand. Meanwhile, in Indonesia, specifically in the city of Padang, resistance to temephos has been identified in the Jati and Mount Pangilun areas.[4] The use of organophosphate substances whose doses are continuously increased can cause toxicity. Therefore, it is necessary to discover alternative larvicides that are effective and have minimal side effects such as those derived from natural materials.

Papaya seeds are a waste material that is often thrown away by people. However, several studies have shown that papaya seed contains various compounds such as alkaloid, saponin, tannin and flavonoid which have larvicidal effects.[5] The midgut of Ae. aegypti larvae is tubular and divided into anterior, medial and posterior sections. Midgut lined by cylindrical epithelial cells which are supported by basal membrane. These epithelial cells have round nucleus, heterogeneous and basophilic cytoplasm. The cell surface contains brush border (microvilli) that helps expand the surface area for nutrient absorption in the larvae.[6]

Research has shown that permot leaf plant (Passiflora foetida) contains active compounds such as alkaloid, flavonoid and saponin which are lethal to larvae and cause damage to the midgut of Ae. aegypti larvae.[6] Meanwhile, other studies also reported that starfruit (Averrhoa bilimbi) extract has larvicidal effects due to its content of saponins, tannins, and terpenoids.[17] These compounds are also found in papaya seed. Therefore, the author is interested in studying the effect of papaya seed extract on the histopathology features of the midgut in Ae. aegypti larvae.

Methods

The design of this study was based on an authentic experimental approach, with the use of a post-test control group as the only intervention. The investigation took place between June and October 2024. The subjects of this research were larvae derived from Ae. aegypti eggs that were acquired from Universitas Putra Malaysia (UPM). The minimum required sample size for this research was established according to the WHO guidelines from 2005 titled “Guideline For Laboratory and Field Testing of Mosquito Larvicides,” which mandates at least 25 larvae for each treatment group. The study included a total of 6 treatment groups along with 5 repetitions, necessitating 750 larvae in total. The samples were chosen based on certain inclusion and exclusion criteria. The inclusion criteria specified that the larvae must be Ae. aegypti and reach the third instar stage, be healthy, and show active movement. Conversely, the exclusion criteria ruled out any larvae that did not successfully develop. This study received ethical clearance from the Research Ethics Committee at the Faculty of Medicine, Andalas University, with approval number 415/UN. 16. 2/KEP-FK/2024.

Preparation of Papaya Seed Extract

The tools and materials used in making papaya seed extract included blender, 60-mesh sieve, analytical balance, suction funnel, filter paper, rotary evaporator, digital scales, measuring cylinders, oven, container, aquadest (5 L) and ethanol 96%. Raw materials consisted of 500 g papaya seeds. Papaya seeds were obtained from the waste of Penang papaya. Initially, the seeds underwent sorting and were rinsed with fresh water. Afterwards, they were left to dry in the air until they were entirely moisture-free. Once dried, the seeds were blended into a fine powder and sieved using a 60 mesh sieve. A total of 500 grams of papaya seed powder were weighed and put into a container, soaked in 96% ethanol in a ratio of 1: 3 for 4 days, a process known as maceration. Then, the ethanol mixture was filtered using filter paper and suction funnel. The separation of ethanol and the chemical compounds in the papaya seed was performed using rotary evaporator for 2 hours. The resulting extract was then oven dried at 40°C to produce a thick extract.

Procedure for Rearing Aedes aegypti Larvae

The tools and materials for rearing Ae. aegypti larvae included Ae. aegypti larvae (instar III–IV), fish food, plastic dropper pipettes, plastic containers, tilt cloth and wooden sticks. Egg hatching was conducted by filling a plastic

container halfway with mineral water. Ae. aegypti egg were placed in the the container and covered using a tilt cloth. The temperature, pH and humidity were controlled to ensure simultaneous hatching, maintaining a temperature of 25-30°C, water pH between 5.8-8.6, and humidity 80-90%.8,9 Once hatched, fish food which has been ground finely with a mortar and sprinkled a little into a plastic container as nutrition for the larvae. The water in the plastic container was replaced daily. Next, identification of the third instar Ae. aegypti larvae was performed based on specific characteristic: S shaped body, size of 4-5 mm, has large and fat siphon on the last abdominal segment, prominent spines on the thorax, dark brown siphon, and a resting position forming an angle at the water surface. The abdominal side of the 8th segment is present comb teeth which is regular with a shape like a thorn or trident.[7]

Procedure for Giving Papaya Seed Extract to Larvae

Plastic cup measuring ±350 ml were prepared. The cups in the negative control group received 200 mL of aquades. 200 ml of aquades combined with 0.02 mg/L of temephos 1,25% was used as the positive control group. Papaya seed extract was dissolved at concentrations of 2 mg/ml (P1), 2.5 mg/ml (P2), 3 mg/ml (P3), and 3.5 mg/ml (P4) for the treatment groups. up to 200 ml in aquades. Total of 25 test larvae were placed into each cup, and the cups were covered with tilt cloth. The mortality of the larvae was observed by gently probing them with a flexible stick to check their movement. Dead larvae were separated from the cup and preserved in 30 ml bottles containing 10% formalin.

Preparation and Examination of Midgut Histopathology in Larvae

The tools and materials for preparation and examination of midgut histopathology in larva included microtome, glass slides, cover glasses, light microscope, formalin 10%, paraffin, ethanol 100%, hematoxylin-eosin staining, xylol, and alcohol (70% and 96%). Steps for preparing and examining the histopathology of larval midgut.

Preparation of Tissue Slides

The larvae tissue was processed using the paraffin method. The steps included tissue processing: fixation in 10% formalin for 0-3 hours, dehydration in graded ethanol, clearing in xylol, and impregnation with paraffin. With a microtome, the paraffin blocks holding the larvae were sliced transversely to a thickness of 4 to 6 micrometers. Staining with Hematoxylin-Eosin

The slides were dehydrated in 96% alcohol, immersed in hematoxylin-eosin solution for 5-10 minutes, rinsed with water, stained with eosin for 3-5 minutes, washed with graded alcohol (70-96%), dried, immersed in xylene for 2-3 minutes, and cleaned with tissue. Finally, 1-2 drops of entellan were added, and the slides were covered with cover glass.

Observation of Midgut Histopathology

Midgut histopathological changes were observed under a light microscope at 200x and 400x magnifications. The observations included midgut diameter, epithelial cell height, the number of viable epithelial cells, degenerated epithelial cells, brush border damage, and basal membrane damage.

Data Analysis

Larval mortality data were initially tested for normality and homogeneity. If data were normally distributed and homogeneous, One-Way ANOVA was conducted, followed by Bonferroni post hoc test to identify differences among groups. Effective concentrations (LC50 and LC90) were determined through Probit analysis. Histopathology was also tested for normality and homogeneity. Next, the Kruskal-Wallis test was conducted to examine the relationship between papaya seed extract administration and the histopathology profile of the Aedes aegypti larvae midgut. Then, the Mann-Whitney test was performed to determine differences between each treatment group.

Results

The study found that all larvae in the positive control group and all papaya seed extract groups died within 24 hours. In contrast, no mortality was observed in the negative control group. The variables observed and analyzed in the histopathology examination of the midgut of Ae. aegypti larvae included midgut diameter, epithelial cell height, viable epithelial cells, degenerated epithelial cells, brush border damage, and basal membrane damage. Statistical analysis revealed the data were not normally distributed and non homogeneous, so they were analyzed using the Kruskal-Wallis test.

Effect of Papaya Seed Extract on the Mortality Rate of Ae. aegypti Larvae

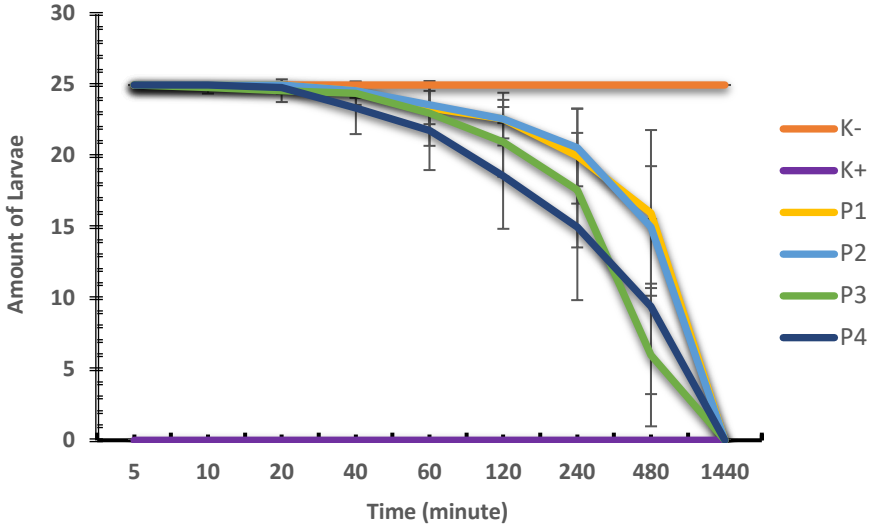


Figure 1. Graph of *Ae. aegypti* Larval Mortality Over Time

Figure 1 shows that all larvae died within 24 hours in the positive control, P1, P2, P3, and P4 groups. The fastest mortality rate was observed in the positive control group. Among the papaya seed extract groups, the highest mortality within 8 hours of exposure occurred in the P3 group, while the lowest mortality was observed in the P1 group.

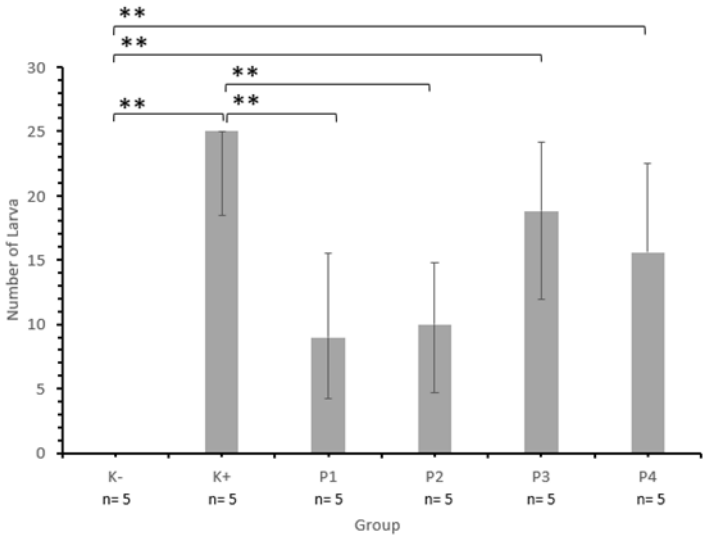


Figure 2. Mortality of Ae.aegypti larvae after exposure to papaya seed extractin the 480th minute. Description: K(-) = negative control (aquades); K(+) = positive control (temefos 1.25%); P1= papaya seed extract 2 mg/ml; P2= papaya seed extract 2.5 mg/ml; P3= papaya seed extract 3 mg/ml; P4= Papaya seed extract concentration 3.5 mg/ml, *p<0.05; **p<0.01 (ANOVA One Way Test with Post hoc Bonferroni)

A notable correlation between the impact of papaya seed extract delivery and the mortality rate of Ae aegypti was revealed by the observation data of larval death. p-value of 0.05 for larvae. With 9 larvae, P1 had the lowest mortality rate in the papaya seed extract group, while P3 had the highest mortality rate, with 18 larvae. The Bonferroni post hoc test for Ae. aegypti larval mortality revealed considerable variations between the positive and negative controls. the positive control and P1, the positive control and P2, the negative control and P3, and the negative control and P4.

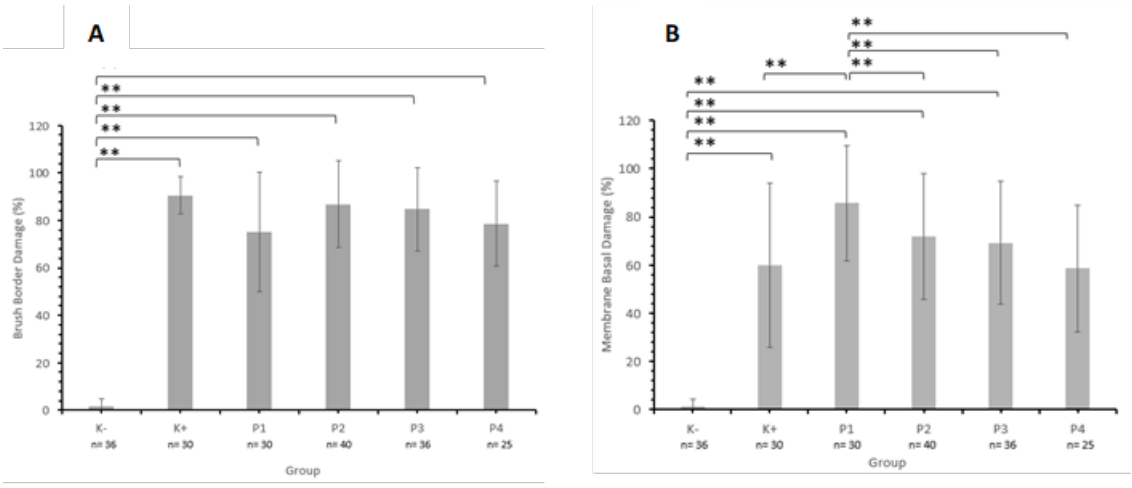
Table 1. LC50 and LC90 of Papaya Seed Extract as Larvicide Against *Ae. aegypti* Larvae within 8 Hours

PROBIT	Probability	95% Confidence Limits for Concentration		
		Estimate	Lower Bound	Upper Bound
	0.5	2.599	-.697	3.450
	0.9	4.776	3.716	40.550

Table 1 illustrates the lethal concentration of papaya seed extract against *Ae. aegypti* larvae, as determined through probit analysis. The LC_{50} was found to be 2.599 mg/ml, while the LC_{90} was 4.776 mg/ml after 8 hours of exposure. These values were statistically significant, as indicated by the narrow confidence intervals, confirming a reliable dose response relationship. The relatively small difference between LC_{50} and LC_{90} suggests that only a modest increase in concentration is required to achieve a high mortality rate. This concentrationd ependent toxicity highlights the consistency of the larvicidal activity of papaya seed extract and strengthens its potential as an effective botanical alternative for vector control.

Effect of Papaya Seed Extract on the Histopathological Features of the Midgut in Ae. aegypti Larvae

After observation and statistical analysis of the histopathology of the midgut of Ae. aegypti larvae, several parameters were examined and measured for each larva, including midgut diameter, epithelial cell height, viable (healthy and intact) epithelial cells, degenerated epithelial cells, brush border damage, and basal membrane damage. After conducting normality and homogeneity tests, the results showed that the data were not normally distributed and not homogeneous. Therefore, the *Kruskal-Wallis test* was performed, followed by the *Mann-Whitney* test to determine differences among the treatment groups**.**



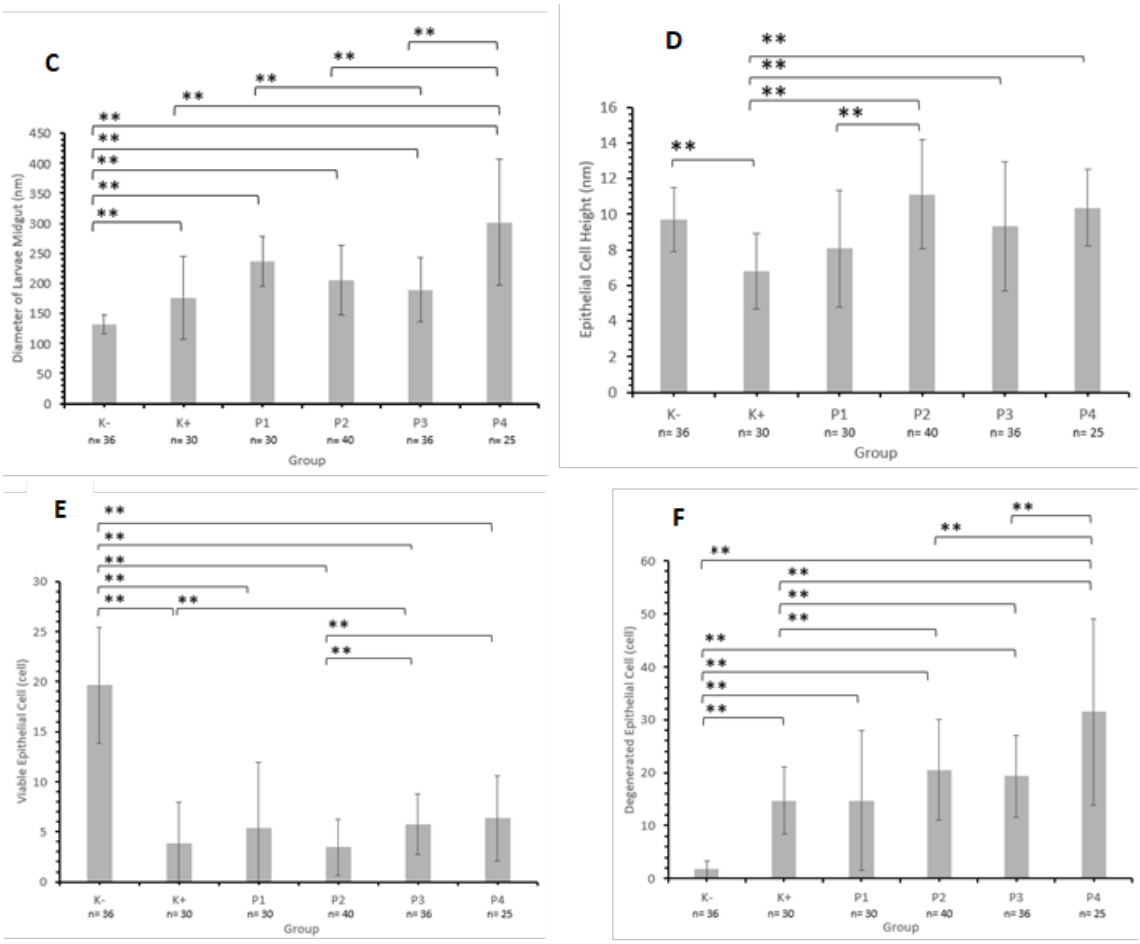


Figure 3. A. Midgut diameter, B. Height of midgut epithelial cells, C. Number of viable cells in midgut, D. Number of degenerated cells in midgut, E. Damage to midgut brush border, F. Damage to the basal membrane of midgut in *Ae.aegypti* larvae after exposure to papaya seed extract for 24 hours. Remarks: K(-) = negative control (aquades); K (+) = positive control (temefos 1.25%); P1= papaya seed extract concentration 2 mg/ml; P2= papaya seed extract concentration 2.5 mg/ml; P3= papaya seed extract concentration 3 mg/ml; P4= papaya seed extract concentration 3.5 mg/ml, *p<0.05;**p<0.01 (*Kruskal-Wallis* and *Mann-Whitney Test*)

Figure 3 presents the observations and measurements of the midgut of Ae. aegypti larvae. Measurements of Midgut diameter revealed that the negative control group had the smallest and most regular diameter at 131 nm, while the P4 group had the largest diameter at 301.52 nm. Measurements of epithelial cell height showed that the negative control group had the shortest epithelial cell height at 6.8 nm, while the P2 group had the tallest epithelial cells at 11.1 nm. The count of viable epithelial cells indicated that the negative control group had the highest number of viable epithelial cells at 19 cells, while the P2 group had the lowest at 3.45 cells. The count of degenerative epithelial cells revealed that the negative control group had the fewest degenerative cells at 1.75 cells, while the P4 group had the highest number of degenerative epithelial cells. Observations of brush border damage showed that the negative control group had the smallest damage area at 1.67%, while the positive control group had the most extensive damage at 90.5%. Observations of basal membrane damage indicated that the negative control group had the smallest damage area at 1.38%, while the P1 group had the largest damage area at 85.67%.

The Kruskal-Wallis test was carried out since the data were found by statistical analysis to be neither normally distributed nor homogeneous. The results indicated that all variables related to midgut observations and measurements of Ae. aegypti larvae showed significant differences across all groups.

The comparison of the histopathology images of the midgut of Aedes aegypti larvae in the negative control group (aquades), the positive control group (1.25% temephos), and the treatment group given papaya seed extract can be observed using a light microscope at 200x and 400x magnifications, as shown in Figure 4 below.

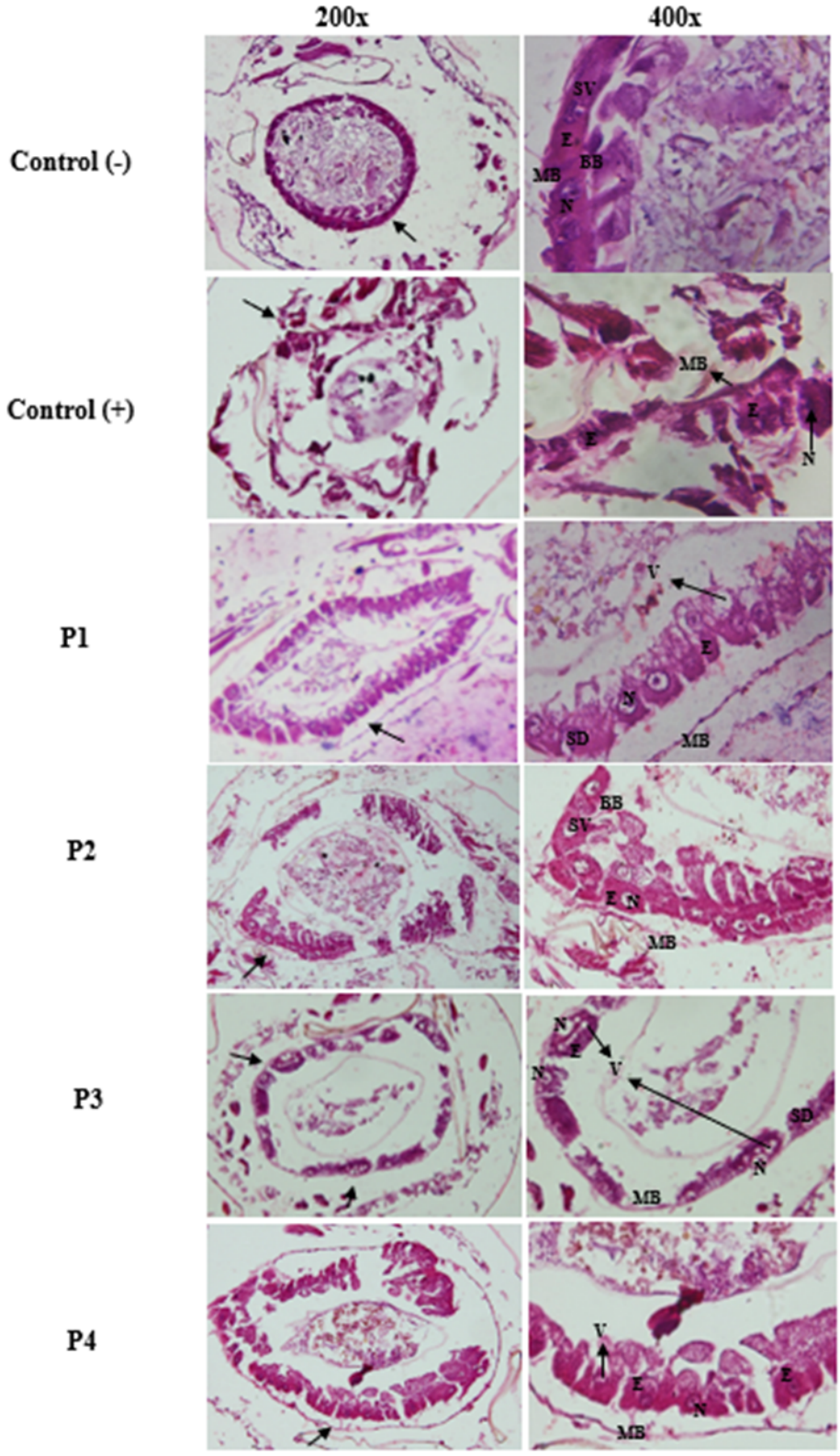


Figure 4. The midgut histopathologicay images of the *Ae. aegypti* larvae stained with HE at 200x magnification

(left) and 400x magnification (right). Legend: E (epithelial cells), N (nucleus), MB (basal membrane), BB (brush border), V (vacuole), SV (viable cells), SD (degenerated cells)

Papaya seed extract induced histopathological changes in the midgut of Aedes aegypti larvae, characterized by a decrease in the number of healthy cells, an increase in degenerated cells, thinning of the midgut mucosal surface, and damage to the midgut membrane. These changes potentially disrupt the digestion and nutrient absorption processes in the midgut of Aedes aegypti larvae.

Discussions

The administration of papaya seed extract can cause the death of Ae. aegypti larvae. All larvae died after being exposed to papaya seed extract for 24 hours at various concentrations, namely 2 mg/ml, 2.5 mg/ml, 3 mg/ml, and 3.5 mg/ml. Meanwhile, in the positive control treatment using 1.25% temephos, all larvae died within the first 10 minutes of exposure. The larvicidal effect of papaya seed extract occurs due to the presence of compounds toxic to the larvae, such as alkaloids, saponins, tannins, and flavonoids.[4]

The most abundant alkaloid in papaya seeds is carpain. This compound is toxic to invertebrates. Alkaloids can inhibit acetylcholinesterase enzyme activity by phosphorylating the serine amino acid at the esteratic site of the enzyme. The neurotransmitter that transmits nerve impulses is acetylcholine. The acetylcholinesterase enzyme typically breaks down acetylcholine into choline and acetic acid. However, when this enzyme is inhibited, acetylcholine accumulates, resulting in continuous stimulation of nerve impulses, causing symptoms such as tremors, convulsions, and uncontrolled movements in insects, eventually leading to death.[7,18] Alkaloids also act as stomach poisons, damaging the digestive tract by inhibiting digestive enzymes necessary for the larvae to digest food.[9] Alkaloids can disrupt the cell membrane and cytoskeletal structure, causing ion leakage and loss of essential substances from the cells.[16]

Saponins have a glycoside structure with hydrophilic and hydrophobic parts. These compounds interact with the larval cuticle membrane, causing cell membrane damage through foam formation and interaction with membrane lipids. When the hydrophobic part interacts with the lipid membrane, it damages the membrane structure and disrupts cell integrity, leading to ion, water, and nutrient leakage. Saponins can also act as antifeedants by binding to proteins and fats in food, causing a bitter taste that suppresses larval appetite. Tannins are polyphenolic compounds that hinder digestion, disrupting larval growth. These compounds bind to proteins and form complexes with cell membrane proteins or essential enzymes.[11] When tannins interact with the cell membrane, they disrupt its integrity, causing ion leakage and loss of vital substances. Papaya seed flavonoids, such as flavonone and dihydroflavonol, are non-polar polyphenolic compounds that act as respiratory toxins in larvae. Flavonoids damage the spiracles and impair respiratory nerve and muscle function, spreading to nerve tissues, leading to sudden central nervous system activity and seizures in larvae.[22] Additionally, flavonoids can inhibit DNA synthesis required for larval development and growth. Disrupted DNA synthesis inhibits protein synthesis, preventing larvae from growing optimally. These compounds also reduce gastrointestinal membrane permeability, affecting nutrient transport and exhibiting juvenile hormone activity that disrupts insect metamorphosis.[9,16]

The histopathological analysis of Ae. aegypti larval Midgut revealed distinct differences between treatment groups. The Midgut, a primary digestive area and insecticide target, showed damage to its structure and function, resulting in inhibited feeding, metabolic disturbances, and larval death. Observations under a microscope revealed that in the negative control group, the Midgut appeared rounded and composed of a uniform columnar epithelial cell layer. These epithelial cells had centrally located, globular nuclei with heterogenous cytoplasm containing granules and basophilic regions. The brush border of epithelial cells consisted of microvilli, which enhance secretion and nutrient absorption, with a flexible basal membrane underneath and a peritrophic membrane surrounding the lumen.[1]

In the positive control group treated with temephos and the papaya seed extract-treated group, significant damage was observed. In the positive control group, discontinuity in the basal membrane and epithelial cells was evident. The epithelial cells changed from columnar to cuboidal, with irregularly widened Midgut diameter

and cytoplasmic vacuoles. In the papaya seed extract group, epithelial cell damage was more pronounced, with degenerated nuclei that appeared pale, enlarged, and lacked nucleoli. Epithelial cells detached from the basal membrane, brush border damage occurred, gaps formed between epithelial cells, and vacuoles were observed in the cytoplasm. Increased extract concentration accelerated the diffusion of toxic substances into Ae. aegypti larvae.

Viable epithelial cell count indicates Midgut epithelial cell health. Healthy Midgut cells result in more viable epithelial cells, whereas deteriorating Midgut conditions increase degenerative epithelial cells, eventually causing lysis and epithelial cell loss. Midgut damage results from toxic substances in papaya seed extract that harm Midgut epithelial cells. Saponins act as larvicides by reducing mucosal membrane surface tension in the digestive tract, causing tract membrane damage. At high doses, saponins irritate the larval digestive tract mucosa.[9]

Alkaloids disrupt cell membranes and cytoskeleton structures, causing cell leakage and death in the Midgut. Flavonoids inhibit larval growth and disrupt nutrient transport by reducing Midgut epithelial membrane permeability.[9,16] Midgut damage impacts digestion, nutrient absorption, ion transport, osmoregulation, and chemical processes (e.g., enzyme formation) and immune mechanisms against pathogens.[17]

Several plants have been reported to possess larvicidal effects, including permot leaf (Passiflora foetida) and starfruit (Averrhoa bilimbi) extract. The larvicidal activity is attributed to toxic compounds capable of killing mosquito larvae. Permot leaf is known to contain alkaloids, flavonoids, and saponins that are lethal to larvae, whereas starfruit contains saponins, tannins, and terpenoids that also play a role as larvicides.[6,17] In this study, papaya seeds were selected as an alternative larvicide because they contain toxic compounds such as alkaloids, saponins, tannins, and flavonoids. Moreover, papaya seeds are generally considered agricultural waste and remain underutilized by the community, yet they have the potential to be processed into a natural larvicide to disrupt the transmission chain of dengue fever vectors.

The study successfully demonstrated that papaya seed extract has a notable impact on the histopathological characteristics of the midgut of Aedes aegypti larvae. As similar studies have not previously been conducted, these findings provide a novel contribution to the field. However, the study was limited by the use of crude papaya seed extract, which made it impossible to determine the specific active compounds responsible for the observed histopathological damage. The results highlight the potential of papaya seeds as an alternative larvicide to replace currently used synthetic larvicides, with the prospect of reducing adverse side effects. Future studies are recommended to fractionate specific active compounds to identify their individual effects on midgut histopathology. Further research is also needed to develop papaya seed preparations into more user friendly formulations, such as ready to use powder, while eliminating the taste and color changes that occur when dissolved in water.

The practical challenges of using papaya seed extract as a larvicide include the availability of raw materials, as sufficient quantities of papaya seeds of consistent quality are required for large-scale production. The content of bioactive compounds may vary depending on the fruit variety, age, or drying method, potentially affecting larvicidal efficacy. Additionally, the stability and storage of the extract are critical, as natural extracts can degrade or lose potency over time. Determining the optimal dose that effectively kills larvae while remaining safe for non target organisms and the environment is also challenging. Furthermore, scaling up the extraction process requires specialized equipment and cost considerations. Environmental factors such as temperature, pH, and water salinity may further influence the extract's effectiveness.

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Declarations of competing interest

No potential competing interest was reported by the authors.

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