

Contact toxicity of ethanolic *Syzygium aromaticum* flower-bud extract against adult *Aedes* and *Culex* mosquitoes as livestock disease vectors: a bottle bioassay

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ABSTRACT

Mosquitoes are important vectors of pathogens, whose bites cause significant health and economic burdens. In the livestock sector, these pests significantly reduce animal productivity and transmit critical veterinary pathogens. Chemical-based repellents pose risks, making plant-derived alternatives like clove (*Syzygium aromaticum*) a promising candidate due to its bioactive compounds. This study evaluated the contact toxicity of ethanolic *Syzygium aromaticum* flower-bud extract against adult *Aedes* and *Culex* mosquitoes using a bottle bioassay. Experimental groups consisted of four treatment concentrations (0.5%, 1%, 3%, and 5%), positive control, and negative control. The study employed a completely randomized design with three replicates of 30 mosquitoes each, totaling 540 mosquitoes assessed hourly for seven hours. Results demonstrated that clove extract at 3% concentration achieved 100% knockdown activity within three hours, while 5% concentration induced complete knockdown activity within the first hour of observation. This observed efficacy indicates contact toxicity rather than repellency, suggesting that the extract warrants further investigation for development as a potential botanical contact insecticide.

INTRODUCTION

Mosquito-borne infectious diseases remain a major challenge in both public and veterinary health in Indonesia. As primary vectors, mosquitoes play a crucial role in transmitting diverse pathogenic agents, including viruses, bacteria, and parasites. Malaria with 543,965 positive cases reported in Indonesia (Ministry of Health of the Republic of Indonesia, 2025). Susilawati et al. (2026), beyond their role in human disease transmission, mosquitoes also contribute to the spread of several animal diseases, such as lumpy skin disease (LSD), Japanese encephalitis, and dirofilariasis (Dayanti et al., 2021; Diptyanusa et al., 2022; Hidayatik et

al., 2025). Additionally, the high incidence of these diseases not only increases the burden on healthcare systems and economic losses due to treatment costs but also affects animal health and livestock productivity, underscoring the importance of effective mosquito population control in livestock mosquito-borne diseases (Rafiq et al., 2024).

One promising candidate for natural repellents is clove (*Syzygium aromaticum*), a locally available Indonesian commodity rich in essential oils and bioactive compounds. Clove leaves contain eugenol, saponins, flavonoids, and tannins (Muhammad et al., 2022). Eugenol, the primary component of clove, not only imparts its distinctive aroma but also exhibits neurotoxic



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effects that disrupt the nervous system and chemoreceptors of insects (Oktari et al., 2022; Sheikh et al., 2021). Additionally, the combined action of saponins, flavonoids, and tannins contributes to cuticle damage, metabolic disruption, impaired nutrient absorption, and toxic effects in mosquitoes (Muhammad et al., 2022).

Although the potential of clove as a natural repellent has been investigated, studies specifically evaluate the effectiveness of ethanolic clove extract against adult mosquitoes using the bottle bioassay method at varying concentrations remain limited, particularly for mosquito populations collected from Yogyakarta. Therefore, this study aimed to evaluate the insecticidal and protective potential of ethanolic clove extract (*Syzygium aromaticum*) at concentrations of 0.5%, 1%, 3%, and 5% as a natural herbal repellent against mosquitoes using the bottle bioassay method. The findings are expected to provide preliminary scientific evidence supporting the potential application of clove flower bud extract as a plant-derived insecticidal agent for mosquito control.

MATERIALS AND METHODS

Study Period and Location

The study was conducted from July to August 2026. Experimental procedures, including egg hatching, mosquito rearing, and repellent activity assays, were carried out at the Entomology Laboratory, Department of Parasitology, Faculty of Veterinary Medicine, Universitas Gadjah Mada. Mosquito genus identification was performed at the Parasitology Laboratory, Faculty of Veterinary Medicine, Universitas Gadjah Mada. Preparation of ethanolic clove extract was conducted at the Pharmaceutics Laboratory Unit II, Faculty of Pharmacy, Universitas Gadjah Mada.

Preparation of Clove Extract

A total of 3 kg dried clove (*Syzygium aromaticum*) was used in this study. Extraction was performed using ethanol as a polar solvent. All extraction procedures were conducted at the Pharmaceutics Laboratory Unit II, Faculty of Pharmacy, Universitas Gadjah Mada.

Mosquito Eggs Collection and Larval Rearing

Mosquito eggs were collected using ovitraps

lined with filter paper as an oviposition substrate. White filter papers were cut to fit the container diameter (500 mL glass beaker) and placed inside ovitraps, which were positioned in potential breeding sites such as stagnant water containers at the Faculty of Veterinary Medicine, Universitas Gadjah Mada. Ovitrap were left for seven days, after which filter papers containing eggs were retrieved, air-dried, and transferred to hatching trays.

Egg hatching was conducted in plastic trays (35 × 27 × 5 cm) filled with clean water. Filter papers containing eggs were submerged, allowing hatching within 2–3 days at room temperature, followed by larval development (Service, 2012). Larvae were fed homogenized chicken liver mixed with water, administered via pipette. Larvae developed into pupae within 5–7 days (Service, 2012). Pupae were subsequently moved to rearing cages. Pupae were transferred individually using pipettes into insectaria for adult emergence.

Adult mosquitoes were maintained in insectaria (19.5 × 19.5 × 24 cm) constructed with wire frames covered by voile fabric, which served as ventilation and access points. Pupae developed into adults within 2–3 days (Service, 2012). Adults were provided with 10% glucose solution absorbed in cotton, administered twice daily as a carbohydrate source (Service, 2012). Mosquitoes were reared until ready for repellent activity assays.

Extract Dilution and Repellent Preparation

Ethanolic clove extract was prepared at four concentrations: 0.5%, 1%, 3%, and 5%. The concentrated extract was diluted with 96% ethanol using the formula $M_1 \times V_1 = M_2 \times V_2$. Test solutions were prepared by diluting 0.05 mL, 0.1 mL, 0.3 mL, and 0.5 mL of concentrated extract to a final volume of 10 mL with 96% ethanol. Malathion (1 mL) served as the positive control, while distilled water (2 mL) was used as the negative control. Each treatment, including the controls, was tested in three independent bottles.

Repellent Assay Using Bottle Bioassay

Repellent activity was assessed using the bottle bioassay method with glass bottles covered by voile fabric for ventilation. Bottles were coated with ethanolic clove extract at concentrations of 0.5%, 1%, 3%, and 5%. The inner surfaces of the glass bottles were coated with the respective

concentrations of ethanolic clove flower bud extract and air-dried at room temperature until completely dry before introducing the adult mosquitoes for the knockdown bioassay according to (Brogdon & McAllister, 1998) (Brogdon & Mcallister, 1998; WHO, 2009, 2016). The same procedure was applied for positive and negative controls.

Each assay was performed in triplicate, with 30 adult mosquitoes per bottle per treatment. Mosquitoes were deprived of blood meals and fed only sugar solution, followed by 24-hour fasting prior to testing to enhance responsiveness (Wahyuni et al., 2022). The 24-hour sugar deprivation period before testing, followed by seven hours of exposure without access to a sugar source, may have introduced physiological stress and contributed to the increased knockdown activity in the negative-control group during the later observation periods.

Observation and Data Analysis

Using knockdown activity consistently for the measured endpoint. Reserve mortality for confirmed deaths and repellency for results obtained from a suitable repellency assay. Data were tabulated and mean knockdown activity percentages were calculated for each treatment. Results were analyzed descriptively and presented as bar charts.

Statistical analysis was performed using one-way ANOVA (IBM SPSS Statistics 25) to determine differences among treatments. Significant results ($P < 0.05$) were further analyzed using Tukey's post-hoc test to identify specific group differences. Mosquito knockdown was expressed as the number and percentage of affected mosquitoes relative to the total number exposed in each replicate. Each treatment consisted of three replicates, with 30 mosquitoes per replicate. Replicate-level responses were considered the experimental units for statistical analysis. Given the binomial nature of the response variable, differences among treatments were evaluated using an appropriate binomial regression framework, subject to model diagnostics. Background knockdown in the negative-control group was considered when interpreting treatment effects. Statistical significance was set at $P < 0.05$, and effect estimates, 95% confidence intervals, and relevant test statistics were reported where

estimable.

Mosquito Genus Identification

Genus identification was conducted on mosquitoes that died during treatments. Morphological characteristics genus level of 540 mosquitoes was examined microscopically at the Parasitology Laboratory, Faculty of Veterinary Medicine, Universitas Gadjah Mada according to (Gyawali et al., 2025). Mosquito specimens that had died were placed in Petri dishes and observed under a dissecting microscope to determine genus-level classification.

RESULTS AND DISCUSSION

Mosquito Identification

The test mosquitoes used in this study originated from eggs collected using ovitraps placed in stagnant water containers at the Faculty of Veterinary Medicine, Universitas Gadjah Mada. Mosquito specimens were examined under a dissecting microscope for genus identification. The identification results confirmed that the test mosquitoes belonged to the genera *Aedes* sp. (Figure 1a) and *Culex* sp. (Figure 1b). *Aedes* sp. specimens exhibited characteristic features such as black-and-white scale patterns on the leg, a lyre-shaped scutum with silver and black markings, and banded legs and abdomen. In contrast, *Culex* sp. specimens displayed shorter palpi relative to the proboscis, dark brownish scales on the thorax, legs, and abdomen, as well as small claws and paired pulvilli on each tarsal segment. These morphological characteristics are consistent with the descriptions provided by (Service, 2012). Morphological examination identified the mosquito specimens as belonging to *Aedes* spp. and *Culex* spp. (Figure 1). However, the numbers and proportions of each genus within individual treatment groups were not documented; therefore, genus-specific distributions and treatment responses could not be determined.

The identification results confirmed that the test mosquitoes used in this study belonged to the genera *Aedes* sp. and *Culex* sp. Both genera are recognized as vectors of numerous human and animal diseases, including dengue fever, chikungunya, yellow fever, Japanese encephalitis, and filariasis (Lopez et al., 2025; Maksum et al., 2024; WHO, 2024; Wulandari et al., 2022). The

use of these genera as test organisms provides valuable insight into the potential application of ethanolic clove extract against mosquitoes of significant importance in both public and veterinary health.

The predominance of *Aedes* sp. and *Culex* sp. in this study is likely related to the collection method, which employed ovitraps placed in containers with stagnant water around the Faculty of Veterinary Medicine, Universitas Gadjah Mada. Both genera are known to exploit a wide range of natural and artificial water sources as oviposition sites. Furthermore, eggs of *Aedes* sp. and *Culex* sp., belonging to the subfamily Culicinae, lack flotation devices and are therefore more resistant to desiccation during ovitrap collection. This characteristic enhances their

likelihood of hatching and developing into adults compared to other genera (Service, 2012). Such traits may explain why these two genera dominated the test mosquito population in this study.

These findings are consistent with Windyaraini et al. (2020) who reported that *Aedes* sp. dominated the mosquito communities in the Universitas Gadjah Mada area (64%) and *Culex* sp. (36%), while *Anopheles* sp. was present at very low levels. The limited presence of *Anopheles* sp. may be attributed to differences in oviposition habitat preferences, as most species of this genus favor relatively clean, vegetated water bodies. Moreover, *Anopheles* eggs are less resistant to desiccation, reducing their survival during ovitrap collection (Service, 2012).



(a)



(b)

Figure 1. Morphological observation of female's mosquitoes: (a) *Aedes* sp., (b) *Culex* sp.

Repellent Activity of Ethanolic Clove Extract Against Test Mosquitoes

This experimental study aimed to evaluate the potential of ethanolic clove (*Syzygium aromaticum*) extract as an herbal repellent at concentrations of 0.5%, 1%, 3%, and 5%. The ethanolic clove extract demonstrated toxic effects on adult mosquitoes, resulting in knockdown activity during exposure. Knockdown activity was observed over a 7-hour period, recorded at hourly intervals, and subsequently converted into percentage values. The knockdown activity data for each concentration of ethanolic clove extract are summarized in Table 1, which presents the combined results from three replicates.

Table 1 illustrates the differences in knockdown activity among adult mosquitoes

exposed to varying concentrations of ethanolic clove extract during the 7-hour observation period. The highest knockdown activity and insecticidal effects were observed at concentrations of 3% and 5%, both of which induced complete knockdown activity (100%) in all test mosquitoes. Knockdown activity in the positive control group was attributed to the toxic effects of malathion (1 mL). In contrast, the negative control group treated with distilled water showed negligible knockdown activity, indicating no significant toxic effect.

Table 1 and Figure 2 demonstrate that in the positive control group, all test mosquitoes died within the first hour of observation, corresponding to 100% knockdown activity. In contrast, the negative control group (distilled water) exhibited minimal, gradual knockdown

activity, with only 16.7% of mosquitoes affected.

At lower concentrations (0.5% and 1%), ethanolic clove extract produced relatively low knockdown activity rates compared to higher concentrations, with 4.4% and 8.9% knockdown activity, respectively. The 3% concentration showed a marked increase in activity, causing complete mortality demonstrated toxic effects (100%) of test mosquitoes within three hours. Meanwhile, the 5% concentration achieved faster results, inducing 100% knockdown activity within the first hour of observation.

Differences in mosquito knockdown activity across treatments were analyzed using one-way ANOVA to assess the statistical significance of the effects at each concentration. One-way

ANOVA indicated an overall difference in mosquito knockdown responses among the six treatment groups ($F(5, 12) = [F\text{-value}], P < 0.001$). The results of the ANOVA analysis are presented in Table 2. The percentage of mosquito knockdown activity across treatments is further visualized in Figure 2, presented as a bar chart to highlight comparative differences among concentrations. The ANOVA results presented in Table 2 revealed significant differences among treatments with varying concentrations of ethanolic clove extract, yielding a significance value of 0.000 ($P < 0.05$). This finding confirms that differences in extract concentration produced statistically significant variations in mosquito knockdown activity.

Table 1. Adulticidal toxic effect activity of ethanolic clove flower buds extract against adult mosquitoes, based on combined replicates (1, 2, and 3)

Treatment	Mosquito knockdown activity by hour (individual count)							Survival (%)	Knockdown (%)	SD
	1	2	3	4	5	6	7			
KP	90	-	-	-	-	-	-	0	100	0.000
KN	0	0	2	0	0	4	9	83.3	16.7	1.732
T1	0	0	4	0	0	0	0	95.6	4.4	1.155
T2	0	0	4	0	4	0	0	91.1	8.9	0.577
T3	8	50	32	-	-	-	-	0	100	0.000
T4	90	-	-	-	-	-	-	0	100	5.774

Notes: KP = Positive control (Malathion 95% 1 mL); KN = Negative control (Distilled water 2 mL); T1 = Treatment 1 (Ethanolic clove extract 0.5%); T2 = Treatment 2 (Ethanolic clove extract 1%); T3 = Treatment 3 (Ethanolic clove extract 3%); T4 = Treatment 4 (Ethanolic clove extract 5%); % = Accumulated percentage values; SD: Standard deviation

P-value ANOVA 0.000 and based on Tukey's post hoc test, the 0.5% and 1% concentrations did not differ significantly from the negative control, indicating that at these concentrations the repellent effect remained low and ineffective in causing mosquito knockdown activity. In contrast, the 3% and 5% concentrations did not differ significantly from the positive control, suggesting that at these concentrations the extract exhibited high effectiveness comparable to malathion. Although the positive control (malathion) and the 3% clove flower bud extract both reached 100% knockdown, their time-dependent responses differed substantially. Malathion achieved complete knockdown within the first hour, whereas the 3% extract produced 8.9% knockdown at one hour and reached 100% only after three hours. Therefore, similar final knockdown percentages should not be

interpreted as evidence of equivalent insecticidal performance. Furthermore, the limited number of replicates and the ceiling effect at 100% constrain the interpretation of non-significant post hoc comparisons.

The increase from 1% to 3% concentration showed a significant difference, marked by a sharp rise in mosquito knockdown activity. However, the difference between the 3% and 5% concentrations was not statistically significant, suggesting that 3% may already be an optimal concentration for achieving effective repellent activity. This study demonstrated that ethanolic clove (*Syzygium aromaticum*) extract exerts toxic effects on adult mosquitoes when tested using the bottle bioassay method. Treatments at 0.5% and 1% concentrations resulted in relatively low knockdown activity rates (4.4% and 8.9%, respectively). In contrast, the 3% concentration

induced complete knockdown activity (100%) within three hours, while the 5% concentration achieved 100% knockdown activity within the

first hour of observation. These findings highlight the potential of ethanolic clove extract as a natural herbal repellent against mosquitoes.

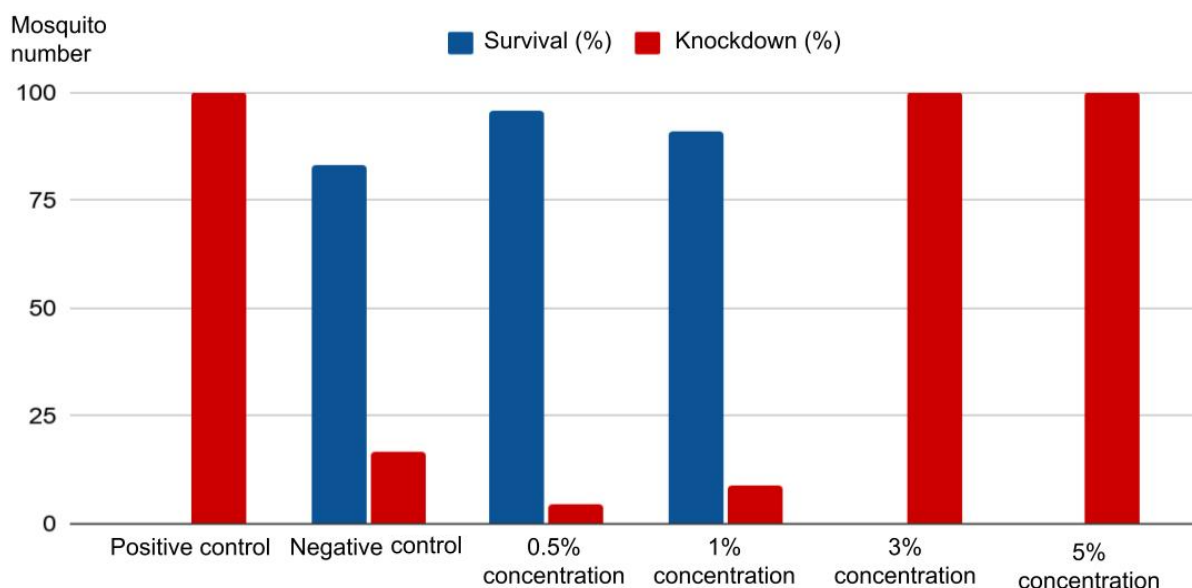


Figure 2. Final percentages of mosquito survival and knockdown following exposure to different concentrations of ethanolic clove extract (0.5%, 1%, 3%, and 5%), compared with positive and negative controls.

Statistical analysis using ANOVA confirmed that concentration variations produced significant differences in mosquito knockdown activity ($P < 0.05$). Tukey's post hoc test further revealed that the 0.5% and 1% concentrations did not differ significantly from the negative control, indicating limited repellent activity at these concentrations. Conversely, the 3% and 5% concentrations did not differ significantly from the positive control, demonstrating high effectiveness comparable to malathion. The significant difference between 1% and 3% concentrations underscores the need to reach an optimal threshold, while the lack of a significant difference between 3% and 5% suggests that 3% may already represent the optimal concentration for repellent efficacy.

The increased knockdown activity observed at higher concentrations is likely attributable to elevated levels of bioactive compounds in the extract. Lower concentrations (0.5% and 1%) may not contain sufficient active compounds to elicit strong biological responses, consistent with the findings of Zulaikha et al. (2019) and Lopez

et al. (2025) who emphasized that repellent effectiveness requires a minimum detectable threshold for mosquito contact exposure.

Although the 5% concentration produced faster knockdown activity than 3%, the lack of a significant difference between the two indicates that increasing concentration beyond 3% does not proportionally enhance effectiveness. This suggests the existence of an optimal threshold, beyond which additional concentration provides only limited concentration-dependent speed. Similar observations were reported by Mapossa et al. (2021) and Lopez et al. (2025) who noted that herbal repellents at certain concentrations yield diminishing returns in terms of protection.

The toxic effects of ethanolic clove extract may be mediated by its bioactive constituents, particularly eugenol, which constitutes 70–90% of clove essential oil (Sheikh et al., 2021). Ethanol, as a solvent, enhances extraction efficiency for both polar and semi-polar compounds, yielding extracts with higher bioactive content (Peiris et al., 2023) than those from other solvents (Bitwell et al., 2023). Eugenol stimulates mosquito

olfactory receptors, triggering avoidance behavior, and acts as a neurotoxin by penetrating the cuticle or digestive tract and disrupting the nervous system (Noya et al., 2022). Previous research also reported the knockdown activity of clove flower extract against mosquitoes, supporting its potential contact toxicity. However, the specific bioactive compounds and underlying biochemical mechanisms responsible for the observed effects were not investigated in the present study (Nopitasari et al., 2014).

Other compounds contribute synergistically: saponins damage the cuticle and cell membranes, flavonoids inhibit respiration and feeding activity, and tannins interfere with metabolism by precipitating proteins, while carvone, fenchone, and terpinen-4-ol inhibit acetylcholinesterase activity, leading to acetylcholine accumulation, impaired nerve transmission, muscle paralysis, and eventual death. The observed knockdown activity may be associated with bioactive compounds commonly found in clove flower buds, particularly eugenol. However, the phytochemical composition of the extract and its potential neurotoxic mechanisms were not investigated in the present study. Therefore, the specific mechanisms underlying the observed knockdown activity remain unclear and require further investigation (Muhammad et al., 2022; Nopitasari et al., 2014; Noya et al., 2022).

This study has several limitations. First, the absence of an ethanol vehicle control limits the ability to distinguish the effects of the clove extract from those of the solvent. Second, the use of mixed *Aedes* and *Culex* cohorts prevents genus-specific interpretation of the observed knockdown activity. Third, the amount of extract deposited per bottle was not adequately characterized, limiting the reproducibility and comparability of the exposure conditions. Furthermore, phytochemical profiling was not performed, preventing identification and quantification of the compounds responsible for the observed effects. The assessment was restricted to a single experimental exposure period, without evaluation of longer-term effects or residual activity.

Comparisons with previous studies should be interpreted cautiously because experimental

conditions may differ substantially. These differences include the plant material used (flower buds or leaves), extraction solvent, concentration units (% w/v or % v/v), mosquito species, and measured endpoints. In particular, studies evaluating mosquito landing or repellency cannot be directly compared with the present bottle bioassay, which assessed knockdown following contact with extract-treated surfaces. Such methodological differences may contribute to variations in reported insecticidal effectiveness.

These findings align with previous studies reporting the efficacy of clove extract against multiple mosquito species. Sritabutra et al. (2013) demonstrated effectiveness against *Aedes* spp. and *Culex* spp., while Rahmawati (2021) reported 100% repellency at 5% concentration against *Aedes* spp. Lopez et al. (2025) further noted that 5% represents an optimal concentration, with no meaningful increase in protection at higher levels. Additionally, Nopitasari et al. (2014) observed rapid knockdown effects in *Culex* spp. at 2.5% and 5% concentrations, attributed to the neurotoxic activity of eugenol.

CONCLUSION

This study evaluated the contact toxicity of ethanolic *Syzygium aromaticum* extract against adult *Aedes* and *Culex* mosquitoes as livestock disease vectors. The 3% and 5% extract treatments achieved 100% knockdown within three and one hours, respectively. These findings demonstrate that clove extract is a potential botanical insecticide to control vectors in livestock housing. However, limitations like unassessed recovery, mixed populations, and lack of vehicle controls require further research. Future studies must evaluate species-specific efficacy, livestock dermal safety, and semi-field performance before practical application in animal production facilities is considered.

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