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Concentration-Dependent Larvicidal Activity of Nutmeg Seed (*Myristica fragrans Houtt.*) Extract against Third-Instar Aedes aegypti Larvae

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Abstract

Dengue prevention continues to depend on integrated vector management, including control of immature Aedes aegypti in water-holding containers. Plant-derived larvicides may provide locally available candidates, but their activity must be established through controlled bioassays before practical use. This study evaluated the concentration-dependent larvicidal activity of nutmeg seed (Myristica fragrans Houtt.) extract against third-instar A. aegypti larvae. A posttest-only controlled experiment involved five groups: 10%, 20%, 30%, and 40% (v/v) ethanolic nutmeg seed extract and an aquadest negative control. Each group comprised three independent vessels containing 25 larvae, yielding 375 larvae in total. Nutmeg seed simplicia was macerated in 96% ethanol for 5 × 24 hours and concentrated using a rotary evaporator. Larval mortality was observed every four hours for 24 hours. Data were analyzed using ANOVA, followed by exploratory least significant difference pairwise comparisons; a Kruskal-Wallis test was applied as a sensitivity analysis. Mortality increased from 0% in the control to 42.7%, 52.0%, 64.0%, and 82.7% at 10%, 20%, 30%, and 40%, respectively. Mean mortality differed significantly among groups, $F(4,10) = 34.753$, $p<0.001$, with a large effect size ($\eta^2=0.933$). The sensitivity analysis supported an overall group difference ($H=11.932$, $p=0.018$). The 40% extract differed significantly from all lower concentrations, whereas differences between 10% and 20% and between 20% and 30% were not significant. Nutmeg seed extract demonstrated concentration-dependent larvicidal activity, with the highest mortality at 40%. However, the absence of a positive control, chemical standardization, lethal-concentration estimates, and non-target safety testing means that household or field application cannot yet be recommended.

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1. INTRODUCTION

Dengue is categorized as an arboviral disease with a rapidly growing burden and expanding geographic distribution. The World Health Organization reported more than four million cases and more than 3,000 deaths from 97 countries in January-July 2025. The risk of transmission is determined not only by the presence of the virus, but also by unplanned urbanization, human mobility, climate change, and environmental suitability for Aedes aegypti as a primary vector (Cattarino et al., 2020; Ryan et al., 2019; World Health Organization (WHO), 2025; Zeng et al., 2021). Increasing temperatures and changing rainfall patterns can expand the seasons and areas suitable for mosquito development. Thus, environmental health interventions need to adapt to local dynamics (Gizaw et al., 2024).

In Indonesia, dengue remains a serious public health problem. The Ministry of Health of the Republic of Indonesia reported 114,720 cases and 894 deaths in 2023. By the 43rd week of 2024, this number had increased to 210,644 cases and 1,239 deaths, spread across 259 districts/cities in 32 provinces (Kemenkes RI, 2024). This large burden emphasizes that case surveillance must go hand in hand with environmental management, reduction of breeding sites, community involvement, and evidence-based vector control (Maipadiapati et al., 2026).

Vector control is one of the fundamental strategies in the global dengue response (World Health Organization (WHO), 2024). Larvicide is commonly used to stop the development of mosquitoes in water containers that cannot be immediately drained or removed. However, repeated exposure to insecticides can select for resistant populations. Resistance to various groups of insecticides has been reported in *A. aegypti* populations in Jakarta, Banjarmasin and other regions in Indonesia, with the involvement of metabolic and target-site mechanisms (Hamid et al., 2017, 2018; Silalahi et al., 2022). Similar phenomena have also been reported in other Southeast Asian countries. Therefore, the rotation of active ingredients and the development of new candidates must be assigned within a resistance management framework, not as a sole replacement for existing interventions (Malijan et al., 2024).

Natural ingredients offer a diversity of bioactive compounds that can work in the nervous, respiratory, integumentary, and digestive systems of insects. Recent studies have revealed that plant extracts, essential oils, terpenes, and nanoformulations can exhibit larvicidal activity, although potency is greatly influenced by plant species, plant part, solvent, extraction method, chemical composition, larval instar, and formulation (Abdulwahab et al., 2024; Huang et al., 2019; Milugo et al., 2021). Natural origin does not automatically imply safety. The effectiveness against vectors should be assessed alongside non-target organism toxicity, stability, residue, and the feasibility of application.

Nutmeg (*Myristica fragrans Houtt*.) is a spice plant native to Indonesia, and its seeds are rich in essential oils and secondary metabolites. The chemical composition may include sabinene, α-pinene, β-pinene, terpinen-4-ol, myristicin, elemisin, eugenol, and other phenylpropanoid compounds, with variations depending on the origin of the material and analysis methods (Ashokkumar et al., 2022; Khairan et al., 2024; Matulyte et al., 2019; Warsito, 2021; Wibowo et al., 2018). The essential oils of nutmeg leaves and fruits have shown larvicidal activity against A. aegypti, while the nonpolar fraction of nutmeg seeds has also shown insecticidal activity against other mosquitoes (Bulu et al., 2019; Carolina & Maman, 2016). Nutmeg-based formulations, including silver nanoparticles synthesized using *Myristica fragrans* fruit peel, have demonstrated larvicidal activity against the dengue vector, *Aedes aegypti*. However, because nanoparticle synthesis involves different plant materials and preparation procedures, their toxicity values cannot be directly compared with those obtained from crude ethanol extracts (Hillary et al., 2025).

Evidence regarding the response of A. aegypti larvae to concentration-based nutmeg ethanol extract in the context of local Acehnese materials is still limited. This study aimed to evaluate the differences in mortality of third-instar *Aedes aegypti* larvae after exposure to 10%, 20%, 30%, and 40% nutmeg extract preparations for 24 h. The research hypothesis was that larval mortality would increase with increasing extract concentration.

2. METHODS

This was a laboratory experimental study with a post-test-only controlled design. The implementation was conducted from March 20 to June 29, 2025. The preparation of simplicia and larval bioassays was performed in Gampong Bayu, Aceh Besar, while the extraction and concentration were performed at the Integrated Laboratory/Microbiology, Environmental Health Department, Poltekkes Kemenkes Aceh. Nutmeg seeds were obtained from South Kluet, South Aceh Regency. The experimental unit was the treatment vessel, not the individual larvae.

To prepare nutmeg seed extract, two kilograms of ripe nutmeg seeds were separated from fresh fruit, washed, cut into pieces of approximately 1.5 cm, and dried in the air without direct exposure to sunlight for at least seven days. The dry material was weighed, ground into simplicia powder, and 200 g of simplicia was macerated using 2,000 mL of 96% ethanol for 5 × 24 h. The maceration results were further filtered, and the filtrate was evaporated using a rotary evaporator until a thick extract was obtained. The study report does not include the yields, chromatographic profiles, or levels of marker compounds; therefore, the treatment concentration in this study was based on the ratio of the extract volume to the final solution volume (v/v).

A. aegypti larvae were obtained from the Public Health Laboratory in Ingin Jaya District, Aceh Besar, Indonesia. The larvae were acclimatized for 24 h and selected at the third instar stage. The five groups consisted of 10% extract (10 mL extract + 90 mL distilled water), 20% (20 mL extract + 80 mL distilled water), 30% (30 mL extract + 70 mL distilled water), 40% (40 mL extract + 60 mL distilled water), and negative control (100 mL distilled water). Each group had three independent vessels, each containing 25 larvae; thus, 15 vessels and 375 larvae were used. The study did not use a standard larvicide positive or solvent control.

Mortality was observed every four hours until 24 h after exposure. Larvae were categorized as dead if they sank, did not move, and did not show a response after mechanical stimulation. The number of dead larvae was recorded on an observation sheet. As the control mortality was 0%, the Abbott correction was not applied. The mortality percentage was calculated as the total number of dead larvae divided by 75 larvae in each group, multiplied by 100%.

Primary data for each vessel were re-analyzed to prepare the article. Mean, standard deviation, frequency, and percentage were used for the description. Differences in the mean mortality among the five groups were analyzed using one-way ANOVA at the $\alpha=0.05$ level. Homogeneity of variance was checked using the median-based Brown-Forsythe/Levene test, while the normality of residuals was assessed using the Shapiro-Wilk test. Effect sizes were calculated using eta squared (η^2). The least significant difference (LSD) test was applied as an exploratory pair analysis, as it was the method applied in the original report. Considering the small number of repetitions and abnormal residuals, the Kruskal-Wallis test was performed for sensitivity analysis.

This study did not involve human participants or vertebrates. The experimental organisms were third-instar larvae of Aedes aegypti, which are classified as invertebrates. The research protocol was reviewed and approved by the Health Research Ethics Committee of STIKES Kendal, Indonesia (ethical approval number

019/EC/KEPK_STIKES_KENDAL/III/2025, dated March 3, 2025). All experimental procedures, including the handling of plant extracts, mosquito larvae, laboratory materials, and post-experimental waste, were conducted in accordance with applicable institutional laboratory biosafety and waste-management procedures.

3. RESULTS AND DISCUSSION

During the 24 h observation period, no deaths were observed in the control group. Mortality increased as the extract concentration increased, from 42.7% at 10% concentration to 82.7% at 40% concentration. No concentration produced 100% mortality.

Table 1. Mortality of *Aedes aegypti* Larvae after Exposure to Nutmeg Extract for 24 Hours

Group	Repetition I	Repetition II	Repetition III	Mean ± SD	Death/ Total (n)	Mortality (%)
Control	0	0	0	0.00 ± 0.00	0/75	0.0
10%	8	12	12	10.67 ± 2.31	32/75	42.7
20%	11	14	14	13.00 ± 1.73	39/75	52.0
30%	12	18	18	16.00 ± 3.46	48/75	64.0
40%	18	22	22	20.67 ± 2.31	62/75	82.7

Note: *Each repetition involved 25 larvae in one vessel; percentages were calculated from 75 larvae per group.*

One-way ANOVA revealed significant differences in mean mortality between groups ($F(4, 10) = 34.753$; $p < 0.001$). Concentration explained approximately 93.3% of the variation in mortality between the experimental units ($\eta^2=0.933$). The variance between groups was relatively homogeneous, but the residuals were not normally distributed. Therefore, the results were confirmed using the Kruskal-Wallis test, which similarly showed an overall difference between the groups.

Table 2. Results of analysis of differences in mortality between groups

Analysis	Statistics	df	p-value	Interpretation
One-way ANOVA	$F = 34.753$	4;10	<0.001	Significant difference
Eta squared	$\eta^2 = 0.933$	-	-	The effect size was very large
Brown-Forsythe/Levene	$F = 0.312$	4;10	0.864	Homogenous variance
Shapiro-Wilk residual	$W = 0.848$	-	0.016	The residuals were not normally distributed
Kruskal-Wallis	$H = 11.932$	4	0.018	Sensitivity analysis was significant

LSD comparison showed that all extract concentrations were significantly different from that of the control. The 40% concentration was significantly different from the 10%, 20%, and 30% concentrations. In contrast, the differences between 10% and 20% and between 20% and 30% were not statistically significant. This finding corrected the interpretation of the original report, which stated that all pairs of concentrations had significant differences (Rahmah, 2025).

Table 3. Pairwise comparison of mortality using the exploratory LSD test.

Comparison	Mean Difference	95% CI	p-value	Significance
10% vs control	10.667	6.545-14.789	<0.001	Significant
20% vs control	13.000	8.878-17.122	<0.001	Significant

Comparison	Mean Difference	95% CI	p-value	Significance
30% vs control	16.000	11.878-20.122	<0.001	Significant
40% vs control	20.667	16.545-24.789	<0.001	Significant
20% vs 10%	2.333	-1.789-6.455	0.236	Not Significant
30% vs 10%	5.333	1.211-9.455	0.016	Significant
40% vs 10%	10.000	5.878-14.122	<0.001	Significant
30% vs 20%	3.000	-1.122-7.122	0.136	Not Significant
40% vs 20%	7.667	3.545-11.789	0.002	Significant
40% vs 30%	4.667	0.545-8.789	0.030	Significant

This study showed a clear concentration-mortality pattern: the higher the concentration of nutmeg extract, the higher the mortality of A. aegypti larvae after 24 h. The 40% concentration produced the highest mortality rate of 82.7% and was significantly different from all lower concentrations. The overall effect was large, although none of the treatments achieved complete mortality in the study. The Kruskal-Wallis results that were in the same direction as the ANOVA results strengthened the conclusion of differences between groups; however, the findings still need to be read as preliminary evidence because only three vessels were available per group.

The activity found is consistent with the report that nutmeg essential oil has a larvicidal effect on *A. aegypti*. Carolina and Maman (2016) obtained LC50 values of approximately 110.1 and 133.8 µg/mL for fruit and leaf oils, respectively. Such findings cannot be directly compared with the 10%-40% concentrations in this study because of the different test materials used: the previous study used distilled essential oils with mass units per volume, while this study used a thick ethanol extract expressed in v/v that had not been standardized. Previous studies have indicated that the nonpolar fractions of *Myristica fragrans* may contribute substantially to insecticidal activity. Recent evidence has shown that nutmeg essential oil, which contains abundant lipophilic volatile compounds, exhibits insecticidal effects against mosquito vectors, supporting the hypothesis that hydrophobic constituents play an important role in mosquito control (Seo et al., 2021).

Nutmeg contains a mixture of monoterpenes and phenylpropanoids, including sabinene, pinene, terpinen-4-ol, myristicin, and elemicin. A previous study conducted in Aceh found myristicin to be one of the main components of nutmeg oil (Khairan et al., 2024), while other studies from other regions showed that the component proportions could change according to variety, place of growth, storage, and extraction method (Ashokkumar et al., 2022; Matulyte et al., 2019; Warsito, 2021; Wibowo et al., 2018). These volatile compounds can interfere with neuromuscular, membrane, and respiratory functions, or insect detoxification enzymes. However, this mechanism cannot be claimed for the extract in this study since quantitative phytochemical screening and examinations for GC-MS, enzymes, or histological changes in larvae were not conducted.

The literature on essential oils shows that larvicidal activity is highly dependent on the composition and interactions between compounds. Terpene combinations can be synergistic or antagonistic, and formulations can alter the bioavailability of active ingredients (Huang et al., 2019; Pandiyan et al., 2019; Sarma et al., 2019). For example, geranial and trans-cinnamaldehyde show increased toxicity when combined and cause changes in larval and pupal morphology (Sittichok et al., 2024). Various essential oils from other plants are also active against *Aedes*, but toxicity values vary widely according to species, test larvae, and bioassay conditions (Martins et al., 2024; Pavela & Govindarajan, 2017). Therefore, the high mortality at a concentration of 40% should be viewed as a basis

for fractionation and dose-response modeling, not as evidence that the crude extract is ready for application.

LSD comparison showed no significant differences between 10% and 20% and between 20% and 30%. Such patterns might reflect overlapping ranges of biological responses, variation between vessels, or low assay power due to the use of only three replicates. In contrast, the 40% concentration was consistently separated from the lower groups. Previous studies on botanical insecticides in Indonesia have also reported increased mortality at higher concentrations, including basil extract against houseflies and *Srigading* extract against *Aedes aegypti* larvae (Chitra et al., 2023; Fakhriadi et al., 2023). However, similar trends did not indicate equal potency because of differences in the extract matrices, target species, and assay designs.

A study on nutmeg lotion repellent on Culex mosquitoes revealed that nutmeg ingredients could also influence the behavior of adult mosquitoes, but the repellent endpoint could not be used to explain larval mortality directly (Arrizqiyani et al., 2020). Similarly, zinc oxide nanoparticles synthesized using M. fragrans showed toxicity against dengue vectors, but the activity was the result of a combination of nanoparticles and phytochemicals, not nutmeg extract alone (Ashokan et al., 2017). This comparison is important because it prevents the discussion from equating test materials that are chemically and biologically different.

Nutmeg is a local commodity with a potential to provide raw materials for regional resource-based larvicides. The development of standardized extracts can support the diversification of active ingredients in vector control, especially when resistance to conventional insecticides increases (Hamid et al., 2018; Silalahi et al., 2022). However, plant-based products should not be directly placed into household water reservoirs. The translational phase must include the identification of marker compounds, stable formulation, comparison with standard temephos or larvicides, non-target organism testing, residue evaluation, and semi-field testing. Reviews of natural larvicides consistently emphasize that effectiveness, ecological safety, cost, and user acceptability should be assessed simultaneously (Abdulwahab et al., 2024; Milugo et al., 2021).

This study had several limitations. The experimental unit comprised only three vessels per group, which limited the estimation of variation and statistical assumption testing. In addition, there was no positive control, solvent control, residual ethanol verification, extract yield, botanical authentication, or chemical profile. The temperature, pH, photoperiod, vessel size, feeding, randomization, and blinding of observers were not fully documented. Moreover, the current study did not calculate LC50/LC90, did not evaluate time-based mortality, only used one instar and 24-hour exposure, and did not assess non-target organism toxicity or field effectiveness. Further studies are recommended to apply adequate numbers of repetitions, a randomized design, positive control, binomial/probit model for lethal concentrations, GC-MS standardization, and semi-field testing before practical recommendations are provided.

4. CONCLUSION

The ethanol extract of nutmeg seeds demonstrated larvicidal activity against third-instar Aedes aegypti larvae, with mortality increasing proportionally with extract concentration after 24 hours of exposure. The highest larvicidal activity was observed at the highest concentration tested, indicating its potential as a promising candidate for further formulation development. However, the extract should not yet be recommended for direct public use. Further studies are needed to determine its lethal concentrations, compare its efficacy with standard larvicides, standardize its active compounds, and

evaluate its safety toward non-target organisms and effectiveness under semi-field conditions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL CONSIDERATIONS

The research protocol was reviewed and approved by the Health Research Ethics Committee of STIKES Kendal, Indonesia (ethical approval number 019/EC/KEPK_STIKES_KENDAL/III/2025, dated March 3, 2025). All experimental procedures, including the handling of plant extracts, mosquito larvae, laboratory materials, and post-experimental waste, were conducted in accordance with applicable institutional laboratory biosafety and waste-management procedures.

AUTHOR CONTRIBUTIONS

Fajriansyah contributed to conceptualization, methodology, study design, supervision, laboratory investigation, and writing of the original draft. Helvina Rahmah contributed to methodology, experimental investigation, preparation of the nutmeg extract, data collection, and data curation. Agus Hendra Al Rahmad contributed to data processing, formal analysis, statistical analysis, validation, and writing (review and editing). All authors contributed to the interpretation of the findings, critically reviewed the manuscript, and read and approved the final version of the manuscript.

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DECLARATION OF ARTIFICIAL INTELLIGENCE USE

During manuscript preparation, ChatGPT Plus was used to support the comprehension of the referenced articles and improve the scientific language and organization. Grammarly was used for grammar checking and paraphrasing, and IBM SPSS Statistics was used for statistical analysis. All outputs were critically reviewed and verified by the authors, who remained fully responsible for the manuscript's accuracy, originality, and integrity.

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