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Larvicidal Activity of Green Spinach (*Amaranthus hybridus* L.) Leaf Extract Against *Aedes aegypti* Larvae

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Abstract. Dengue hemorrhagic fever is a vector-borne disease primarily transmitted by the *Aedes aegypti* mosquito. Vector control strategies commonly include the use of larvicide to reduce mosquito populations at the larval stage. However, long-term reliance on chemical larvicides can contribute to the development of resistance, bioaccumulation, and adverse environmental impacts. In contrast, plant-based larvicides are generally biodegradable and are considered less harmful to non-target organisms. In this study, green spinach (*Amaranthus hybridus* L.) leaves, known to contain alkaloids, flavonoids, saponins, tannins, and terpenoids, were evaluated for their larvicidal activity. An experimental cross-sectional design was applied using five groups: positive control, negative control, and ethanol leaf extract concentrations of 1%, 1.5%, and 2% (five replicates each). Mortality rates were 100% in the positive control, 0% in the negative control, and increased with extract concentration (57% at 1%, 86% at 1.5%, and 100% at 2%). Post hoc Mann–Whitney testing showed significant differences in most group comparisons, with one non-significant comparison. The larvicidal effect is likely attributable to the synergistic action of multiple secondary metabolites present in the plant extract. This study contributes to the limited body of knowledge on the larvicidal potential of *A. hybridus* by demonstrating its concentration-dependent effectiveness against *A. aegypti* larvae, supporting its potential application as an environmentally friendly botanical larvicide.

Keywords: *Aedes aegypti*; *Amaranthus hybridus* L; Dengue hemorrhagic fever; Larvicide; Secondary metabolites

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INTRODUCTION

Dengue hemorrhagic fever (DHF), caused by the dengue virus and transmitted by *Aedes aegypti* mosquitoes, remains a major health issue in Indonesia. In 2023, there were 114,720 cases and 894 deaths, with cases rising to 210,644 and deaths to 1,239 in 2024 (Kemenkes RI, 2025). The highest numbers of cases were reported in West Java (19,338), East Java (9,401), and West Kalimantan (9,003) (Pusat Analisis Keparlemenan DPR RI. 2024). DKI Jakarta saw an increase

from 379 cases in December 2024 to 670 in January 2025 (BPS Provinsi DKI Jakarta, 2025), highlighting the need for effective vector control.

Dengue transmission occurs when an infected female *A. aegypti* mosquito injects virus-containing saliva during feeding, leading to viral spread (Nanaware et al., 2021). Controlling mosquito breeding sites and targeting immature stages is critical in reducing vector density (Repelita, 2024; Siregar et al., 2023). Indonesia's 3M program (draining, covering, and disposing of water-holding containers) and biological controls, like larvivorous fish, aim to limit mosquito populations (Makrufardi et al., 2021; Andriani et al., 2021). Chemical larvicides, while effective, pose risks such as resistance, bioaccumulation, and ecological harm (Meier et al., 2022; Zhou et al., 2025).

Plant-based larvicides are gaining attention as eco-friendly alternatives due to their biodegradability and lower environmental risks (Prabowo et al., 2024). Phytochemicals in plant extracts, such as alkaloids, flavonoids, and saponins, have shown promise in larvicidal activity (Ajaegbu et al., 2023). *A. aegypti* instar III larvae are commonly used in bioassays due to their developed morphology, making them more responsive to bioactive compounds (Utami & Porusia, 2023). Studies have demonstrated that ethanolic plant extracts can achieve significant larval mortality (Wahyuni et al., 2023).

Amaranthus hybridus L. (green spinach) is an abundant plant in Indonesia, widely cultivated for food and other uses (Alhanif et al., 2023; Okwuchukwu et al., 2022). The use of ethanol as a solvent is intended to extract polar compounds, including flavonoids, alkaloids, saponins, tannins, and terpenoids. Its leaf extracts have shown antimicrobial activity due to phytochemicals like alkaloids and flavonoids (Yelfi et al., 2022). Although *A. hybridus* has been reported to contain bioactive compounds such as alkaloids, flavonoids, and saponins with antimicrobial activity, studies evaluating its larvacidal activity against *A. aegypti* larvae remain limited. In particular, the effectiveness of ethanolic leaf extract of *A. hybridus* at low concentrations has not been sufficiently investigated. Therefore, this study was conducted to evaluate its potential as a plant-based larvacide against third-instar larvae of *A. aegypti*. The concentrations of 1.0%, 1.5%, and 2.0%, were selected based on a modification of the dosage range used in a previous study (Wahyuni et al., 2023), in which higher concentrations of ethanolic plant extracts produced 100% larval mortality. Therefore, lower concentrations were chosen in the present study to determine whether *A. hybridus* leaf extract could still produce larvicidal effects at reduced doses. No preliminary test was conducted prior to the main bioassay.

METHODS

Study Design

This study employed an experimental design to investigate the cause–and–effect relationship by evaluating the impact of a specific treatment. Data were collected using a cross-

sectional approach, in which observations and outcomes were recorded at a single point in time. The independent variable was the concentration of *A. hybridus* leaf extract, and the dependent variable was the mortality of third-instar *A. aegypti* larvae.

Sample

This study used third-instar *A. aegypti* larvae. Samples were allocated into five groups: a positive control (Temephos 0.02 mg/L), a negative control (distilled water), and three treatment groups induced with *A. hybridus* leaf ethanol extract at 1%, 1.5%, and 2%. The number of replications was determined using Federer's formula, so each group was replicated five times. Following the WHO larvicide testing standard of 20 larvae per treatment (World Health Organization, 2005), the total sample size was 500 larvae. Primary data were collected directly by the researcher, and purposive sampling was applied with inclusion criteria of active, 4–5 mm *A. aegypti* third instar larvae, and exclusion criteria of non- *A. aegypti* larvae, dead larvae, non-third-instar larvae, or larvae outside the 4–5 mm size range.

Tools and Materials

The tools used in this study included an oven, analytical balance, blender, microscope, cover glass, stirring rod, filter paper, dropper pipette, measuring pipette, beaker, measuring cup, funnel, dark glass bottle, jar, test tube rack, test tube, tray, plastic bowl, and stick. The material used for this study consisted of mature *A. hybridus* leaves, approximately two months old. The test organisms were third-instar *A. aegypti* larvae obtained from the Entomology Laboratory of BBLKM East Jakarta. Chemicals used included distilled water, 96% ethanol, Temephos (0.02 mg/L), concentrated CH_3COOH, concentrated H_2SO_4, 2% $K_2Cr_2O_7$, Mayer's reagent, Dragendorff's reagent, 10% NaOH, 10% $FeCl_3$, 1 N HCl, and concentrated HCl.

Preparation of Green Spinach Ethanol Extract

Mature *A. hybridus* L. leaves were washed thoroughly under running water and oven-dried at 45 °C for 8 hours until completely dry. The dried leaves were then pulverized, and 100 g of the powder was extracted by re-maceration using 96% ethanol at a 1:10 (w/v) ratio. The mixture was tightly sealed and stored in a glass bottle for 24 hours, then filtered through filter paper. The residue was repeatedly re-macerated with fresh 96% ethanol until the resulting filtrate became clear. The combined liquid extract was concentrated by evaporation in an oven at 60 °C until a viscous extract was obtained. The final extract was stored in a tightly closed glass container and protected from sunlight.

Extract Characterization: Yield, Residual Ethanol, and Phytochemical Screening

The percentage of extraction yield was determined by calculating the weight of the viscous extract (g) divided by the weight of the simplicia (g). The formula is as follows as described by Syamsul et al. (2020):

$$\%\ Yield = \frac{weight\ of\ viscous\ extract\ (g)}{weight\ of\ simplicia\ (g)} \times 100\%$$

To ensure the extract was free of residual ethanol. 1 mL of the extract was reacted with 2 drops of concentrated H_2SO_4 and 1 mL of 2% $K_2Cr_2O_7$. A positive result (ethanol present) was indicated by a color change from orange to bluish.

Qualitative Phytochemical Screening to Identify Major Secondary Metabolites

For alkaloids, 1 mL of extract was mixed with 1 mL of 1 N HCl and 9 mL of distilled water, heated in a water bath for 2 min, and filtered. The filtrate was tested using Mayer's reagent (3 drops filtrate + 3 drops reagent; positive if a yellowish-white precipitate forms) and Dragendorff's reagent (3 drops filtrate + 3 drops reagent; positive if an orange precipitate forms). For flavonoids, 1 mL of extract was treated with 2–4 drops of 10% NaOH; formation of a yellow-to-yellow-brown color indicated a positive result.

For saponins, 1 mL of extract was mixed with 10 mL of hot distilled water, cooled, and vigorously shaken for 10 seconds; persistent foam of approximately 1–10 cm indicated saponins. For tannins, 1 mL of extract was added with 1–2 drops of 10% $FeCl_3$; a dark brown/greenish-black color indicated tannins. For terpenoids, 1 mL of extract was treated with 3 drops of concentrated HCl and 1 drop of concentrated H_2SO_4; formation of a red or purple color indicated a positive result.

Preparation of Green Spinach Leaf Extract Working Solution

The concentrated extract obtained from the re-maceration process was prepared into working solutions of 2%, 1.5%, and 1%, each tested with the designated number of replications. Serial dilutions were prepared using the standard dilution equation. For the 2% solution, 2 mL of 100% extract was pipetted and diluted with distilled water up to 100 mL. For the 1.5% solution, 1.5 mL of 100% extract was diluted to 100 mL. For the 1% solution, 1 mL of 100% extract was diluted up to 100 mL. The positive control was prepared using Temephos (0.02 mg/L) by dissolving Temephos 0.02 mg/L to obtain a final volume of 100 mL with distilled water. The negative control consisted of distilled water only.

Preparation of Third-Instar Larvae

Total of 500 third-instar *A. aegypti* larvae were used. Larvae were produced by colony rearing. Adults were maintained in cages, and females were blood-fed with guinea pig blood to induce egg development. Eggs were collected on filter paper placed in water containers and stored in a dry, tightly sealed container. Third-instar larvae were identified by a 4–5 mm body length and a dark-brown siphon with trident comb scales. Before testing, larvae were acclimated in 20 mL of distilled water for 30 min. Rearing and testing were conducted at 25–27 °C and 70–80% relative humidity.

Larvicidal Assay

The process of holding larvae using 20 mL of distilled water, larvae were put into a bowl for 30 minutes, after which the remaining distilled water and working solution were added according to the concentration of each extract until it reached 100 mL. Dead larvae were recorded; larval death was indicated by the larvae not responding when exposed to light. Calculation of dead larvae was carried out after 24 hours of treatment.

Larvae Mortality Calculation

Larval mortality was recorded to determine the percentage mortality of *A. aegypti* larvae. Larvae were classified as dead if they did not respond to gentle probing with a blunt stick, showed no movement when exposed to flashlight illumination, and were observed resting at the bottom of the solution. The percentage mortality at 24 hours was calculated using the mortality percentage formula from Yuliana et al., (2021). The formula is as follows:

$$\%\ Mortality = \frac{number\ of\ dead\ larvae}{total\ number\ of\ larvae} \times 100\%$$

Data Analysis

This study data consisted of numerical counts of larval mortality. Statistical analyses were performed using SPSS version 26.0 for Windows to determine the significance of differences among group means. Data distribution was assessed using the Kolmogorov–Smirnov normality test, and variance homogeneity was evaluated using Levene's test. If the data were normally distributed and homogeneous, between-group differences were analyzed using one-way ANOVA, followed by the Least Significant Difference (LSD) post-hoc test to identify the most effective larvicidal concentration. If the data were not normally distributed, the Kruskal–Wallis test was applied, followed by Mann–Whitney post-hoc comparisons to determine which concentration showed greater larvicidal effectiveness. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSIONS

A total of 100 grams of dried *A. hybridus* leaf powder was extracted with 96% ethanol using the re-maceration method. The extraction yielded a 35.85% yield. The ethanol-free screening was conducted to confirm that the *A. hybridus* leaf extract was free from residual ethanol. This indicates that the *A. hybridus* leaf extract did not contain detectable ethanol. Qualitative phytochemical screening was performed based on observable color changes and precipitate formation. This screening aimed to identify bioactive compounds with potential as natural larvicides. The metabolites assessed included alkaloids, flavonoids, saponins, tannins, and terpenoids. The following table (Table 1) is the result of the extract test.

Table 1.
Green Spinach Leaf Extract Assay

Assay	Results	Interpretation
Ethanol	No colour changes from orange to bluish	-
Alkaloid (Mayer's)	White sediment	+
Alkaloid (Dragendorff's)	Orange sediment	+
Flavonoid	Brownish yellow	+
Saponin	Foam (height 1.4 cm)	+
Tannin	Blackish green	+
Terpenoid	Red	+

Note: (+) = positive result; (-) = negative result

Larvae of *A. aegypti* were exposed to an ethanol extract of *A. hybridus* leaf as a natural larvicide for 24 hours. Mortality outcomes were then recorded and are presented in Table 2.

Table 2.
Larval Mortality Percentage (%) of all Groups after 24 Hours of Treatment

Groups (treatment)	Replicates					Average	Mortality percentage (%)	SD
	I	II	III	IV	V			
Positive control (Temephos 0.02 mg/L)	20	20	20	20	20	20	100	0
Negative control (distilled water)	0	0	0	0	0	0	0	0
A. hybridus ethanol extract 1%	12	11	11	13	10	11.4	57	1.14
A. hybridus ethanol extract 1.5%	18	16	17	17	18	17.2	86	0.83
A. *hybridus* ethanol extract 2%	20	20	20	20	20	20	100	0

Figure 1 shows that the positive control (Temephos at 0.02 mg/L) killed all larvae in every replication, with a mean mortality of 20 larvae. In contrast, the negative control (distilled water) caused no larval death (0%). Treatment with 1% *A. hybridus* leaf extract resulted in an average mortality of 11.4 larvae (57%), while 1.5% produced a mean mortality of 17.2 larvae (86%). The 2% extract killed all tested larvae (100%), matching the positive control. The highest mortality was observed at 2% (100%).

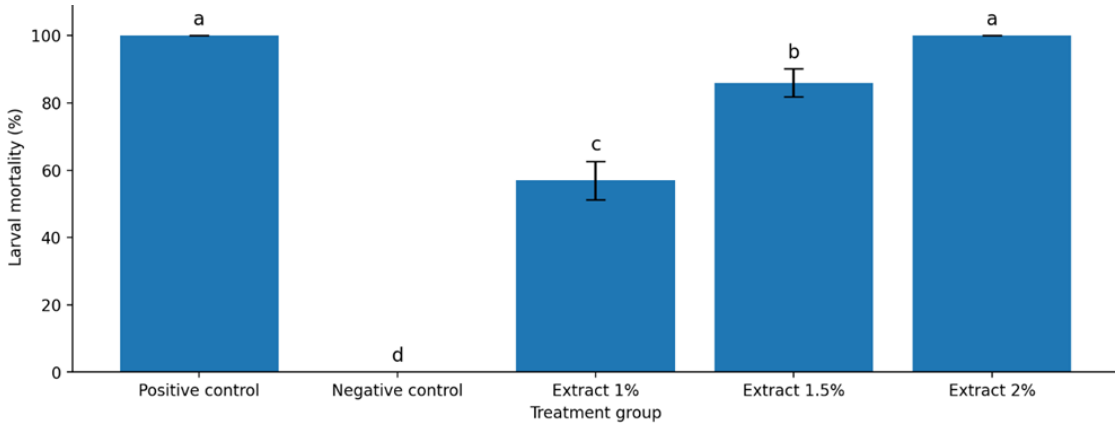


Figure 1. *Aedes aegypti* larvae mortality after 24 hours of treatment of all groups with Mann-Whitney and post-hoc analysis. Different letters annotate significance ($p<0.05$).

Statistical analysis indicated significant differences among all groups ($p < 0.05$), whereas one comparison was not significant ($p > 0.05$), namely between the positive control and the 2% ethanol extract group ($p = 1.000$), as shown in Figure 1. This indicates that larval mortality in the 2% extract treatment was comparable to that achieved with Temephos 0.02 mg/L. Figure 2 shows third-instar A. *aegypti* larvae used in this study.

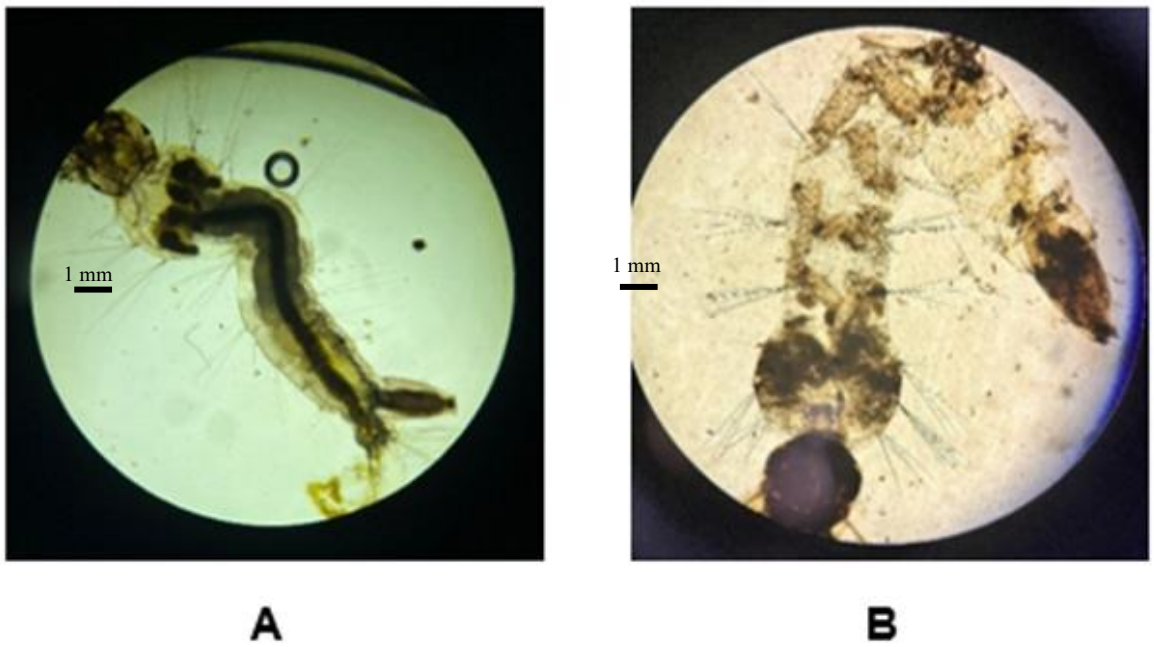


Figure 2. Microscopic images of third-instar *A. aegypti* larva. (A) Before treatment with *A. hybridus* leaf extract. (B) After treatment with *A. hybridus* leaf extract. Magnification: [10×4]

Natural larvicides represent a promising alternative to chemical larvicides that are persistent in the environment and potentially harmful to humans, as larval-stage control can reduce the emergence and spread of adult mosquitoes (Tutik et al., 2020). *A. hybridus* leaf contains secondary metabolites such as alkaloids, flavonoids, saponins, tannins, and terpenoids. These secondary metabolites may act synergistically as natural larvicidal agents against *A. aegypti* (Sittichok et al., 2024).

In this study, third-instar *A. aegypti* larvae were used because they exhibit complete morphological development and larger body size, making them more suitable for larvicidal exposure and consistent with WHO standards (Adrianto et al., 2024). In contrast, fourth-instar larvae may rapidly develop into pupae, a resting stage that does not require feeding (Hawkes & Hopkins, 2021), whereas first- and second-instar larvae are smaller and less morphologically developed, limiting observation and experimental reliability (Adrianto et al., 2024). Before bioassays, larvae underwent an acclimatization (holding) step in 20 mL of distilled water for 30 minutes to stabilize movement and minimize potential bias from abrupt environmental changes (Chaiphongpachara et al., 2020). Experiments were conducted at 25–27°C and 70–80% relative

humidity, conditions aligned with the optimal development of *A. aegypti* larvae (Mujiarto et al., 2024).

Treatments in this study consisted of ethanol extract of *A. hybridus* leaf at 1%, 1.5%, and 2%, with Temephos (0.02 mg/L) as the positive control and distilled water as the negative control. These controls were included to verify the expected efficacy of the standard larvicide and to confirm that the test medium did not induce mortality (Widianingtiyas et al., 2025). Kruskal–Wallis analysis indicated significant differences among most groups, except that the 2% extract group did not differ from the positive control, suggesting that 2% was the most effective concentration because it produced larval mortality comparable to Temephos. This pattern was consistent with mortality percentages, where 2% and the positive control achieved 100% mortality, followed by 1.5% (86%) and 1% (57%), while the negative control remained at 0%.

The concentration–response trend likely reflects higher levels of active secondary metabolites in more concentrated extracts, resulting in greater larvicidal activity (Wahyuni et al., 2023), implying that ethanol extract of *A. hybridus* leaves may be more efficient, achieving 100% mortality at 2% within 24 hours, plausibly due to its combined content of alkaloids, flavonoids, saponins, tannins, and terpenoids.

Mechanistically, alkaloids and terpenoids can disrupt neural transmission by altering impulse regulation associated with acetylcholine-mediated muscle activity (Anin et al., 2023; Câmara et al., 2024; Kerage et al., 2019). Under normal conditions, acetylcholinesterase terminates neurotransmission by hydrolyzing acetylcholine (Trang & Khandhar, 2023). Inhibition of this enzyme can lead to acetylcholine accumulation, unstable signaling, and sustained respiratory muscle spasms that culminate in larval death (Costa et al., 2023; Halisa et al., 2022). Flavonoids may additionally target Noppera-bo (Nobo), an enzyme involved in ecdysteroid biosynthesis that regulates hatching, molting, and metamorphosis; binding of flavonoids to Nobo can inhibit its activity and disrupt ecdysteroid production, thereby impairing larval development before pupation (Ebihara & Niwa, 2023; Inaba et al., 2022).

Saponins, as natural surfactants, may damage lipid membranes, increase membrane permeability, and induce larval lysis (Kaihena & Ukratalo, 2021; Sari & Sumadewi, 2021), and they may also interfere with molting by reducing sterol absorption required for 20-hydroxyecdysone synthesis (Shakeel et al., 2025). Tannins may contribute to mortality by forming tannin–protein complexes in the digestive tract, reducing protein digestibility and absorption, and thereby inhibiting growth and inducing starvation (Putra & Sjofjan, 2021; Samtiya et al., 2020). Additionally, tannins can reduce lipid and carbohydrate absorption through complex formation, limiting nutrient availability and further slowing development, which may ultimately lead to mortality under prolonged exposure. (Kurniawan & Zahra, 2021; Molino et al., 2023; Petchidurai et al., 2023).

Collectively, these mechanisms support the observed high larvicidal efficacy of the 2% ethanol extract of *A. hybridus* leaves and its potential application as a natural larvicide against *A. aegypti*. Future work should therefore test broader concentration ranges and multiple observation times to derive LC50/LC90 and LT50, standardize and quantitatively screen the extract, and validate mechanisms using biochemical and histological assays. This study can be followed by evaluating non-target safety and environmental persistence and confirming performance through formulation development and semi-field or field trials.

CONCLUSION

The ethanol extract of green spinach (*A. hybridus* L.) leaves demonstrated larvicidal activity against A. *aegypti* after 24 hours of exposure. Larval mortality increased with concentration, rising from moderate mortality at 1% (57%) to high mortality at 1.5% (86%) and reaching complete mortality at 2% (100%), while the negative control showed 0% mortality and the positive control produced 100% mortality. Statistical analysis confirmed that the 2% extract showed no significant difference compared with the positive control, indicating that this concentration provided the strongest and most effective larvicidal effect under the study conditions. Overall, the findings support the potential use of *A. hybridus* leaf ethanol extract as a natural larvicide, with 2% identified as the most effective concentration in this experiment. Future studies should test a wider concentration range and multiple observation times to determine LC50 and LC90 values and standardize the phytochemical content of the extract. Evaluation of non-target safety and semi-field or field trials is also needed to confirm its practical use as a larvicide.

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AUTHOR CONTRIBUTION STATEMENT

HR: Conceptualization, Methodology, Formal analysis, Writing original draft. **AR:** Conceptualization, Investigation, Data curation, Visualization, Validation. **AS:** Supervision, Project administration, Writing, review & editing, Funding acquisition. **AF**: Investigation, Resources, Writing, review & editing. All authors have read and agreed to the published version of the manuscript.

ETHICAL APPROVAL STATEMENT

This study uses invertebrate animals (*A. Aegypti*) obtained from the Entomology Laboratory of BBLKM East Jakarta as the research subjects; therefore, ethical approval is not required. The

research was carried out in accordance with good laboratory practice and the WHO guidelines for laboratory and field testing of mosquito larvicides

DECLARATION OF GENERATIVE AI USE

During the preparation of this work, the authors used Claude (Anthropic) in order to improve readability, grammar, and language editing, and to help draft the recommendations for future research in the Conclusion. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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