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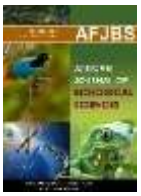

The Potential of Tulsi Ethanol (*Ocimum tenuiflorum*) Extract as a Natural Larvicide on *Aedes aegypti* Larvae

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ABTRACT

Indonesia is an archipelago with a tropical climate. This condition facilitates the spread of dengue fever, causing infections every year. Based on the Bali Provincial Health Profile has seen an upward trend in the Incidence Rate (IR) of dengue fever, with rates of 22.4 (2018), 137.3 (2019), and 278.6 (2020) per 100.000 population so that in 2020, Bali contributed the highest IR when compared to other provinces in Indonesia. Cases of vector resistance to synthetic larvicides have also been reported in various previous studies. The purpose of this study is to analyze the potential of ethanol extract of Tulsi plant (*Ocimum tenuiflorum*) as a natural larvicide on *Aedes aegypti* instar III larvae. This research involved creating Tulsi extract using the maceration method and testing its larvicidal activity against *A. aegypti* larvae in a laboratory setting. Extract was obtained by maceration method using 96% ethanol solvent and tested at concentrations of 0.03%, 0.06%, 0.12%, 0.24%, and 0.48%. Each concentration underwent three replicates over 24 hours. The results showed that as the concentration of Tulsi extract increased, there was a significant increase in the death of *A. aegypti* larvae with significant differences based on the Kruskal Wallis test (P value < 0.05). The 50% lethal concentration value (LC_{50}) was determined to be 0.135%, while the 99% lethal concentration value (LC_{99}) was determined to be 0.25%. This research succeeded in proving the potential of Tulsi extract as a larvicide made from natural ingredients. Further research is needed to be able to create practical alternative larvicide formulations based on Tulsi.

KEYWORDS: Natural larvicide, Tulsi, *Ocimum tenuiflorum*, *Aedes aegypti*

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INTRODUCTION:

Indonesia is an archipelagic country crossed by the equator. With its tropical climate, this condition supports the biodiversity that lives in the archipelago. This certainly has various impacts, one of which is causing Indonesia to become a country with a risk of dengue hemorrhagic fever infection, which can occur throughout the year (Megawati et al., 2017). Dengue Hemorrhagic Fever is an infectious disease that is transmitted through blood-sucking arthropod vectors (mosquitoes). These viruses are known as arthropod-borne-viruses (arboviruses), and the *Aedes aegypti* mosquito has been known to spread 4 types of arboviruses, namely the viruses that cause yellow fever, zika, dengue, and chikungunya (Souza-Neto et al., 2019). The vector will be infected throughout its life due to sucking the blood of vertebrates that have the dengue virus, and the virus will develop in the vector's body without causing disease (Brooks et al., 2013).

Urban conditions with poor environmental quality and sanitation, high levels of population density, and a lack of public understanding of how to eradicate mosquito nests can trigger explosive dengue hemorrhagic fever epidemics, causing *Kejadian Luar Biasa* (KLB) or, in English, Extraordinary Events, during the rainy season. An extraordinary event is an epidemiologically significant increase in morbidity and mortality in an area within a certain time (Permenkes, 2004). This problem will certainly have an impact on life in the region, especially Bali, where most of its economy relies on the tourism sector. In epidemiological research in 2018, from a sample of foreign tourists who visited Bali between 2015 and 2017, of the 201 tourists suspected of being infected with the dengue virus, 133 people were confirmed to have had the infection by NS1 or RT-PCR examination (Masyeni et al., 2018). In 2018 to 2020, based on the Bali Province Health Profile, it is found that there is a trend of increasing frequency of dengue fever based on the Incidence Rate (IR) indicator of 22.4 (2018), 137.3 (2019), and 278.6 (2020) per 100,000 population so that in 2020 Bali contributed the highest IR compared to other provinces in Indonesia (Dinkes Bali, 2021; Kemenkes, 2021).

Various steps have been taken to reduce the incidence of dengue hemorrhagic fever through increased vector eradication efforts by draining, covering, and burying unused used items and sowing larvicide in standing water. This step is the primary way to limit the spread of vectors as research on dengue vaccines and antiviral drugs is in the development stage (Hamid et al., 2017). Indonesia has been using larvicides to control *A. aegypti* since 1970 (Ahmad et al., 2009), during which time cases of vector resistance to larvicides have been found in various regions of Indonesia (Hamid et al., 2017; Haziqah-Rashid et al., 2019; Putra et al., 2016). These adverse effects occur because of excessive use and inappropriate dosage over a long period of time (Putra et al., 2016). So, it is necessary to use alternative larvicides that are safe for the environment and effective in reducing the number of *A. aegypti* larvae.

Many plants have potential and are stated to have larvicidal effects. These plants were chosen as alternative larvicidal agents because they have specific effects and are environmentally friendly (Ghosh et al., 2012). One of the plants that has a larvicidal effect and can grow in tropical areas of Indonesia is a plant from the genus *Ocimum*, which is included in the Lamiaceae family (Ghosh et al., 2012; Upadhyay, 2017). This plant is known as "Tulsi" or "Kuru-kuru" by Indonesian people, in English, it is better known as "Holy basil"(Malav et al., 2015; Ravi et al., 2012).

Tulsi (*Ocimum tenuiflorum*) is a plant that is sacred in Indian Hindu tradition and is often used for religious purposes. Tulsi is described as having anti-inflammatory, analgesic, antipyretic, antidiabetic, hepatoprotective, hypolipidemic, antistress, and immunomodulatory effects. These various effects arise from the metabolite content of the *Ocimum* genus plants, such as camphor, methyl eugenol, eugenol, limonene, thymol, myrcene, cineole, and caryophyllene (Upadhyay, 2017). The use of Tulsi in Indonesian society is only limited to a spice, whereas if Tulsi plant can be extracted with the right method it can provide more value (Sopianti & Sary, 2018). Tulsi can be extracted using the maceration method because it will not damage the desired secondary metabolite compounds due to the heating process. Ethanol was chosen as the right solvent to obtain secondary metabolites because it has a good extraction rate for compounds such as flavonoids and phenolics and is safe for the environment, food, and medicines (Hakim & Saputri, 2020).

In research conducted by Firmansyah et al. (2019), it was found that basil leaf extract (*Ocimum sanctum*), which is a relative of the Tulsi plant in the same genus, has larvicidal capabilities against *A. aegypti* instar IV larvae with a basil leaf extract concentration of 0.66% for lethal concentration. 50% (LC_{50}) and 1.38% for a lethal concentration of 90% (LC_{90}) within 24 hours (Firmansyah et al., 2019). This finding is also supported by research by Ikhsanudin et

al. (2021), which obtained LC_{50} values of 4405,803 ppm and LC_{90} of 6080,714 ppm in third instar *A. aegypti* larvae within 24 hours (Ikhsanudin et al., 2021).

Until now, testing of the larvicidal ability of Tulsi extract has never been carried out. In this study, the researchers will analyze the potential of Tulsi extract as a natural larvicide against *A. aegypti* larvae. The plant contains various metabolites that can be influenced by geographical factors, thus allowing for results that are different from those of existing research.

MATERIAL AND METHODS:

This research is an experimental study with a posttest-only controlled group design. Tulsi is obtained from Candi Baru, Gianyar Regency. The extraction process was carried out in the Biomedical Lab, Faculty of Medicine and Health Sciences, Warmadewa University, using the maceration method. Based on the World Health Organization (WHO) Guidelines for Laboratory and Field Testing of Mosquito Larvicides in 2005, the maximum standard concentration requirement for larvicides is 1% (WHO, 2005), so the concentration of Tulsi ethanol extract to be used in this research is determined as follows: 0.03%, 0.06%, 0.12%, 0.24 %, and 0.48%.

The extraction process begins with drying Tulsi plants using sunlight until a constant dry weight is obtained, the simplicia is then finely ground with a blender and macerated using 96% alcohol in a ratio of 1 (powder): 5 (96% alcohol) in an aluminum foil covered container. The first maceration process was carried out for 48 hours which was then filtered to become the first filtrate. The filtered pulp from the first maceration was macerated again for 24 hours and then filtered to produce the second filtrate, the same thing was also done to obtain the third filtrate. The pulp from the second maceration will be macerated again for 24 hours according to the second maceration procedure to produce the third filtrate. The filtrate that has been obtained (filtrate 1, 2 and 3) is then evaporated solvent using a rotary evaporator at 50□ in dark conditions to produce a crude extract.

A total of 20 *Aedes aegypti* instar III larvae were used for each concentration in the test and control groups. The sample size was determined based on WHO recommendations in Procedures for insecticide and bacterial larvicide testing (WHO, 2016). Larvae are selected when they are active; if they appear inactive or deformed, they will not be used. Tulsi extract was weighed according to the specified concentration and added 100 ml of water in a measuring flask. Pour the solution into a 300 ml container and then put 20 *A. aegypti* instar III larvae into each test container and leave it for 24 hours without being given food. After 24 hours, observe the larvae that do not move even though it has been stimulated (dead) with a stick on all test containers. This study was repeated 3 times in each test and control group (WHO, 2016).

To determine the correlation between the administration of various concentrations of Tulsi ethanol extract and the death of third-instar *A. aegypti* larvae, the One-Way ANOVA test was carried out to determine if the data was normally distributed. If the data distribution is abnormal, the Kruskal-Wallis test is performed. A Probit Analysis test is carried out to determine the lethal concentration of 50% (LC_{50}) and lethal concentration of 99% (LC_{99}).

RESULT:

Data on the results of the Tulsi extract larvicide test can be seen in Table 1 and Figure 1 below. Based on Table 1, the administration of Tulsi extract to the test group shows that the higher the concentration of the extract, the higher the larval mortality rate. The lowest rate was found at an extract concentration of 0.03%, with as many as one larva (1.67%) and the highest mortality rate was at a concentration ≥ 0.24% as many as 60 larvae (100%). Meanwhile, no larval deaths were found after 24 hours of observation in the control group that was not given Tulsi extract.

Table 1. Mortality rate of third instar *Aedes aegypti* larvae after 24 hours

Repetition	Concentration Groups					
	Control	0.03%	0.06%	0.12%	0.24%	0.48%
I	0	0	3	7	20	20
II	0	1	2	4	20	20
III	0	0	4	6	20	20
Total	0	1	9	17	60	60
Mean	0	0.33	3	5.67	20	20
Percentage of Mortality (%)	0%	1.67%	15%	28.3%	100%	100%

Figure 1 shows the percentage of mortality of third instar *Aedes aegypti* larvae in each test and control group after 24 hours. The graph shows an increase in *A. aegypti* larval mortality along with increasing extract concentration in the treatment group. The graph shows a significant increase in the death rate in the concentration range of 0.12% to 0.24%, which allows a lethal concentration of 50% (LC_{50}) and a lethal concentration of 99% (LC_{99}) to be in that concentration range.

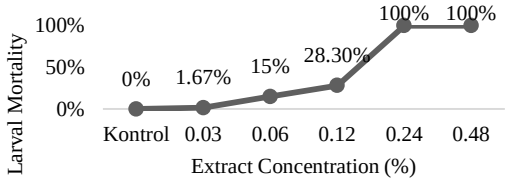

Figure 1. Mortality graph of third instar *Aedes aegypti* larvae

The test data does not meet the requirements of the One-Way ANOVA test (Sig. > 0.05), the data shows an abnormal distribution (Sig. < 0.05). Therefore, statistical analysis was continued with the Kruskal-Wallis test. The Kruskal-Wallis test results show the Asymp. Sig. < 0.05 (*P* =.005), thus there is a difference in the percentage of death of *A. aegypti* larvae in the test group, it can also be said that each concentration has a different ability to kill larvae.

Results of the estimated effective concentration against *A. aegypti* instar III larvae using Probit Analysis are shown in Table 2. Tulsi extract shows the ability to kill 50% of *A. aegypti* instar III larvae (LC_{50}) at an extract concentration of 0.135% with a concentration range of 0.106% to 0.188% and the ability to kill 99% of *A. aegypti* instar III larvae (LC_{99}) at an extract concentration of 0.25% with a concentration range of 0.194% to 0.421%.

Table 2. Results of Probit Analysis

LC	Estimated Concentration	95% Confidence Interval (Lower -Upper Bound)
50%	0.135 %	0.106 % - 0.188 %
99%	0.25 %	0.194 % - 0.421 %

DISCUSSION:

Controlling disease vectors with biological methods is an alternative approach to eradicating mosquito larvae. This method prioritizes predatory organisms or toxin-producing organisms, such as plants containing certain phytochemicals that can kill mosquito larvae (Permenkes, 2017). These plants are widely distributed in Indonesia in the Poaceae and Lamiaceae families and are supported by many previous studies on the larvicidal activity of the *Ocimum* genus Tulsi plant species (Astriani & Widawati, 2017).

Simplicia tulsi is extracted using the maceration method using 96% ethanol solvent with the aim of dissolving polar and non-polar secondary metabolites. The maceration method was chosen because it is a simple process and does not use a heating process during the extraction. Simplicia that is soaked in a solvent will undergo a breakdown of the cell membrane due to the pressure difference between the intracellular and extracellular. This condition causes secondary metabolites in the plant cytoplasm to be attracted to the solvent. This event will continue to repeat until a concentration balance is reached between the solution in the intracellular and extracellular (Dewatisari, 2020).

In comparison with other types of solvents such as chloroform, ethanol solvents could attract secondary metabolites with higher concentrations, even though they only produce less diversity of secondary metabolites (Sintya et al., 2023). Therefore, the solubility of the chosen solvent greatly affects the concentration level of secondary metabolites resulting from the extraction process (Hariantha et al., 2024).

This study used concentrations of Tulsi extract with variations of 0.03%, 0.06%, 0.12%, 0.24% and 0.48%. The use of various concentration quantities to determine the lethal concentration that causes death in the test population in a toxicity study, requires the use of four to five different dose concentrations (WHO, 2016). This dose range must be wide enough to produce a varied response so that it is possible to accurately observe the effect of different concentrations on mortality and determine the lethal concentration value. As an illustration, the dose in this study

started from the smallest concentration, which was then increased by multiplying by two until the dose concentration was high enough to kill the entire test population (Wirasuta & Niruri, 2006).

Crude extract obtained by extraction method using ethanol solvent produces a blackish green extract and has a strong aroma. At concentrations of 0.03% and 0.06%, the crude extract dissolved looked watery, not so concentrated, and had a not so strong aroma. Whereas at a concentration of 0.12%, the extract solution looked more concentrated and tended to have a sharp aroma. The same thing also happened at higher concentrations (0.24% and 0.48%), the results of the extract solution were increasingly concentrated and had the strongest aroma. In the control group, there was no larval mortality. The group with a concentration of 0.03%, the larval mortality rate was 1.67%. The group with a concentration of 0.06% had a larval mortality rate of 15%. The mortality rate increased to 28.3% in the 0.12% concentration group. Finally, in the groups with concentrations of 0.24% and 0.48%, the larval mortality rate was 100%. This data shows that Tulsi extract has a lethal effect on *Aedes aegypti* instar III larvae which is getting greater as the concentration of the extract increases.

Furthermore, a normality test was conducted to assess whether the data distribution was normally distributed or not and to select the analysis method. The Kolmogorov-Smirnov and Shapiro-Wilk test results show a value of $P = .006$ and $P = .000$ in each of these tests even though the data has been transformed using natural log. Based on the findings of these values, the test data did not meet the requirements of the One-Way Anova test ($P > 0.05$) and the data showed an abnormal distribution ($P < 0.05$), therefore the statistical analysis continued with the alternative test. Kruskal Wallis test was conducted to determine the difference in the average mortality of *A. aegypti* instar III larvae in all test groups. The Kruskal Wallis test results show that each concentration has a different level of effectiveness in killing *A. aegypti* larvae, as indicated by the Asymp.Sig. < 0.05 ($P = .005$). This indicates a variation in the percentage of larval mortality among the tested groups. Furthermore, there were at least two Tulsi extract concentration groups that showed significant differences in the mean number of larval deaths. This confirms that the concentration of the extract affects its effectiveness in killing larvae.

The Probit Analysis test was next conducted to determine the effectiveness of a larvicide by estimating the concentration required to kill 50% and 99% of *A. aegypti* instar III larvae within 24 hours. The test results showed a lethal concentration 50% (LC_{50}) value of 0.135% and a lethal concentration 99% (LC_{99}) value of 0.25%. Many previous research reports on the larvicidal activity of *Ocimum* genus plant extracts can be compared with this study. Research conducted by Firmansyah et al. (2019) found that basil leaf extract (*Ocimum sanctum*), has a larvicidal effect on *A. aegypti* instar IV larvae. In the study, the concentration of basil leaf extract was 0.66% for LC_{50} and 1.38% for LC_{90} (Firmansyah et al., 2019).This finding was supported by research conducted by Ikhsanudin et al. (2021), which obtained an LC_{50} value of 4405.803 ppm and an LC_{90} of 6080.714 ppm on *A. aegypti* instar III larvae (Ikhsanudin et al., 2021). The WHO guidelines for mosquito larvicide testing set a maximum concentration standard of 1% for larvicides. The results of this study have LC_{50} and LC_{99} values that are far below the maximum limit recommended by WHO. This indicates that the ethanol extract of Tulsi plant is not only effective but also in accordance with WHO concentration standards (WHO, 2005).

The study's findings indicate that the LC_{50} and LC_{99} values for Ocimum *tenuiflorum* extract are lower than those reported in the studies by Firmansyah et al. (2019) and Ikhsanudin et al. (2021), suggesting a higher potency of the extract in this research (Firmansyah et al., 2019; Ikhsanudin et al., 2021). The difference in lethal concentration values when compared to other studies can be influenced by the phytochemical components of plants used that come from different geographical areas, allowing plants to produce secondary metabolite products that adjust their environment and geography even though they are still in the same genus (Qaderi et al., 2023). As an example of the differences in secondary metabolite findings, in Sopianti & Sary (2018) research on secondary metabolite screening of *Ocimum tenuiflorum* leaves obtained from Bengkulu City, compounds of flavonoids, tannins, phenols, saponins, steroids and essential oil were obtained as the extracts (Sopianti & Sary, 2018), while in a similar study using Tulsi samples obtained from Gianyar Regency, Bali, secondary metabolites were found in the form of terpenoid-alkaloids, benzene, sesquiterpenes, alkylbenzene, phenolic, and phthalate esters (Sintya et al., 2023).

The lethal concentration value is also influenced by differences in the extraction procedures. The concentration of secondary metabolites obtained using 70% ethanol solvent will be lower than using 96% ethanol solvent. The difference in different concentration levels is due to the lower water content in 96% ethanol so that it can attract more secondary metabolites that are polar and non-polar compared to using 70% ethanol solvent (Yunita & Khodijah, 2020). The use of 95% ethanol solvent in the research of Sintya et al. (2023) showed that eugenol (terpenoid-alkaloid) was only found using ethanol solvent and became a secondary metabolite that had a high

concentration with a peak area of 17.20 (Sintya et al., 2023). This data shows that ethanol solvents could attract secondary metabolites with higher concentrations (Sintya et al., 2023) and the use of ethanol solvents with different concentration levels will affect the concentration of secondary metabolites resulting from maceration (Yunita & Khodijah, 2020) so that with a higher phytochemical composition at a lower extract concentration leads to lower lethal concentration value. Based on Koraag et al. (2016), the lower the LC_{50} concentration value of an extract, the higher the toxicity of the plant (Koraag et al., 2016).

Differences in phytochemical composition may explain the potency of plant extracts in killing mosquito larvae as these phytochemical constituents have different mechanisms of action to cause larval mortality. The entry of secondary metabolites can be through ingestion and absorption by the intestinal wall (stomach poison) or through the porous skin on the larval body surface which is then spread throughout the larval body (contact poison) (Corradini et al., 2011). During the test process, larvae will receive relatively nonspecific toxic effects on various phytochemical molecular targets. Secondary metabolites will affect larval physiology in various ways, such as affecting the synthesis, storage, release, binding, and re-absorption of neurotransmitters, as well as affecting the activation of receptors and the function of enzymes involved in signal transduction pathways (Rattan, 2010).

The results of secondary metabolite analysis from Firmansyah et al. (2019), Ikhsanudin et al. (2021), and Sintya et al. (2023) show that there is alkaloid, saponin, flavonoid, terpenoid, phenol and tannin content in the ethanol extract in each study (Firmansyah et al., 2019; Ikhsanudin et al., 2021; Sintya et al., 2023). The mechanism of action of these secondary metabolites can resemble synthetic larvicides that are widely circulated in the community. Alkaloids have two dominant mechanisms: nicotine, which affects acetylcholine receptors, and veratrin, which affects membrane sodium channels (Brenner & Stevens, 2022). Saponins can inhibit proteolytic action, which causes a decrease in digestive enzyme activity, triggers alteration of the larval midgut, and interferes with food absorption in larvae (Firmansyah et al., 2019; Murugan et al., 2012). Flavonoids can reduce the activity of digestive enzymes and intestinal proteins, thereby inhibiting insect growth (Rattan, 2010). Terpenoids are secondary metabolites that are most commonly found in plants and are the dominant constituents of essential oils. The terpenoid content in essential oils does not harm mammals, birds, and fish (Andrade-Ochoa et al., 2018). Terpenoids work by disrupting the nervous system, such as when using pyrethroids (monoterpene esters) and phenolics, which can be found in essential oils in the form of simple phenols and in the form of tannins (Rattan, 2010). Tannins work by reducing the activity of digestive enzymes and intestinal proteins, thereby inhibiting insect growth (Rattan, 2010). Based on these data, it can be assumed that variations in secondary metabolite content in Tulsi extract, mainly alkaloids, saponins, flavonoids, terpenoids, phenols, and tannins, act as larvicides in *A. aegypti* larvae through neurotoxicity and growth inhibitory mechanisms.

CONCLUSION:

Tulsi extract was able to cause the death of *Aedes aegypti* larvae as the concentration increased with a significant difference based on the Kruskal-Wallis test (P value <0.05), with a 50% lethal concentration (LC_{50}) value of Tulsi (*Ocimum tenuiflorum*) extract against *A. aegypti* larvae of 0.135 % and the lethal concentration 99% (LC_{99}) value of Tulsi (*Ocimum tenuiflorum*) extract against *A. aegypti* larvae is 0.25%. This research succeeded in proving the potential of Tulsi extract as a larvicide made from natural ingredients. Further research is needed to be able to create practical alternative larvicide formulations based on Tulsi.

CONFLICT OF INTEREST:

None declared

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