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Larvicidal Activity of Red Betel Leaves (*Piper ornatum*) Ethanolic Extract Against Mosquito Larvae

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Abstract: Larvae are the initial carriers of dengue hemorrhagic fever (DHF) and culex, making them significant in disease transmission. Excessive chemical larvicides pose serious risks to human health, thus driving the development of biological alternatives like red betel leaf extract. This study aimed to assess the efficacy of red betel leaf extract in larval control. Using 300 larvae, concentrations of 5%, 6%, and 7% were tested with three replicates over 24 h. The bioassay involved placing the larvae into a plastic container filled with the extract. It was found that ethanol extract from Piper ornatum leaves contained alkaloids, tannins, saponins, flavonoids, and terpenoids. After 24 h, results revealed 83% mortality at 5%, 98% at 6%, and 100% at 7% of extract concentration. Consequently, the 7% concentration showed the fastest efficacy in killing mosquito larvae (100% in 50 min). The LC50 value, representing the concentration at which 50% of larvae are killed, was determined to be 0.04%. Additionally, the LT50 value, indicating the time required to kill 50% of larvae (based on the LC50 value), was found to be 3.34 hours. In conclusion, red betel leaf extract demonstrated promising larvicidal activity against mosquito larvae. Future research is anticipated to prove its safety via in vitro and in vivo test.

Introduction

Larvae are one of the first vectors that cause dengue hemorrhagic fever (DHF). DHF is a disease caused by the *Aedes aegypti* mosquito, transmitted through its bite and caused by dengue infection. This dengue fever disease is spread in tropical areas and is influenced by weather and temperature. In recent years, this number of cases has been getting higher data in 2010, the total number of cases reached 2.2 million people and increased in 2016 to a total of 3.37 million people. (1). Currently cases have spread to 427 districts/cities in 34 provinces of Indonesia. A total of 661 people died and 95,893 suffered from dengue fever (2). The spread of this disease is considered very fast and widespread, so graphic mapping is needed to prevent and control dengue fever (3).

The government has made significant efforts to reduce the risk of dengue fever by using chemical larvicides to destroy larvae. These larvae typically hatch in water where female mosquitoes lay their eggs. Abate powder is a commonly used chemical larvicide among the public. However, the use of chemicals to eliminate larvae poses significant dangers to human health and can lead to resistance (4). As a result, biological control methods are now widely adopted and continuously developed as alternatives to chemical larvicides (5). Betel leaf is widely utilized in traditional medicine and herbal remedies due to its diverse medicinal properties, one of which is its potential as a larvicide. *Piper betle* extract has been reported to effectively kill *Aedes Aegypti* Sp larvae at a concentration of 10% (6). *Piper crocatum* extract is also reported to have an LC50 value at a concentration of 9.91 ppm against *Aedes aegypti* mosquitoes (7). The essential oil from *Piper crocatum* has also been proven to kill mosquito larvae within 3 hours with just a concentration of 0.1% (8).

Piper ornatum, also known as red betel leaf, is a tropical plant species native to Southeast Asia. It is recognized for its vibrant red-colored leaves, which

distinguish it from the more commonly known betel leaf (*Piper betle* and *Piper crocatum*). Based on the larvicidal and anti-mosquito effectiveness of the aforementioned *Piper betle* species, *Piper ornatum* also holds similar potential due to its similar secondary metabolite content. Besides, research on the larvacide effect of this species has not been reported yet. Therefore, we conducted an anti-*Aedes aegypti* larva activity test on ethanol extracts from Piper ornatum at concentrations of 5%, 6%, and 7% for durations ranging from 10 to 1440 min.

Experimental Section

Materials

Red betel leaves (*Piper ornatum*) were collected from Lubuk Linggau, South Sumatera, and identified by Herbarium Universitas Andalas with notification letter number of 331/K-ID/ANDA/VI/2022. The materials used were 96% Ethanol (JK Care, Jakarta, Indonesia), Mayer reagent (Nitra Chemical, Indonesia), Dragendorf reagent (Nitra Chemical, Indonesia), Distilled water (Onelab, Indonesia), Ferric chloride (Nitra Chemical, Indonesia), Magnesium Staearat (Nitra Chemical, Indonesia), Hydrochloric acid (Nitra Chemical, Indonesia), Sulfuric acid (Merck, Germany), Acetic Anhydrous (Merck, Germany), Chloroform (Merck, Germany), and Temephos (Abate, BASF Coatings GmbH, Germany).

Simplicia and Powder Preparation

The collected red betel leaves were wet-sorted to separate them from any dirt or foreign objects in the simplicia material. Subsequently, they were washed under running water, drained, and dried through sun-drying. The dried red betel leaves (simplicia) were then sorted again in their dried state and weighed. Following this, the leaves were powdered using a blender and sieved through a 60-mesh sieve.

Extraction

Crushed red betel leaves, totaling 1 kg, were placed into a maceration container. Subsequently, 4 liters of 96% ethanol solvent were added until the leaves were completely submerged. The sealed maceration container was then stored for 3 days, shielded from sunlight and intermittently stirred. Following this period, the mixture underwent filtration to separate the dregs from the sample phytrate. The obtained dregs underwent additional extraction using an equal amount of 96% ethanol, a process that was repeated twice. The resulting ethanol extract was collected and concentrated using a Rotary Evaporator at a temperature of 50°C until a thick extract was obtained. Further concentration was achieved in a water bath set at 50°C, culminating in a dense, concentrated extract, from which the yield of the extraction results was calculated. The extract was then phytochemically screened.

Lost on Drying Test

The evaporator cup was heated at 105°C for 10 min. Two grams of simplicia were then weighed and recorded. The sample was placed in an oven for 15 to 30 min, cooled in a desiccator, and weighed again. This process was repeated until a constant weight value was obtained.

Larvacide Activity Test

Mosquito larvae were collected from larval nursery center Baturaja, Palembang (300 in total) were divided into 5 groups (3 replications) using a plastic dropper pipette and filtered to prevent water dilution. Each larva was placed into a plastic cup containing different concentrations of red betel leaf extract: 5%, 6%, 7%, positive control (temephos 1%), and negative control. This process was left for 24 h, after which the number of dead larvae was counted. The percentage of larval mortality was then calculated. Dead larvae are larvae that do not move and their bodies shrivel.

Result

Extract Yield, Lost on Drying and Phytochemicals

The extract yield obtained in this study was 6.8% with an LoD of 1.8%. The results of the phytochemical screening test for red betel leaf extract can be seen in table 1 below.

Table 1. Secondary metabolites found in the ethanolic extract of red betle leaves.

No	Compound	Reagent	The Result	Criteria	Present
1	Alkaloid	Mayer	White precipitate	White precipitate	+
		Dragendorf	Red brick precipitate	Red brick precipitate	+
2	Saponin	Hot water	Foaming	Foaming	+
3	Tanin	Ferric Chloride	Blackish green	Blackish green	+
4	Flavanoid	Aquadest+mg+HCl	Yellow color	Yellow color	+
5	Terpenoid	Chloroform + acetate acid anhydrous + sulfuric acid	Chocolate ring formed	Chocolate ring formed	+
6	Steroid			Green blue	-

Table 2. Larvicide effect of the ethanolic extract of red betle leaves.

Extract Concentration	% Death of Aedes aegypty Larvae Instra III								
	Larval Death Time in Minutes								
	10	20	30	40	50	60	120	180	1440
5%	0%	6%	15%	23%	30%	40%	56%	68%	83%
6%	6%	13%	25%	35%	41%	53%	70%	80%	98%
7%	28%	45%	56%	70%	75%	90%	100%	100%	100%
Positive control	35%	55%	75%	90%	100%	100%	100%	100%	100%
Negative control	0%	0%	0%	0%	0%	0%	0%	0%	0%

The research conducted focused on the activity of red betel leaf extract (Piper ornatum) in killing Aedes aegypti mosquito larvae. The extract was tested at concentrations of 5%, 6%, and 7%, each replicated 3 times. In each cup, 20 Aedes aegypti mosquito larvae were placed and observed for 24 h, with time intervals recorded at 10, 20, 30, 40, 50, 60, 120, 180, and 1140 min. The data collected from these observations were then organized and presented in Table 2.

The LC50 test is a value that shows the concentration of toxic substances which can cause up to 50% death in the test larvae. Meanwhile, LT50 is the time that can result in up to 50% death in the test larvae. The following is a table of LC50 analysis for each hour and LT50 for each concentration. Based on the relationship between extract concentration and the percentage of larval deaths after 3 h (the highest R-squared value), the equation y = 1600x–13.4 was obtained. Based on the equation, the LC50 (in 3 h) was determined to be 0.04% and LT50 for this concentration (based on equation generated from Table 4) was 200 min or 3.34 h.

Tabel 3. LT50 of the ethanolic extract of red betle leaves against Aedes aegypti larvae.

No	Extract Concentration	Time (min)
1	5%	73.95
2	6%	57.51
3	7%	25.98

Discussion

The phytochemical analysis of red betel leaves collected from Lubuk Linggau City indicated the presence of several compounds. Alkaloids were detected using Mayer and Dragendorf reagents, resulting in the observation of white and brick-red precipitates, respectively. Saponins were confirmed by observing foam formation when using distilled water as a reagent. Tannins were detected using $FeCl_3$ reagent, showing blackish-green observations, while flavonoids were identified through observations of yellow color when using Aquadest, magnesium, and HCL reagents (10).

Similarly, a previous study conducted by Moerfieh and Supomo (2011) analyzed red betel leaves collected from Minahasa Regency, North Sulawesi, yielding comparable results (11). The analysis revealed the presence of flavonoids, saponins, alkaloids, and tannins, consistent with the findings from Lubuk Linggau City. This consistency suggests that the compound content of the red betel leaves remains consistent across different regions.

The LOD tests conducted was to establish a maximum limit or range for the amount of compounds lost during the drying process, a crucial consideration in maintaining extract quality. The standard drying loss value typically falls below 11.00% (9). In our study, the determination of drying loss parameters for the ethanol extract of betel leaves yielded a value of 1.8%. This indicates that the extract complies with the specified requirement of being below 11%, ensuring its quality.

Table 2 displays the mortality rates of test larvae across different concentration groups. In the 5% concentration group, an average of 16 larvae died, accounting for 83% mortality within 1440 min (24 h). At a concentration of 6%, the average death increased to 19 Aedes aegypti larvae, resulting in a mortality rate of 98% within the same time frame. The 7% concentration led to the highest mortality, with an average death of 20 larvae (100%) within 120 min (2 h). This suggests that as the concentration of red betel leaf extract increases, its larvicidal effect also increases.

The results indicate that the 7% concentration achieved the highest mortality rate. This concentration meets the criteria for a botanical insecticide, as effective mortality typically ranges from 10% to 95% of the total test larvae, as referenced in (12). Larvicides are crucial in controlling disease vectors like mosquitoes. According to guidelines from the Ministry of Health, a larvicide is considered effective if it can eliminate 80% or more of the test larvae (13).

The LC50 results reveal that red betel leaf extract effectively kills 50% of Aedes aegypti mosquito larvae at a concentration of 0.04% (in 3 h). The LC50 interval during observation (10 min – 24 h) ranges from 0.04%

to 0.087%. This indicates that the faster you want the larvae to die, the higher the concentration required. The LT50, indicating the time needed to kill 50% of the test larvae, demonstrates that a concentration of 5% requires 73.95 min, while a 7% concentration requires 57.51 min. The reduction in LT values from 5% to 7% concentration stems from the increased concentration causing higher toxicity, thereby accelerating larval mortality (15).

The research findings revealed distinct effects on mosquito larvae when exposed to red betel leaf extract. Observations showed that larvae immersed in the extract exhibited blackened bodies, a result of digestive disorders and cell wall damage caused by tannins. These tannins create complex bonds with proteins in enzymes and substrates, inhibiting enzyme activity (16). Additionally, larvae displayed stiffness and a pale yellow color due to alkaloids. Alkaloids, even in low doses, trigger chemical reactions in larvae during metabolism, hindering growth hormone function and potentially causing larval death (17).

Furthermore, flavonoids act as stomach poisons, leading to larval mortality (18). Seizures and paralysis were observed in larvae, attributed to cell membrane damage and the infiltration of numerous toxins into the larval body. Saponin, another compound present in the extract, inhibits the acetylcholinesterase enzyme, reduces enzyme activity during digestion, and hampers larval absorption processes. This can lead to anorexia and damage to the larvae's body warts due to fluid loss (19).

Alkaloids function as insecticides by inhibiting acetylcholinesterase enzyme activity. They also synergize with triterpenoid compounds to hinder cell mitosis. Moreover, they stimulate endocrine glands to release juvenile hormones, disrupting metamorphosis and causing abnormal larval death (20). This effect is intensified as mosquito larvae are capable of absorbing substantial amounts of these toxic substances (21).

While this study provides valuable insights into the potential of red betel leaf extract as a natural larvicide, there are several limitations that need to be addressed. One of them is the narrow focus of the research on testing the larvicidal effects against *Aedes aegypti* only. Future studies could broaden the scope of testing to include other mosquito species as a step towards gaining a more comprehensive understanding of the extract's effectiveness as a larvicide. Additionally, it is important to identify and further study the mechanism of action of the extract on mosquito larvae, including its impact on the environment and non-target organisms.

Conclusion

In conclusion, the research findings demonstrate that red betel leaf extract (*Piper ornatum*) contains alkaloid, tannin, saponin, flavonoid, and terpenoid, and exhibits significant activity as a natural larvicide. The LC50 value, representing the concentration at which 50% of larvae are killed, was determined to be 0.04%. Additionally, the LT50 value, indicating the time required to kill 50% of larvae, was found to be 3.34 hours. These results affirm the effectiveness of red betel leaf extract as a potent larvicide.

Declarations

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Conflict of Interest

The authors declare no conflicting interest.

Data Availability

The unpublished data is available upon request to the corresponding author.

Ethics Statement

Not applicable.

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Not applicable.

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