Identification of Active Compounds and Effectiveness Test of 96% *Tithonia diversifolia* Ethanol Extract on Mortality of *Aedes aegypti* Mosquito

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Abstract

Dengue Hemorrhagic Fever (DHF) remains a major public health problem in tropical regions, with Asia accounting for 70% of global cases and Indonesia reporting a massive 90,269 cases in 2024. Vector control is the primary mitigation strategy due to limitations in available therapies and vaccines, yet reliance on chemical insecticides poses ecological toxicity and resistance risks, driving the need for safer bioinsecticide alternatives. Tithonia diversifolia is known to contain active phytochemical constituents with insecticidal potential. This true experimental study used a Post Test Only with Control Group design conducted from August to September 2025 involving 25 Aedes aegypti per group with four replications. Leaf simplicia were extracted using 96% ethanol, followed by alcohol-free verification, qualitative phytochemical screening, and formulation into spray preparations at concentrations of 5%, 10%, 15%, 20%, and 25%. Mortality was assessed over 24 hours, and statistical evaluation included univariate, bivariate, and probit analyses to determine LC_{50}, LC_{90}, LT_{50}, and LT_{90}. Extraction produced a 12.53% yield containing saponins, flavonoids, phenols, tannins, alkaloids, and steroids. Mosquito mortality increased with rising concentrations, with the highest effectiveness observed at 25%. The Kruskal–Wallis test confirmed significant differences between groups, while LC_{50} and LC_{90} values were 2.77% and 5.37%, respectively. Although the highest mortality was observed at 25%, the 20% concentration was considered optimal because it met WHO efficacy standards while using a lower extract concentration.

Keywords: Active compounds, aedes aegypti, insecticide, tithonia diversifolia

Identifikasi Senyawa Aktif dan Uji Efektivitas Ekstrak Etanol 96% Daun Kembang Bulan (*Tithonia diversifolia*) Terhadap Mortalitas Nyamuk *Aedes Aegypti*

Abstrak

Demam Berdarah Dengue (DBD) merupakan masalah kesehatan utama di wilayah tropis, dengan Asia menyumbang 70% kasus global dan Indonesia mencatat 90.269 kasus pada tahun 2024. Upaya pengendalian terutama difokuskan pada eliminasi vektor karena keterbatasan terapi dan vaksin. Penggunaan insektisida kimia menimbulkan dampak toksik bagi lingkungan serta risiko resistensi, sehingga diperlukan alternatif bioinsektisida yang aman. *Tithonia diversifolia* diketahui mengandung senyawa aktif yang berpotensi sebagai insektisida. Penelitian *true experimental* ini menggunakan desain *Post Test Only with Control Group* yang dilakukan pada Agustus–September 2025 menggunakan 25 ekor *Aedes aegypti* per kelompok dengan empat kali pengulangan. Simplisia daun kembang bulan diekstraksi menggunakan etanol 96%. Ekstrak kemudian diuji bebas alkohol, dilakukan uji fitokimia kualitatif, dan diformulasikan menjadi sediaan spray dengan konsentrasi 5%, 10%, 15%, 20%, dan 25%. Uji mortalitas dilakukan selama 24 jam. Analisis statistik dilakukan menggunakan analisis univariat, analisis bivariat, serta analisis probit untuk menentukan LC_{50}, LC_{90}, LT_{50}, dan LT_{90}. Hasil ekstraksi dengan etanol 96% menghasilkan rendemen ekstrak sebesar 12,53% dengan kandungan aktif berupa saponin, flavonoid, fenol, tanin, alkaloid, dan steroid. Pada pengujian efektivitas insektisida terhadap nyamuk *Aedes aegypti,* mortalitas nyamuk meningkat sesuai konsentrasi, dengan efektivitas tertinggi pada 25%. Uji *Kruskal–Wallis* menunjukkan perbedaan bermakna antar perlakuan. Nilai LC_{50} dan LC_{90} masing-masing 2,77% dan 5,37%. Nilai LT_{50} tercepat adalah 3 jam pada konsentrasi 25% dan LT_{90} 12 jam pada konsentrasi 25%. Secara keseluruhan, ekstrak etanol daun kembang bulan terbukti efektif sebagai bioinsektisida, terutama pada konsentrasi 20%.

Kata kunci: *Aedes aegypti*, daun kembang bulan, insektisida, senyawa aktif

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Introduction

Dengue Hemorrhagic Fever (DHF) is a significant public health problem in tropical and subtropical regions. The endemic distribution of dengue covers more than 100 countries across various WHO regions, with Asia accounting for approximately 70% of global cases.[1] In Indonesia, there were 90,269 cases and 651 deaths due to dengue in 2024. These data emphasize that dengue remains a serious threat requiring continuous surveillance and sustained intervention.[2]

Prevention and control strategies for dengue primarily focus on interrupting the transmission cycle through vector control, considering the limited vaccine coverage and the fact that current therapy is still supportive and symptomatic.[3] Another limiting factor is the inadequate access to healthcare facilities in several regions. Endemic areas of *Aedes aegypti* that are distant from healthcare services tend to have higher morbidity, complication rates, and mortality. Current government programs include the Mosquito Breeding Site Eradication campaign (Pemberantasan Sarang Nyamuk/PSN) using the 3M Plus approach (draining, covering, and recycling water containers), as well as fogging.[3]

Another commonly applied measure by the community to disrupt mosquito reproduction is the use of chemical insecticides and larvicides.[4] Frequently used chemical insecticides and larvicides include organophosphate, organochlorine, carbamate, and pyrethroid groups. Some of these substances are toxic to non-target organisms and may potentially damage the environment and disrupt ecosystems, especially when used indiscriminately. For example, carbofuran, an insecticide belonging to the carbamate group, is toxic not only to insects but also to birds and mammals. If the concentration of carbofuran increases in the environment, the risk of mortality in birds and mammals also rises. Therefore, environmentally friendly insecticidal and larvicidal agents that remain effective in disrupting the mosquito reproductive cycle are urgently needed.[5]

One plant with potential as a natural insecticidal agent is the Mexican sunflower (*Tithonia diversifolia*).[6] The leaves of this plant are known to contain alkaloids, terpenoids, saponins, tannins, and polyphenols. Alkaloids generally induce neurogenic toxicity by disturbing synaptic transmission; terpenoids and volatile components act as toxins to the respiratory system; saponins damage the larval epithelial membrane, disrupt osmoregulation, and facilitate the entry of other substances into the mosquito body; while tannins and polyphenols bind to proteins and inhibit the activity of several vital enzymes in the mosquito body. [7,8,9]

Figure 1. *Tithonia diversifolia*
Sources: West Java Conservation Trust Fund

To maximize the production of active compounds in the Mexican sunflower (*Tithonia diversifolia*), an appropriate extraction method is required. The extraction method selected in this study was maceration using 96% ethanol as the solvent, followed by qualitative phytochemical screening to identify the active compounds contained in the leaves of *Tithonia diversifolia*. Subsequently, a spray formulation was prepared and tested on mosquitoes.[10]

Several studies have also demonstrated that the leaf extract of *Tithonia diversifolia* exhibits larvicidal and ovicidal activity against *Aedes aegypti* by inhibiting larval development and reducing egg viability, which is associated with its secondary metabolite content.[11] Nevertheless, scientific evidence regarding the insecticidal effectiveness of *Tithonia diversifolia* extract at the adult stage remains limited, even though adult *Aedes aegypti* mosquitoes are the primary vectors of dengue virus transmission and represent a crucial target for breaking the transmission cycle. This limitation highlights the need for further research to evaluate the potential of *Tithonia diversifolia* as an effective bioinsecticide against adult mosquitoes.

Therefore, this study aims to identify the active compounds present in the 96% ethanol extract of *Tithonia diversifolia* leaves and to evaluate its effectiveness in spray form against the mortality of *Aedes aegypti* mosquitoes.

Method

This study was employed a true experimental design using a Post-Test Only with Control Group Design, conducted in the Zoology Laboratory, Botany Laboratory, and Organic Chemistry Laboratory at the Faculty of Mathematics and Natural Sciences, Lampung University. The research was carried out from August to September 2025 using 25 mosquitoes for each treatment group, with four replications per treatment. The equipment used included pipettes, thinwall rectangle boxes, spray bottles, mosquito cages measuring 30 × 30 cm, paper cups, and cotton. The research materials consisted of *Aedes aegypti* eggs, 3 kg of *Tithonia diversifolia* leaves processed into simplicia, 96% ethanol, water, distilled water, 0.01% cypermethrin insecticide, sugar water, fish pellets, and filter paper.[12]

Preparation of *Tithonia diversifolia* Leaf Simplicia

Fresh *Tithonia diversifolia* leaves roughly weight 3 kg were washed thoroughly and dried at room temperature for approximately seven days. The leaves were dried until they turned brownish and became easily crushed by hand. The dried leaves were subsequently ground using a blender to obtain simplicia powder.[13]

Preparation of 96% Ethanol Extract of *Tithonia diversifolia* Leaves

Extraction of the simplicia was conducted using the maceration method with 96% ethanol at a 1:10 ratio.[14] The simplicia powder was soaked for 24 hours at room temperature, after which the macerate was separated and the residue was remacerated twice using fresh solvent. All macerates were combined and evaporated using a rotary evaporator to obtain a concentrated extract suitable for phytochemical testing. This procedure ensured optimal phytochemical yield as the active component for the study.

Alcohol-Free Test

A total of 1 ml of concentrated extract was placed into a test tube, followed by the addition of 2 drops of H_2SO_4 and 2 drops of glacial acetic acid, then heated. This procedure aimed to detect the presence or absence of ethanol in the extract. The extract was considered ethanol-free if no characteristic ester odor of ethanol was detected.

Phytochemical Screening

Phytochemical screening was performed to identify bioactive secondary metabolites in the 96% ethanol extract of *Tithonia diversifolia* leaves. The preliminary screening provided an overview of the compound classes present in the sample as a studied plant material. The test was conducted by reacting the wet extract with specific reagents and observing the resulting color changes. The observations were interpreted qualitatively to analyze secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, and saponins.[15]

The phytochemical procedures performed at the Organic Chemistry Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Lampung, followed the methods described by Kartikasari et al. (2022)[16], which include seven types of tests targeting different classes of bioactive compounds. Each test assessed the presence of saponins, steroids, terpenoids, tannins, alkaloids, flavonoids, and phenolics. The treatments in these seven tests were evaluated qualitatively based on observable color changes.

Preparation of Insecticidal Spray Formulation

Formulation began with the preparation of a stock solution at a concentration of 25%. The stock solution of *Tithonia diversifolia* leaf extract was then homogenized with the solvent to produce concentrations of 5%, 10%, 15%, 20%, and 25%. Each formulation was transferred into spray bottles and sealed tightly, with a total volume of 50 ml per concentration.[17]

Figure 2. Insecticide spray preparation

Insecticidal Effectiveness Test

The experimental procedure began by preparing mosquito cages containing 25 mature Aedes aegypti mosquitoes. The insecticide effectiveness test was conducted in accordance with the World Health Organization guidelines for laboratory testing of insecticides against adult mosquitoes. The Aedes aegypti mosquitoes used in this study were obtained from a laboratory-reared colony, with the species identity and strain clearly specified. The diluted *Tithonia diversifolia* leaf extract was prepared in a spray bottle and applied onto the filter paper on all sides of the cage at a volume of 5 ml for each concentration and treatment group. Following spraying, each group was observed for 24 hours, and the number of dead mosquitoes was recorded. Mortality percentage was calculated using the formula:

$$\%\ Mortality = \frac{(\text{number of dead mosquitoes})}{(\text{number of mosquitoes tested})}\ x\ 100\%$$

All treatments were performed in four replications to obtain more representative results.

Statistical Analysis

Collected data were analyzed using univariate, bivariate, and probit analysis. In the univariate analysis, data were presented as means and standard deviations. The bivariate analysis employed a One-Way ANOVA followed by a Post Hoc LSD test. If the assumptions for ANOVA were not met, the alternative Kruskal–Wallis test was used, followed by a Mann–Whitney test with a 95% CI. Probit analysis was then conducted to determine LC_{50} and LC_{90} (Lethal Concentration) as well as LT_{50} and LT_{90} (Lethal Time), each with a 95% CI.

Result

Fresh *Tithonia diversifolia* leaves weighing 3 kg were dried and processed into simplicia powder. A total of 500 grams of simplicia produced 62.67 grams of extract, yielding a rendement of 12.53%. The rendement obtained in this study satisfied the standard criteria for raw material suitability, which requires a value above 10%. High-quality simplicia is characterized by very low contamination levels, absence of fungal growth, fine texture, and a moisture content of less than 10%.

The 96% ethanol extract of *Tithonia diversifolia* leaves was analyzed qualitatively to identify its active compounds, and the results are presented in Table 1.

Table 1. Qualitative Phytochemical Screening Results

No	Type of Qualitative Phytochemical Screening	Result	Description
1	Saponin	+	Presence of foam
2	Steroid	+	Blue/purple/green coloration
3	Terpenoid	-	Red/yellow coloration
4	Tannin	+	Dark bluish coloration
5	Alkaloid	+	Whitish-brown coloration
6	Flavonoid	+	Reddish-brown coloration + foam
7	Phenolic	+	Dark bluish coloration

An alcohol-free test of the *Tithonia diversifolia* leaf extract was conducted to ensure the absence of residual ethanol following the extraction process. In this study, the extract did not exhibit any ester odor, indicating that solvent evaporation occurred optimally. Thus, the resulting extract did not contain a meaningful level of ethanol originating from the maceration process.

Table 2. Mean death of *Aedes aegypti*

Groups Intervention	Replication 1			Replication 2			Replication 3			Replication 4			Mean death n_m	Mortality (%) %	
	N	n_m	%	n	n_m	%	N	n_m	N	n_m	%	n			N
K-	25	0	0	25	0	0	25	K-	25	0	0	25	0	0	25
5%	25	15	60	25	15	60	05	5%	25	15	60	25	15	60	05
10%	25	18	72	25	17	68	25	10%	25	18	72	25	17	68	25
15%	25	20	80	25	21	84	25	15%	25	20	80	25	21	84	25
20%	25	24	96	25	24	96	25	20%	25	24	96	25	24	96	25
25%	25	25	100	25	24	96	25	25%	25	25	100	25	24	96	25
K+	25	25	100	25	25	100	25	K+	25	25	100	25	25	100	25

N = total number of Aedes aegypti mosquitoes tested per group.
nm = number of dead mosquitoes.
% = mortality percentage.

Based on the findings presented in Table 2, the Tithonia diversifolia leaf extract demonstrated bioinsecticidal activity against Aedes aegypti, with observable mortality across all tested concentrations. The 25% concentration produced the highest bioinsecticidal effect compared with the other concentrations. In the negative control group (distilled water), no mosquito mortality was observed in any replication, whereas in the positive control group (cypermethrin), total mortality occurred, with all 25 mosquitoes in each replication dying, resulting in a mortality rate of 100%.

Figure 3. Insecticide test result of Tithonia diversifolia extract at 20% concentration with a 96% mortality rate.

Based on the Shapiro–Wilk test, the mortality data of *Aedes aegypti* at concentrations of 10%, 15%, 20%, and 25% demonstrated significance values greater than 0.05, indicating that the data were normally distributed. In contrast, the 5% concentration showed a significance value of 0.001 ($p < 0.05$), indicating a non-normal distribution. A Kruskal–Wallis test was subsequently performed to assess whether significant differences existed among the concentration groups of *Tithonia diversifolia* extract with respect to mosquito mortality. The test produced a significance value of 0.002, confirming a statistically significant difference among the groups.

The Post Hoc LSD test revealed that the 15%, 20%, and 25% concentrations demonstrated significant differences compared with the negative control group. Additionally, the 2%, 10%, and 15% concentrations showed significant differences compared with the positive control group.

Probit analysis indicated that the LC_{50} and LC_{90} values, representing concentrations required to cause 50% and 90% mortality, respectively, at 24 hours, were 2.77% (equivalent to 2.77 mg/L) and 5.37% (equivalent to 5.37 mg/L). The 5% concentration required the longest time to achieve 50% mortality (LT_{50}), occurring at 15 hours, whereas the 25% concentration required only 3 hours to reach the same mortality level.

Furthermore, the 5% concentration required 24 hours to achieve 50% mortality (LT_{50}), while the 25% concentration required 12 hours to reach 50% mortality.

Discussion

The raw material preparation process began with drying and pulverizing the leaves into simplicia. From a total of 500 grams of simplicia subjected to extraction, 62.67 grams of 96% ethanol extract were obtained, yielding a rendement of 12.53%. This value indicates that the extraction process was efficient and met the standard criterion for acceptable raw

material rendement, which is greater than 10%[16]. High-quality simplicia plays a crucial role in optimizing rendement, characterized by low levels of contamination, absence of fungal growth, homogeneous texture, and a moisture content below 10%. These characteristics help maintain the stability of bioactive components during extraction and enhance the storage life of simplicia.[18,19]

Identification of secondary metabolites in the 96% ethanol extract of *Tithonia diversifolia* leaves revealed the presence of alkaloids, flavonoids, tannins, saponins, steroids, and phenolics. Alkaloids exert neurotoxic effects through acetylcholinesterase inhibition and disruption of nerve impulse transmission. Flavonoids induce oxidative stress, damage cellular membranes, and demonstrate antifeedant properties. Tannins inhibit digestive enzymes and weaken structural protein integrity. Saponins increase membrane permeability and disrupt the digestive epithelium, leading to impaired homeostasis. Meanwhile, steroids and phenolics affect membrane stability and enhance oxidative stress, resulting in metabolic damage and subsequent apoptosis[20,21].

The combination of these bioactive metabolites produces a synergistic effect that strengthens the toxicity of the extract against *Aedes aegypti*, positioning *Tithonia diversifolia* leaves as a promising candidate for development as a bioinsecticide[23].

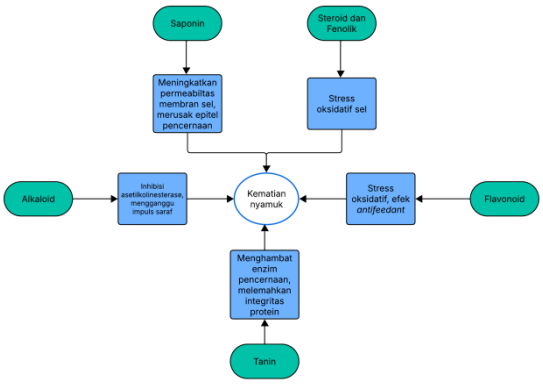

Figure 4. Mechanism of active substance of *Tithonia diversifolia* leaf extract as an insecticide

The identification of active metabolites was complemented by an alcohol-free test to ensure that the extract used was completely devoid of residual ethanol following the maceration and evaporation processes. In this study, the extract did not exhibit the ester-like odor typically indicative of ethanol presence, thereby confirming that the evaporation procedure was effective. The absence of meaningful ethanol content in the extract affirms that the final product is safe for bioactivity testing without solvent-related contamination and ensures that the observed effects originate solely from the phytochemical components of *Tithonia diversifolia* [6].

Administration of the 96% ethanol extract of *Tithonia diversifolia* demonstrated a consistent increase in adult *Aedes aegypti* mortality in accordance with rising concentrations. Mortality ranged from 56% at a 5% concentration to 95% at a 25% concentration within 24 hours. The 20% and 25% concentrations met the WHO efficacy standards (80–95%).[22] Thus, both concentrations exceeded the minimum effectiveness threshold. These findings align with previous studies reporting that higher concentrations of *Tithonia diversifolia* extract leaf exhibit a positive correlation with mosquito mortality across various developmental stages.[10]

Statistical analysis revealed significant differences in mortality among treatment groups based on the Kruskal–Wallis test, supported by Mann–Whitney pairwise comparisons. Mortality at the 20% and 25% concentrations showed significant differences compared with the negative control and no significant differences compared with the positive control, indicating that their effectiveness approximates that of synthetic insecticides. Moreover, mortality at 20% did not differ significantly from 25%, suggesting that both doses represent effective concentrations suitable for bioinsecticide application.

The LC_{50} value of 2.77% and LC_{90} value of 5.37% indicate strong toxic potential, falling within the toxic category (1%–10%) according to the classification by Ismatullah et al. (2014)[21]. In addition to concentration, the time-to-death parameters (LT_{50} and LT_{90}) showed that increasing extract concentration significantly accelerated mortality. At the 5% concentration, LT_{50} was recorded at 15 hours and LT_{90} at 24

hours, while at 25%, LT_{50} decreased to 3 hours and LT_{90} to 12 hours. These effects are attributed to active compounds such as saponins, flavonoids, and alkaloids, which disrupt mosquito physiological systems by damaging the digestive tract, impairing respiration, and interfering with the nervous system. All lethal time values remained within the WHO toxicity testing standard of 24 hours.[24]

Overall, the combined of lethal concentration and lethal time parameters provide a comprehensive depiction of the effectiveness and speed of action of *Tithonia diversifolia* leaf extract as a bioinsecticide[7]. Higher concentrations produced faster and greater lethal effects. Based on statistical analysis, both 20% and 25% concentrations of the 96% ethanol extract yielded optimal results. However, the 20% concentration is considered more optimal as it represents the lowest concentration exceeding the efficacy threshold. Additional advantages of *Tithonia diversifolia* extract include environmental friendliness and potential to reduce resistance risks commonly associated with synthetic insecticides. Nevertheless, the use of higher concentrations must consider potential impacts on non-target organisms; therefore, further studies are required to evaluate the effects of the 96% ethanol extract of *Tithonia diversifolia* on other mosquito species, non-target organisms, and ecological outcomes.

Conclusion

The maceration process of *Tithonia diversifolia* leaf using 96% ethanol yielded 62.67 grams of extract with a rendement of 12.53%. The alcohol-free test confirmed that the 96% ethanol extract contained no meaningful levels of ethanol. Qualitative phytochemical screening showed that the extract tested positive for active compounds including flavonoids, phenols, tannins, saponins, alkaloids, and steroids. The 96% ethanol extract of *Tithonia diversifolia* leaf proved effective as a bioinsecticidal agent against *Aedes aegypti*, with the most effective concentration identified at 20%. Further studies are recommended to evaluate its field effectiveness, formulation stability, and potential effects on non-target organisms.

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