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# THE EFFICACY OF ETHANOL EXTRACT OF BILIMBI LEAVES (*Averrhoa bilimbi* L.) AS A LARVICIDE FOR DENGUE FEVER VECTOR *Aedes aegypti* L.

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## ABSTRACT

Dengue hemorrhagic fever (DHF) continues to be a significant health problem worldwide, including in Indonesia. Controlling the vector mosquito, *Aedes aegypti*, is crucial to reduce transmission and incidence rates of dengue. The widespread use of synthetic chemical insecticides can lead to environmental problem, non-target organism poisoning, and the development of resistance. An alternative approach to vector control is the use of natural insecticides, which contain active compounds derived from plants. One such potential option is the bilimbi leaf (*Averrhoa bilimbi*) due to its content of alkaloids, flavonoids, tannins, and saponins, which exhibit activity against insects. This study aims to investigate the efficacy of ethanol extract from the bilimbi leaf as a larvicide against dengue hemorrhagic fever vector *Ae. aegypti*. Bilimbi leaf extract was prepared using ethanol solvent. Larvicidal assays were conducted using six concentrations: 0.5%, 1%, 1.5%, 2%, 2.5%, 4%, and a positive control (1% temephos) and a negative control (distilled water). Larval mortality was assessed at 6, 24, and 48 hours post-exposure. The highest larval mortality was observed at the 4% concentration, reaching 17.33% (6 hours post-exposure), 76% (24 hours post-exposure), and 100% (48 hours post-exposure). The $LC_{50}$ and $LC_{90}$ values were 19.66% and 236.35% (6 hours), 1.79% and 5.75% (24 hours), and 0.99% and 2.15% (48 hours), respectively. The phytochemical screening showed the presence of saponins, steroids, tannins, alkaloids, flavonoids, and phenolics, all of which were strongly positive. However, terpenoids were negative. These bioactive compounds are known for their potential insecticidal properties. This natural insecticide offers a promising

alternative for vector control strategies, potentially mitigating the environmental and health concerns associated with synthetic insecticides.

**Keywords**: Efficacy, larvicide, *Averrhoa bilimbi* extract, *Aedes aegypti*

## ABSTRAK

Demam denggi berdarah (DHF) terus menjadi masalah kesihatan yang ketara di seluruh dunia, termasuk di Indonesia. Mengawal nyamuk vektor, *Aedes aegypti* adalah penting untuk mengurangkan kadar penularan dan kadar insiden demam denggi Penggunaan meluas racun serangga kimia sintetik boleh membawa kepada masalah alam sekitar, keracunan organisma bukan sasaran, dan perkembangan rintangan. Pendekatan alternatif untuk mengawal vektor ialah penggunaan racun serangga semula jadi, yang mengandungi sebatian aktif yang berasal daripada tumbuhan. Salah satu pilihan yang berpotensi adalah daun Belimbing Buluh (*Averrhoa bilimbi*) kerana kandungan alkaloid, flavonoid, tanin, dan saponin, yang menunjukkan aktiviti melawan serangga. Kajian ini bertujuan untuk mengkaji keberkesanan ekstrak etanol daripada daun Belimbing Buluh sebagai larvasid terhadap vektor demam denggi berdarah *Ae. aegypti*. Ekstrak daun belimbing buluh disediakan dengan menggunakan pelarut etanol. Ujian larvisidal dijalankan menggunakan enam kepekatan: 0.5%, 1%, 1.5%, 2%, 2.5%, 4%, dan kawalan positif (1% temephos) dan kawalan negatif (air suling). Kematian larva dinilai pada 6, 24, dan 48 jam selepas pendedahan. Kematian larva tertinggi diperhatikan pada kepekatan 4%, mencapai 17.33% (6 jam selepas pendedahan), 76% (24 jam selepas pendedahan), dan 100% (48 jam selepas pendedahan). Nilai $LC_{50}$ dan $LC_{90}$ masing-masing ialah 19.66% dan 236.35% (6 jam), 1.79% dan 5.75% (24 jam), dan 0.99% dan 2.15% (48 jam). Saringan fitokimia mengesahkan kehadiran saponin, steroid, tanin, alkaloid, flavonoid, dan fenolik, yang kesemuanya menunjukkan reaksi positif yang kuat. Namun demikian, kehadiran terpenoid tidak dikesan. Bioaktif ini diketahui mempunyai sifat insektisida. Racun serangga semula jadi ini menawarkan alternatif yang menjanjikan untuk strategi kawalan vektor, yang berpotensi mengurangkan masalah alam sekitar dan kesihatan yang berkaitan dengan racun serangga sintetik.

**Katakunci**: Keaktifan, larvisidal, ekstrak *Averrhoa bilimbi*, *Aedes aegypti*

## INTRODUCTION

Vector-borne diseases are diseases caused by pathogens transmitted through vector insects. One of the significant vectors is the *Aedes aegypti* mosquito. *Ae. aegypti* mosquito is the primary species responsible for transmitting DENV virus, which causes dengue fever (DF) (Socha et al. 2022; World Health Organization 2020). In 2023, more than 6 million cases of dengue fever (DF) were reported globally, with over 6,000 related deaths. Data from 2022 indicates that Indonesia recorded 143,266 dengue cases, with an incidence rate (IR) of 52 per 100,000 inhabitants and a case fatality rate (CFR) of 0.86%. The incidence rate of dengue in 2022 was the highest compared to the data from the previous five years (European Centre for Disease Prevention and Control 2024; Ministry of Health Republic of Indonesia 2023).

The control of dengue vector can be achieved through various methods, including the use of insecticides, biological agents, and environmental physical control (World Health Organization 2009). In Indonesia, many communities prefer to use insecticides due to their ease of use and practicality. Insecticides are substances that have the ability to kill insects, making them widely used in efforts to control vector populations (Gan et al. 2021). The most

used insecticides in Indonesia are synthetic insecticides derived from chemical compounds. The use of synthetic insecticides is generally effective because they can rapidly kill insects; however, they can cause pollution due to their persistent nature, difficult to degrade in the environment, and potential to poison non-target organisms (Pathak et al. 2022). Furthermore, improper use of synthetic insecticides can lead to serious issues, such as the development of insecticide resistance, which can complicate vector mosquito population control, making it more difficult to reduce disease incidence rates (Directorate for Prevention and Control of Vector-Borne and Zoonotic Diseases Republic Indonesia 2018; Gan et al. 2021).

One alternative to replace synthetic insecticides is natural insecticides, which are derived from plant-based active compounds (Ayilara et al. 2023; Sayono et al. 2024). Natural insecticides can be a preferred choice because they are environmentally safe, non-toxic to non-target organisms, including animals, and their residues can naturally degrade. Plant-active compounds, which are secondary metabolites, have been proven to exhibit various activities against insects (Ayilara et al. 2023; Isman 2017; Lengai et al. 2020). One plant with the potential as a natural insecticide is bilimbi leaf (*Averrhoa bilimbi*).

Bilimbi is a plant with various health benefits, both from its leaves and its fruit. Unlike the fruit, which is commonly consumed, bilimbi leaves are rarely utilized by the community and are often left unused. However, bilimbi leaves contain phytochemical compounds similar to those found in the fruit, such as alkaloids, flavonoids, tannins, and saponins (Alhassan & Ahmed 2016; Setyawan et al. 2021). These phytochemical compounds are known to have insecticidal activity (e.g., alkaloids can affect nerve transmission in insects, inhibit insect growth and development (Ma et al. 2020; Mbata et al. 2013), saponin disrupt insect cell membrane and synthesis of steroids from ecdysis, phenols affect insect physiological processes (Salminen & Karonen 2011), indicating that the underutilized bilimbi leaves have potential as a natural larvicidal insecticide against *Ae. aegypti*. Therefore, this research objective was to investigate the larvicidal efficacy of ethanol extract from bilimbi leaves (*A. bilimbi*) toward the larvae of dengue vector, *Ae. aegypti* mosquito and to investigate the phytochemical compound of the extract.

## MATERIALS AND METHODS

This study investigates the effect of ethanolic bilimbi leaves extract against laboratory strain *Ae. aegypti* larvae. The research was conducted at the Microbiology and Parasitology Laboratory, Faculty of Medicine, University of Lampung.

### Samples Collection and Extraction

Bilimbi leaves were collected from various locations in the Lampung Province, Indonesia, followed by sorting, washing, drying, and dry sorting. The leaves were then blended to obtain a simplicia powder, which was sieved using a 40-mesh sieve to produce a fine powder. The simplicial powder of the leaves was soaked in 96% ethanol and left to macerate for approximately 24 hours while being stirred occasionally. After 24 hours, the mixture was filtered using a flannel cloth to separate the residue from the filtrate. This process was repeated three times to maximize extraction. The collected filtrate was then evaporated using a vacuum evaporator at 60°C, followed by further concentration using a water bath until a thick extract was obtained.

**Larvacidal Bioassay**
The method in this study follows the guidelines set by the World Health Organization (WHO 2005). A total of 25 third or fourth-instar laboratory strain *Ae. aegypti* larvae were introduced into 240 mL plastic cups containing 200 mL of extract solution at predetermined concentrations. The larvae used in this study were obtained from laboratory strain eggs, reared by IPB University, Indonesia. The concentrations used were 0.5% (5,000 ppm), 1% (10,000 ppm), 1.5% (15,000 ppm), 2% (20,000 ppm), 2.5% (25,000 ppm), and 4% (40,000 ppm). Positive control (1% Temephos) and negative control (distilled water) were also included in the study. The experiment was conducted with three replicates under controlled environmental conditions of temperature and humidity. Each replicate consisted of a total of 200 larvae.

**Mortality Assessment**
Larvicidal efficacy was determined based on the percentage of larval mortality observed at 6 hours, 24 hours, and 48 hours. Larvae were considered dead if they exhibited no movement and did not respond when touched with a stick or exposed to a flashlight. The observed results were then calculated using the following formula (WHO 2005):

Percentage of Larval Mortality
= (Number of dead larvae / Total number of larvae tested) × 100%

**Data Analysis**
The results were further analyzed using the Mann-Whitney test to determine the significance of each treatment and probit analysis to estimate the lethal concentration for 50% ($LC_{50}$) and 90% ($LC_{90}$) mortality. A chi-square test was also performed to assess how well the probit model predicts the probability of an even based on the dose. Statistical analyses were conducted using IBM SPSS Statistics software version 29.0.2.0.

**Gut Damage Observation**
Larvae that died as a result of the treatments were subsequently observed directly by placing them on a microscope slide and examining them under a binocular microscope with a 40x objective lens magnification to assess potential gut damage caused by the extract.

**Phytochemical Screening**
The phytochemical content in the ethanol extract of bilimbi leaves was also analyzed using qualitative testing following the methods by Kartikasari et al. (2022) and Tiwari et al. (2011), with modification. Saponin detection was performed using the foam test method, which involved observing foam formation after shaking the solution with the addition of distilled water. Steroid and terpenoid detection were conducted by adding glacial acetic acid and $H_2SO_4$ (Liebermann-Burchard test), where the presence of steroids was indicated by a blue color change, while terpenoids exhibited a red-purple or red-orange color. Phenol and tannin detection involved the addition of 5% $FeCl_3$ (for phenols) and 10% $FeCl_3$ (for tannins). The presence of phenols was marked by a blue color change, while tannins were identified by a dark blue-black or brownish color accompanied by precipitate formation. Alkaloid detection was carried out using Mayer's reagent, with a positive result indicated by the formation of a yellowish-white precipitate. Flavonoid detection was performed by adding magnesium powder (Mg) and concentrated HCl, with positive results characterized by the formation of red, yellow, or orange colors.

## RESULTS

This study investigates the larvicidal potential of ethanol extract of bilimbi leaves against *Ae. aegypti* larvae by assessing mortality rates at different concentrations over exposure periods of 6, 24, and 48 hours. Each treatment group consisted of 25 larvae with three replications to ensure reliable and reproducible results. The efficacy results of the ethanol extract of bilimbi leaves against *Ae. aegypti* larvae are presented in Table 1.

Table 1. Mean % Mortality of *Ae. aegypti* larvae after treatment with ethanol extract of bilimbi leaves

| Treatment | Mortality (%±SD) | | |
|---|---|---|---|
| | 6-hr | 24-hr | 48-hr |
| Ethanol Extract of Bilimbi Leaves | | | |
| 0.5% | 2.67±2.31$^{a}$ | 6.67±2.31$^{a}$ | 17.33±4.62$^{a}$ |
| 1% | 4.00±0.00$^{a}$ | 24.00±0.00$^{b}$ | 50.67±5.77$^{b}$ |
| 1.5% | 10.67±2.31$^{b}$ | 42.67±2.31$^{c}$ | 62.67±2.31$^{c}$ |
| 2% | 14.67±2.31$^{bc}$ | 62.67±2.31$^{d}$ | 86.67±2.31$^{d}$ |
| 2.5% | 16.00±0.00$^{c}$ | 65.33±6.11$^{de}$ | 100.00±0.00$^{e}$ |
| 4% | 17.33±2.3$^{c}$ | 76.00±4.00$^{e}$ | 100.00±0.00$^{e}$ |
| Negative control (distilled water) | 0.00±0.00$^{d}$ | 0.00±0.00$^{f}$ | 0.00±0.00$^{f}$ |
| Positive control (temephos 1%) | 100.00±0.00$^{e}$ | 100.00±0$^{g}$ | 100.00±0.00$^{e}$ |

Note: Values followed by the same lowercase letter indicate no significant difference in the Mann-Whitney test ($P<0.05$).

The results indicate that the highest percentage of larval mortality was observed in the treatment with the highest concentration, 4%, at both 6 hours and 24 hours of exposure. At 48 hours of exposure, the highest mortality rate was observed at concentrations of 2.5% and 4%, both resulting in 100% mortality. At the 6-hour observation, the mortality rates were 17.33% and 76% respectively. In the negative control, no larval mortality was observed up to 72 hours, while in the positive control, 100% mortality was observed at every time point.

Based on the Mann-Whitney test results, at 6 hours after treatment, the 2% concentration produced a larval mortality percentage that was not significantly different from the 1.5% and 2.5% concentrations. The 2.5% concentration also showed no significant difference in mortality percentage compared to the 4% concentration. At 24 hours after treatment, the 2.5% concentration did not show a significant difference in larval mortality compared to the 2% and 4% concentrations. At 48 hours after treatment, the larval mortality percentage at the 2.5% concentration was not significantly different from the 4% concentration and the positive control.

The toxicity of the ethanol extract of bilimbi leaves was determined through probit analysis to obtain the lethal concentration (LC) values. The lethal concentrations used are $LC_{50}$, the concentration that kills 50% of the test larval population, and $LC_{90}$, the concentration that kills 90% of the test larval population. The results of the probit and chi-square analysis of the efficacy of the ethanol extract of bilimbi leaves can be seen in Table 2.

Table 2. Probit and chi-square analysis of the efficacy of ethanol extract of bilimbi leaves (*A. bilimbi*) against *Ae. aegypti.*

| Treatment | $LC_{50}$ (LCL-UCL)* | $LC_{90}$ (LCL-UCL)* | $R^2$ | Regression Equation | Chi-Square |
|---|---|---|---|---|---|
| 6-hour treatment | 19.66(8.53-267.98) | 236.35(44.32-50438.78) | 0.81 | y = -1.51+1.11x | 0.99 |
| 24-hour treatment | 1.79(1.60-2.20) | 5.75(4.58-7.97) | 0.96 | y = -0.67+2.59x | 0.99 |
| 48-hour treatment | 0.99(0.89-1.08) | 2.15(1.91-2.48) | 0.94 | y = -0.02+3.21x | 0.49 |

Note: *confidence interval 95%

The lethal concentration values ($LC_{50}$ and $LC_{90}$) at 6 hours of extract treatment were found to be 19.66% and 236.35%, respectively, with a regression equation of y = -1.51 + 1.11x and $R^2$ = 0.81. At 24 hours of treatment, the $LC_{50}$ was 1.79% and the $LC_{90}$ was 5.75%, with a regression equation of y = -0.67 + 2.59x and $R^2$ = 0.96. At 48 hours of treatment, the $LC_{50}$ was 0.99% and the $LC_{90}$ was 2.15%, with a regression equation of y = -0.02 + 3.21x and $R^2$ = 0.94. Additionally, a chi-square analysis was conducted, yielding results of less than 0.05 in each case.

The dead test larvae were then observed under a microscope to examine any potential damage to their gut. The following images were obtained during microscopic observation (Figure 1).

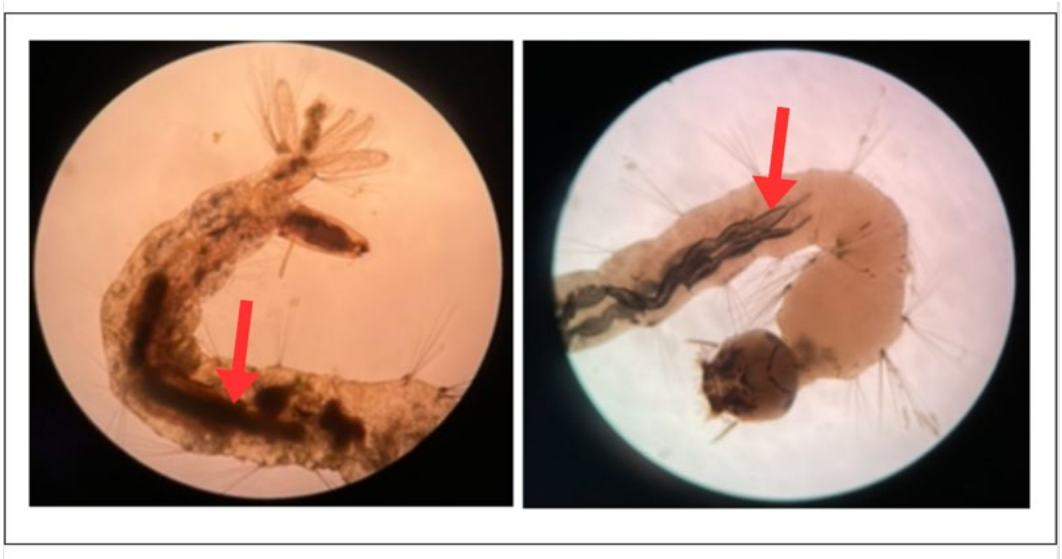

Figure 1. *Ae. aegypti* larvae that died due to treatment with ethanol extract of bilimbi leaves (*A. bilimbi*). The arrow indicates the damaged gut in the larva. Magnification 40x.

The ethanol extract of bilimbi leaves contains various phytochemical compounds, as shown by qualitative phytochemical analysis (Table 3). The phytochemical constituents known to have insecticidal activity include flavonoids, steroids, tannins, alkaloids, flavonoids, and phenolics.

Table 3. Phytochemical content of ethanol extract of bilimbi leaves (qualitative)

| Content | Result | Interpretation |
|---|---|---|
| Saponins | +++ | Strongly positive |
| Steroids | +++ | Strongly positive |
| Terpenoids | - | Negative |
| Tannins | +++ | Strongly positive |
| Alkaloids | +++ | Strongly positive |
| Flavonoids | +++ | Strongly positive |
| Phenolics | +++ | Strongly positive |

## DISCUSSION

Natural insecticides that utilize the activity of phytochemical compounds in plants have the potential to become alternative methods for controlling insect vectors of diseases. This study utilized ethanol extract from bilimbi leaves (*A. bilimbi* L.) as a larvicide against the dengue fever vector *Ae. aegypti*. The current study used third or fourth-instar larvae of *Ae. aegypti* due to the fact that the larvae have fully developed structures, including the digestive tract. Additionally, these instar larvae exhibit active physical and metabolic activity, allowing them to respond effectively to the presence of toxic substances in their environment, including insecticides.

Based on the treatment results using the ethanol extract of bilimbi leaves against *Ae. aegypti* larvae, it was found that there was an increase in the percentage of mortality with increasing concentration and exposure time (Table 1). The higher the concentration, the higher the mortality percentage, due to the increased amount of phytochemical compounds interacting with the tested larvae. The negative control consisted of distilled water without any treatment, resulting in no mortality of the test larvae, indicating that the larval mortality observed in the concentration series was solely due to the extract treatment. Additionally, a positive control was used, consisting of 1% temephos, a synthetic chemical insecticide. Positive control showed 100% of larvae mortality (Table 1). Temephos is widely used for larval control in Indonesia and can be found in pharmacies under the brand name Abate. Temephos is a larvacide from the organophosphate family (Martínez-Mercado et al. 2022). It has neurotoxic properties that inhibits the enzyme acetylcholinesterase, leading to the accumulation of acetylcholine, which causes hyperexcitation and nerve fatigue, ultimately resulting in larval death (Aroniadou-Anderjaska et al. 2023; Martínez-Mercado et al. 2022). The abundance of phytochemical compounds results in a higher toxic effect, leading to the death of a greater number of larvae. Additionally, with longer exposure time, more larvae died, as prolonged exposure to the toxin strengthens its toxic activity, causing higher larval mortality. Based on the probit analysis, the results suggest that as the treatment time increases, the $LC_{50}$ and $LC_{90}$ values of the ethanol extract of bilimbi leaves decrease (Table 2). A smaller LC value indicates a higher toxicity of the treatment substance. Besides the LC values, the chi-square test results also show that the probit model provides a good fit to the observed data, indicating that the model is a reasonable representation of the dose-response relationship. By visual observation, the tested *Ae. aegypti* larvae were also observed to be damaged especially in the internal organs (Figure 1), indicated by the white or transparent color, and damage to the gut, which is the digestive tract in the larvae.

The qualitative phytochemical analysis (Table 3) reveals that the ethanol extract of bilimbi leaves includes a range of phytochemical compounds. Qualitative test showed that the ethanol extract of bilimbi leaves contains saponins, tannins, flavonoids, alkaloids, steroids, and phenolics compounds. These compounds may have similar or different modes of action, enabling them to synergistically kill the test larvae. Flavonoids are capable of inhibiting key enzymes involved in the metabolic processes of larvae, such as acetylcholinesterase and ATPase, thereby disrupting the nervous system and energy metabolism of the larvae, leading to paralysis and death (Neupane et al. 2019; Perumalsamy et al. 2015). Flavonoids can also induce oxidative stress through the formation of reactive oxygen species (ROS) in the larvae. The abundance of ROS results in cellular damage, including lipid peroxidation, protein denaturation, and DNA damage. This oxidative damage disrupts cellular function, ultimately leading to larval death (Huang et al. 2024). Additionally, flavonoids, saponins, and phenols can damage the cell membranes of larvae, resulting in increased membrane permeability. This can cause leakage of essential ions and molecules, leading to cell death (Catelan et al. 2015; Pereira et al. 2024; Procópio et al. 2015). This is further supported by the microscopic observations shown in Figure 1, where internal organ damage in the test larvae is indicated by the white or transparent color and damage to the gut, which is the digestive tract of the larvae.

Similar to flavonoids, alkaloids and terpenoids also act as neurotoxins capable of inhibiting enzymes like acetylcholinesterase. Terpenoids can also affect the gut and cuticle of the larvae, reducing their feeding ability and hindering digestion processes. This disruption can lead to starvation and death in the test larvae (Cruz-Castillo et al. 2023; Wahedi et al. 2024). Steroids present in plants are known as phytosterols. Phytosterols are responsible for intracellular cholesterol transport. This disruption hinders the insect's ability to utilize cholesterol, leading to detrimental effects on their development and survival (Du et al. 2012; Larson et al. 2010). Phytosteroids can mimic or inhibit insect hormones, particularly ecdysteroids, which are crucial for insect molting and development. By disrupting these hormonal pathways, phytosteroids can prevent normal growth and reproduction, leading to the insect's death (Arif et al. 2022; Das et al. 2021). Tannins can interfere with the digestion process of larvae, inhibiting their ability to metabolize nutrients. Furthermore, a study by Farahat et al. (2021) showed that tannins can affect hormonal regulation, resulting in delayed development and abnormal growth patterns in *Culex pipiens* larvae.

Based on the content and mode of action of these phytochemical compounds, the ethanol extract of bilimbi leaves can generally cause death by acting as a neurotoxin, damaging cell membranes, and disrupting the metabolism of *Aedes aegypti* larvae.

## CONCLUSION

The present study screened the phytochemical compounds of ethanolic extract of bilimbi leaves (*Averrhoa bilimbi*) and assessed its toxicity against the larvae of *Ae. aegypti* mosquito. Ethanolic extract of bilimbi leaves consists of saponins, steroids, tannins, alkaloids, flavonoids and phenolic compounds that may contribute to the mortality of *Ae. aegypti* larvae. The study observed that larval mortality increased with higher concentrations over time. The findings indicate that the toxicity of the substance increases over time, with the $LC_{50}$ and $LC_{90}$ values becoming significantly lower as exposure duration extends. This suggests a time-dependent effect on larval mortality. Ethanolic extract of bilimbi leaves offers a promising alternative for vector control strategies as natural insecticides, potentially mitigating the environmental and health concerns associated with synthetic insecticides.

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## AUTHORS DECLARATIONS

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**Conflict of Interest**
The authors declare that they have no conflict of interest.

**Ethics Declarations**
This research protocol was reviewed and obtained ethical approval from Tanjung Karang Health Polytechnic Ethics Committee, Lampung, Indonesia, with ethical approval No. 521/KEPK-TJK/VIII/2024.

**Data Availability Statement**
All data generated or analysed during this study are included in this published article.

**Authors' Contributions**
Suryadi Islami (SI) conceptualized the research and designed the experiments. Afriyani (AF) and Ervina Damayanti (ED) participated in the extraction process and phytochemical analysis testing. Luhut Uli Arto Nainggolan (LUAN), Agaphe Suluh Brahmantio (ASB), and Ilyas Prabamukti (IP) collected, sorted, and prepared bilimbi leaves for the extraction process. Ridwan Hardiansyah (RH), Nabylly Aghna Bachtiar (NAB), and Muhammad Umar Abdullah Al Faruq (MUAAF) maintained the laboratory strain mosquitoes. SI, LUAN, ASB, IP, RH, NAB, and MUAAF participated in the efficacy testing and supports submission to journals. SI and Atikah Fitria Muharromah (AFM) participated in data interpretation. SI, AFM, AF, and ED wrote and revised the paper. All authors read and approved the manuscript.

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