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(RESEARCH ARTICLE)

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Effectivity test of ethanol extract of black pepper (*Piper nigrum*) against mortality of *Aedes aegypti* and its effect on midgut morphological changes

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Abstract

Aedes aegypti is the vector of Dengue Hemorrhagic Fever (DHF). Dengue vector management usually depends on synthetic larvicides. However, it can lead to insect resistance and leave chemical pollutant residues. Therefore natural larvicides are needed for their environmentally friendly and do not induce insect resistance. *Piper nigrum* fruit is expected to contain secondary metabolites beneficial as larvicides to *Aedes aegypti*. This study aims to test the effectivity of the ethanol extract of *Piper nigrum* fruit on the mortality of *Aedes aegypti* larvae and determine the effect of extract exposure on larval midgut morphological changes. The study used a completely randomized design with five experimental groups and four repetitions as follows: K (aquades + 0% extract), P1 (0.25% extract), P2 (0.50% extract), P3 (0.75% extract), P4 (1% extract). Data analysis used one-way ANOVA and continued to the LSD Post hoc test. The extract's effectiveness was analyzed using the probit test and LC_{50} confirmation. Changes in the morphology of larval midgut were observed descriptively and presented in the form of tables and figures. The results showed that the 1% treatment resulted in the highest larval mortality of 93% and the lowest in the 0.25% treatment of 43%. The LC_{50} value was 2,666 based on the probit analysis. The ethanol extract of *Piper nigrum* fruit affected midgut morphological changes of *Aedes aegypti* larvae characterized by peripheral membrane damage, microvilli thinning, epithelial cell swelling and vacuolation, and basement membrane damage.

Keywords: *Piper nigrum*; Biolarvicide; Mortality; Midgut of *Aedes aegypti*

1. Introduction

73,518 cases of DHF were reported, leading to the death of 705 people in 2021 [1]. The *Aedes aegypti* is the vector of the Dengue virus, which causes dengue hemorrhagic fever (DHF) in humans. *Aedes aegypti* live and breed in tropical and subtropical climates, especially in urban and semi-urban areas [2].

DHF countermeasure management mainly depends on its vector control [3]. *Aedes* vector control using Temephos powder larvicide to break its life cycle. However, using synthetic larvicides in the long term can cause resistance effects, risk of toxicity at high doses, and environmental pollution residues [4]. Therefore, finding effective and safer alternative larvicides, such as bio larvicides, is crucial.

Biolarvicides are composed of bioactive essential ingredients in plant secondary metabolites, which act as vector controllers because of their toxicity towards insects. Plant secondary metabolites such as steroids, alkaloids, phenolics, terpenoids, and essential oils have insecticide activity, mosquito repellent, and adulticide [5]. Biolarvicide does not cause environmental pollution because nature can quickly decompose its materials. In addition, bio larvicides have low toxicity to non-target insects [6].

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Piper nigrum has been recognized for thousands of years as a beneficial plant [7]. *Piper nigrum* contain potential phytochemicals for pharmacological purposes. *Piper nigrum* can be utilized starting from the roots, leaves, stems, and fruit. The phytochemical content of *Piper nigrum* fruits is indicated to affect *Aedes aegypti* larvae's mortality, making it potentially natural larvicidal.

Midgut is the body part representing the interconnection between the larva and its environment. Understanding the histology and physiology of midgut is very important to learn larvicide's action mechanism and effectiveness [8]. Plant bioactive compounds are toxic by disrupting the digestive system and corroding the intestines. The midgut section of mosquito larvae is the leading site of the digestive process and is one of the larvicides' target organs [9].

2. Material and methods

2.1. Preparation of *Aedes aegypti* Larvae

Aedes aegypti eggs hatched in 30x15 cm trays filled with water for 1-2 days. The hatched larvae are fed with mashed fish pellets and reared for 3-5 days until they pass the first to third instar phases. It is ready to be used as a test object when the larvae have reached the third instar phase.

2.2. Extraction of *Piper nigrum* Fruit

Piper nigrum fruit is mashed using a blender to become powder (simplicia) as much as 500 gr. Simplicia was macerated with 96% ethanol by putting it into a dark bottle with a ratio of 1:10 and left for 24 hours. The maceration extract was filtered using filter paper to isolate the filtrate from sediment. The filtrate was evaporated using a rotary evaporator at 40 °C to separate the extract from the ethanol solvent and obtain sterling black pepper fruit extract [10].

2.3. Qualitative Phytochemical Testing of *Piper nigrum* fruits

Phytochemical testing aims to confirm the content of secondary metabolites in the ethanol extract of *Piper nigrum* fruits. The qualitative phytochemical test follows Harborne's (1987) and Robinson's (1995) procedures.

2.3.1. Saponin Testing

0.5 mL of sample + 5 mL of distilled water, then shaken for 30 seconds. Positive results were indicated by the presence of foam.

2.3.2. Steroid Testing

0.5 mL sample + 0.5 mL glacial acetic acid + 0.5 mL H_2SO_4. A positive result is indicated by the sample color alteration to blue or purple.

2.3.3. Terpenoid Testing

0.5 mL sample + 0.5 mL glacial acetic acid + 0.5 mL H_2SO_4. A positive result is indicated by the sample color alteration to red or yellow.

2.3.4. Tannin Testing

1 mL sample + 3 drops of 10% $FeCl_3$ solution. A positive result is indicated by the sample color alteration to bluish-black.

2.3.5. Alkaloid Testing

0.5 mL of sample + 5 drops of chloroform liquid + 5 drops of Mayer's reagent (1 g of Kl dissolved in 20 mL of distilled water, added 0.271 g of $HgCl_2$ until dissolved). A positive result is indicated by the sample color alteration to brownish-white.

2.3.6. Flavonoid Test

0.5 mL sample + 0.5 g Mg powder + 5 mL concentrated HCl by degree. A positive result is indicated by the sample color alteration to red/yellow and the presence of foam.

2.4. Mortality Test of *Piper nigrum* against *Aedes aegypti* Larvae.

Preparing 200 ml *Piper nigrum* fruits extract at concentrations of 0.25%, 0.50%, 0.75%, and 1% and control solution (0% extract). Then, 25 instar III *Aedes aegypti* larvae were put into each container and soaked for 24 hours. Larval mortality was calculated at 30, 60, and 1440 minutes (24 hours) [9]. Dead larvae are marked by sinking to the bottom of the plastic cup and do not respond to stimulation. The percentage of larval mortality is calculated by the Abbott's formula [13].

2.5. Tissue Processing of *Aedes aegypti* Larvae.

Larva was fixated in a 10% formaldehyde solution for 24 hours. Then dehydrated using graded ethanol (70%, 80%, 96%, 100%) and xylol for 24 hours to clear the alcohol. Larvae were infiltrated by liquid paraffin for 30 minutes. Put the larvae in the paper boxes containing liquid paraffin until they formed blocks. Paraffin blocks were cut crosswise, forming paraffin tape, using a microtome by a 4-6 µm thickness. Then, staining paraffin tape with Hematoxylin - Eosin [14]. The larval midgut preparations were observed in the damage of the polytropic membrane, epithelial cells, and basement membrane [15].

2.6. Data analysis

Larval mortality was analyzed using an analysis of variance (ANOVA). LSD Post hoc test was used to obtain differences in effect between treatments on larval mortality. To determine the black pepper extract concentration that causes half-maximal mortality against *Aedes aegypti* (LC_{50}) a probit analysis in SPSS statistical software was used. Observations in the midgut morphological changes were analyzed descriptively and presented in tables and figures.

3. Results and discussion

3.1. Phytocompounds of *Piper nigrum* Fruits

Piper nigrum contains phytocompounds such as saponins, terpenoids, tannins, alkaloids, and flavonoids, which potentially act as larvicides against *Aedes aegypti* larvae, as in Table 1.

Table 1 Phytocompounds in ethanol extract of *Piper nigrum*

No.	Phytocompounds	Observation	Result
1.	Saponin	The test solution forms a foam	+
2.	Steroid	No color alteration occurs	-
3.	Terpenoid	The test solution turns yellow.	+
4.	Tannin	The test solution turns black.	+
5.	Alkaloid	The test solution forms two layers, transparent and brown.	+
6.	Flavonoid	The test solution turned yellow, and foam was found.	+

Note: (+) indicates the presence of phytocompounds; (-) no phytocompounds were found.

3.2. Anti-larvicidal Activity

The anti-larvicidal activity of the ethanol extract of *Piper nigrum* fruits against *Aedes aegypti* larvae described by the percentage of mortality rate (Figure 1) and LSD Post-Hoc test in 24 hours. While the LC_{50} values in 24 hours obtained through probit analysis are presented in Table 2.

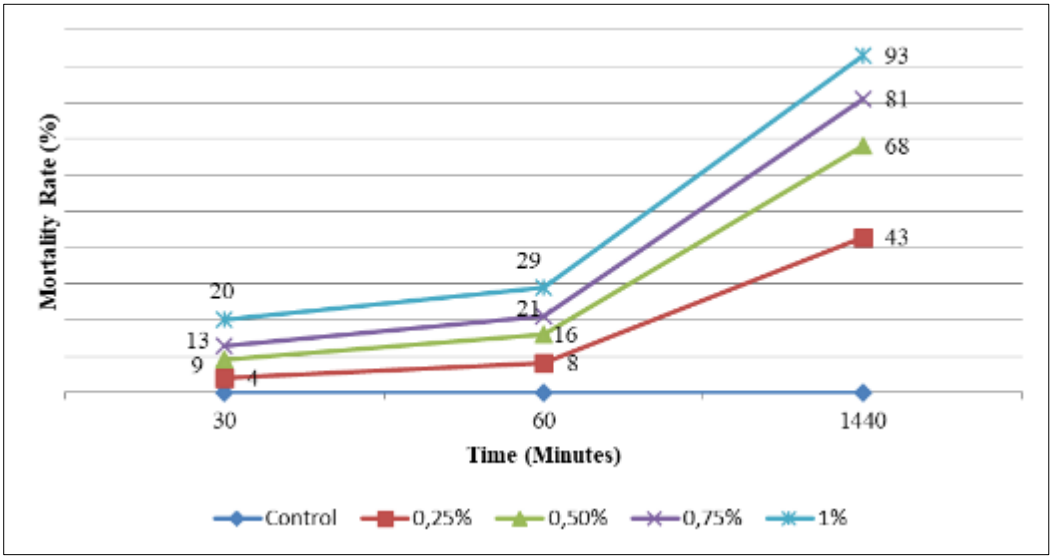


Figure 1 *Aedes aegypti* larval mortality against ethanol extract of *Piper nigrum* fruits

Table 2 LSD Post-Hoc Analysis of Larvae Mortality in 24 Hours

Extract Concentration	N	Larval Mortality (Average±Dev. St.)
0% (Control)	4	0±0.000 (a)
0.25%	4	10,75±0.957 (b)
0.50%	4	17,00±0.816 (c)
0.75%	4	20,25±0.957 (d)
1%	4	23,25±0.957 (e)

Note: Numbers followed by different letters indicate significantly different values between groups (α=5%)

Referring to the data in Figures 1 and Table 2 it can be assumed that the chance of larval mortality will increase with the length of exposure time. After 24 hours of exposure, the ethanol extract of *Piper nigrum* at concentrations shows an increase in the average mortality rate against *Aedes aegypti* larvae. The highest larval mortality reached 23 individuals (93%) at 1%, and the lowest was 11 individuals (44%) at 0.25%. Each concentration shows different effects on larval mortality. It shows the potential bio larvicides of the ethanol extract of *Piper nigrum* fruits against *Aedes aegypti* larvae.

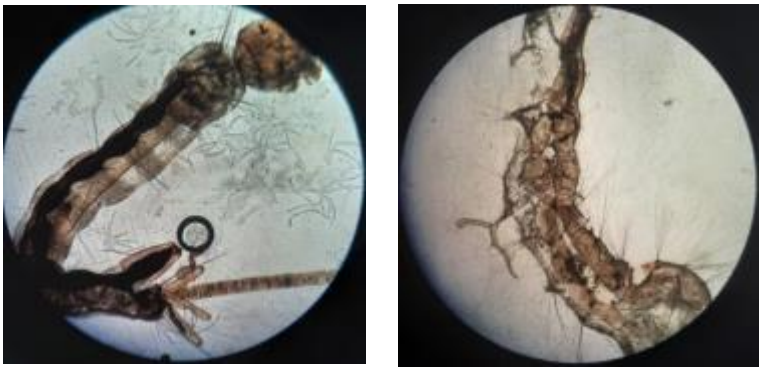
Table 3 LC_{50} ethanol extract of *Piper nigrum* fruits against *Aedes aegypti* Larvae

Lethal Concentration	95% Convidence Interval		
	Estimation	Lower Limit	Upper Limit
LC_{50}	2.666	2.409	2.907

Referring to Table 3 it shown that ethanol extract of *Piper nigrum* fruits has low LC_{50} value against *Aedes aegypti* Larvae. LC_{50} value indicates the effectiveness of black pepper extract against *Aedes aegypti* larvae. The lower the LC_{50} value, the better the effectiveness of the larvicide. Nurhaifah & Sukesi (2015) state that as one of the plants with high potential as bio larvicide, sweet orange (*Citrus sinensis*) has an average LC_{50} value of 0.731%.

3.3. Morphological Changes of Midgut of *Aedes aegypti* Larvae

Observation of body morphology of *Aedes aegypti* larvae treated with ethanol extract of *Piper nigrum* fruits experienced physical damage compared to control larvae (Figure 2). The normal *Aedes aegypti* larvae (Figure 1a) show an intact body without damage and active movement. The *Aedes aegypti* larvae exposed to the test extract (Figure 1b) experienced external body irritation and showed no response or movement.

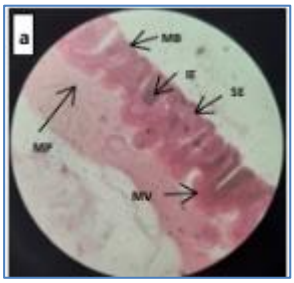
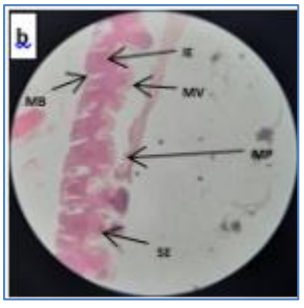


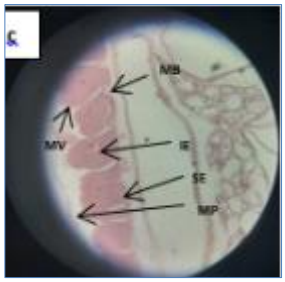
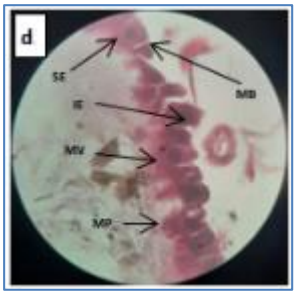
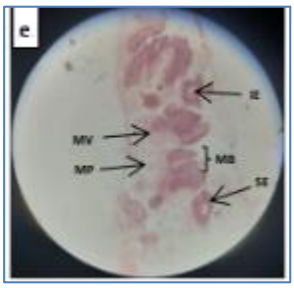
a b

Figure 2 (a) *Aedes aegypti* larvae (control); (b) *Aedes aegypti* larvae after exposed to the test extract

Observation shows that the midgut of *Aedes aegypti* larvae is composed of round nucleated epithelial cells located in the middle, attached to the basement membrane, has a layer of microvilli covering the epithelial cells, and a peripheral membrane that limits the lumen of the midgut. Comparison of normal midgut tissue and after exposure to the test extract showed damage in epithelial cells, basement membranes, peripheral membranes, and microvilli (Table 4).

Table 4 Effect on Ethanol Extract of *Piper nigrum* Fruits against Morphological Changes of Midgut

Extract Concentration	**Midgut Morphology**	
0%	The peritrophic membrane is thick, the basement membrane is in order, the epithelial cells and cell nuclei are in order, and the microvilli are thick.	
0,25%	The peritropic membrane is mildly damaged, the basement membrane is in order, epithelial cells and cell nucleus are in order, and microvilli are thinning.	

0,50%	The peritrophic membrane is damaged, the basement membrane is in order, the epithelial cells become swollen, cell vacuolation is found, and microvilli begin thinning.	
0,75%	The peritrophic membrane is damaged, some epithelial cells begin to detach from the basement membrane, the basement membrane is damaged, the epithelial cells are swelling, and microvilli thinner.	
1%	The peritrophic and basement membranes were damaged, the epithelial cells detached from the basement membrane, the epithelial cells were disorganized and vacuolized in the epithelial cells' nucleus, and a few microvilli were found.	

Ethanol extract of Black Pepper Fruit (*Piper nigrum*) initiates midgut injury by damaging the peritrophic membrane. The peritropic membrane is a semi-permeable, non-cellular matrix structure that lines the midgut lumen and protects midgut cells from toxic substances entering food [16]. Saponin in Black Pepper's phytocompounds can disrupt permeability and allow the poison to enter microvilli and epithelial cells. As a result, the tension in the matrix surface decreases, leading to a peritrophic membrane defect.

On the surface of the epithelial cells, thousands of microvilli protrude into the intestinal lumen, forming a brush-like layer that increases the apical area of the membrane for enzyme secretion and digestive absorption [17]. Microvilli play a role in protecting the digestive tract from ingested toxic substances. Toxins that enter the midgut, such as tannins, cause irritation and thinning of the microvillus, leading to an obstacle to absorbing nutrients and protein.

When the phytocompounds penetrate the epithelial cells, various defense mechanisms to reduce damage effects are made, including cell swelling and vacuolization. Costa et al. (2012) stated that insect midgut epithelial cells exposed to plant phytocompounds showed protrusions of cytoplasmic towards the lumen on the apical surface of epithelial columnar cells, indicating that these cells were involved in the process of apocrine secretion and apoptosis. The vacuolization of the cytoplasm and cell nucleus indicates an internal cell defense against poison penetration.

Alkaloid toxicity can lead to membrane degradation and epithelial cell damage. Damage to the epithelial cells and their surroundings leads to a decrease in the membrane surface pressure and damage to the basement membrane. Mading et al. (2018) state that epithelial cells' detachment from the basement membrane and the nucleus protrusion into the lumen is due to the toxicity of plant phytocompounds. It is a self-defense mechanism to neutralize lumen toxins and cellular cytoplasm toxins.

4. Conclusion

The ethanol extract of black pepper (*Piper nigrum*) causes 93% larvae mortality by 1% concentration, with an LC_{50} value of 2,666. *Piper nigrum* causes changes in midgut morphology characterized by damage to peripheral membrane cells, microvilli, epithelial cells, and basement membrane. Adding hours of observation and positive controls such as

temephos (abate) is suggested to compare the effectiveness of bio larvicides with synthetic larvicides against *Aedes aegypti*.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors declare there is no conflict of interest

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