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Larvicidal activity of granulated pharmaceutical products using Indonesian holy basil leaf extract

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ABSTRACT

Ocimum sanctum Linn, known as holy basil, is a larvicide, which is relatively safe compared to synthetic insecticides. This study **investigates** the larvicidal activity of a granule formulation of Indonesian holy basil leaf extract against **third larval instar of *Aedes aegypti***. The extract of holy basil leaves was obtained by a maceration process with 96% ethanol. The granule was formulated with various concentrations of holy basil leaf extract, including F1 (2000 ppm), F2 (4000 ppm), and F3 (6000 ppm). The extract contained terpenoid, alkaloid, saponin, flavonoid, and polyphenol compounds. The extract granules had a moisture content of 3.01%, flowability of 1.51 seconds, and dispersion time of 1.09 seconds. The mortality rates of mosquitos treated with the different formulation groups were significantly different from positive control with values of 25.33% (F1), 50.67% (F2), and 90.67% (F3). In conclusion, the granulated formulation of holy basil leaf extract has a larvicidal LC₅₀ of 4405.803 ppm and LC₉₀ of 6080.714 ppm. Therefore, a granulated pharmaceutical product derived from holy basil leaf extract could be developed as a potent larvicide to control dengue fever.

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1. INTRODUCTION

Dengue fever, a viral infection, has been rapidly spread by the *Aedes aegypti* mosquito and has infected 2.5 billion people worldwide. The WHO reports that there are currently 50 million cases of dengue fever annually. The tropical climate in Indonesia is a significant factor in the reproduction of the mosquito vectors of dengue virus (DENV) such as *Aedes aegypti* and *Aedes albopictus*. In most regions of Indonesia, DENV is endemic with a mortality rate of 1% [1], [2].

Vaccinations and antivirals can be used as strategic therapeutic management tools to control dengue fever. However, these strategic developments are still under appraisal and are yet to be applied in large human populations. Mosquito vector control is an important alternative for reducing dengue fever incidence. The control of mosquito vectors is carried out to terminate the chain of infection by eradicating mosquito larvae and thereby reducing the incidence of mosquito bites [3], [4]. Use of synthetic larvicides may lead to vector resistance and environmental damage [5]. Furthermore, the use of organophosphate larvicides may induce various diseases in humans, such as cardiovascular disease, male impotence, nervous system disorders, dementia, non-Hodgkin lymphoma, disorders of pregnancy duration, and neurological problem in children [6]. Hence, natural and environmentally safe vector control products are urgently required [7].

An Indonesian plant that could potentially be developed as a larvicide is the holy basil plant. Holy basil leaf contains several chemical compounds that are toxic for mosquito larvae, such as flavonoids, saponins, tannins, and eugenols [8], [9]. Previous study stated that the ethanolic extract of holy basil leaf has larvicidal activity against the third larval instar of *Aedes aegypti* with an LC₅₀ value of 1290.39 ppm and an LC₉₀ value of 3173.53 ppm [10], [11]. In this study, the larvicidal activity was performed on the crude extract of holy basil leaf. To the best of our knowledge, no larvicidal pharmaceutical products have been developing from holy basil leaf. Therefore, we formulated a granule product of holy basil extract and evaluated its larvicidal activity against the third larval instar of *Aedes aegypti*. Granulation improves the surface texture, porosity, wettability in order to increase disintegration and solubility of active substances [12]. Furthermore, the granulation process can produce large particles from the active substance that may increase the flowability and accuracy of dosage [13], [14]. Overall, this study aims to develop holy basil leaf into a potent and easy-to-use larvicide with high activity.

2. RESEARCH METHOD

2.1. Materials

Holy basil leaves (*Ocimum sanctum* Linn) were collected from Kulonprogo District, Special District of Yogyakarta Province, Indonesia. The plant sample was identified in the Laboratory of Plant Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Ahmad Dahlan. *Aedes aegypti* at the third larval instar were provided by the Laboratory of Entomology, Faculty of Public Health, Universitas Ahmad Dahlan. The granule was composed of lactose, amylum manihot, magnesium stearate, polyvinylpyrrolidone (PVP) which were obtained from Brataco, Indonesia. Temephos was obtained from BASF, Indonesia. All ingredients were of standard analytical grade.

2.2. Methods

2.2.1. Preparation of extract

Samples of holy basil were dried in a shady place. The dried holy basil leaf was ground into a soft powder. The powder was dried once more in a shady place at room temperature (27-30°C) in the afternoon. 650 g of dried holy basil leaf powder was processed by maceration in proper ethanol solvent. Furthermore, it was poured into a 500 mL glass beaker and mixed for 180 minutes per days at 200 rpm. The mixing process was done at a temperature of around 25-28°C. Finally, the solvent was evaporated using a rotary evaporator (IKA RV 8 V) to obtain the dense mass extract.

2.2.2. Organoleptic evaluation of extract

Organoleptic evaluation of the extract was performed by observing colour, odour, taste, and consistency. The evaluation of extract consistency was done using a density parameter by pressing the extracted sample between the thumb and the index finger, and assigning a subjective density score.

2.2.3. Identification of chemical compound of extract

Screening of chemical compounds present in the extract of *Ocimum sanctum* Linn was done to determine the plant's active substances. We evaluated the presence of ethanols, terpenoids, alkaloids, saponins, flavonoids, and polyphenols [15], [16]. The holy basil leaf extract was poured into a test tube for each experiment. Classes of chemical compounds were identified as follows:

a) Identification of ethanols

5 ml extract was added by 1 ml NaOH 1N slowly. After three minutes, added 0.1 ml of PVP. If the extract contains ethanol, thus iodoform odour will spread, and yellow sediments will be visible within 30 minutes [17].

b) Identification of terpenoids

Two drops of holy basil extract were placed into the test tube, which have been added 2-3 drops of acetic anhydride solution. Furthermore, it was stirred slowly until dry while being added 1-2 drops of concentrated sulphuric acid. Terpenoid was identified if the green or a bluish colour visible in the test tube [16].

c) Identification of alkaloids

0.5 g of the extract was poured into a test tube and added 2 ml of ethanol 96% while being stirred. The next stage was adding HCL 2N and heating on a water bath. After the mixture cooled, then it was filtered and added some drops of Mayer's reagent. The sample was observed until sedimentation being formed [18].

d) Identification of saponins

0.2 g of the extract was dissolved into 5 ml of distilled water and was heated it until boiling. After cooling, the solution was filtered and 3 ml of distilled water was added to the filtrate while shaking. Saponins could be identified in the extract if a stable foam was formed [19].

e) Identification of polyphenols

200 mg of basil leaf extract was dissolved in 10 ml of solvent for 20 minutes in a water bath. Furthermore, the filtrate was cooled and three drops of FeCl₃ were added. Polyphenols were indicated by a colour change to bluish-green [20].

2.2.4. Granulated formulation from holy basil extract

In our study, the treatment groups were allocated based on the different concentrations of the holy basil leaf extract by 2000 ppm (F1), 4000 ppm (F2), and 6000 ppm (F3). The negative control group used a placebo without holy basil extract. A positive control group contains active substances of temephos. The additional ingredients needed for formulating the granules consist of filler and binder. Lactose was used as the filler, whereas amylum, magnesium stearate, and PVP were the binder. The composition of granules forming consists of 50% active substances and 50% excipients, as shown in Table 1.

Table 1. The formula of holy basil extracts granules

Ingredients	Placebo	F1	F2	F3
Extract ethanol of holy basil leaf (ppm)	0	2000	4000	6000
Lactose (%)	45	45	45	45
Amylum (%)	3	3	3	3
Magnesium stearate (%)	1	1	1	1
PVP (%)	1	1	1	1

2.2.5. Physical characteristics test of holy basil leaf extract granules

Moisture content evaluation was performed by applying the Moisture Balance Toledo. 1000 mg granules of holy basil leaf ethanolic extract were placed into the apparatus with a temperature set at 105°C for 15 minutes. A good percentage of moisture granules is 3-5% [21]. As much as 25 g granules were placed into a funnel with a valve at the bottom of the apparatus. The bottom valve was opened; thus, the granules could flow down to be contained in a glass beaker. Flow time test was done by recording the time needed for the granule to flow [22]. 400 mg of granules were poured into a glass, thus approximately adding 1000 mL of water. The time needed for the granule to be dispersed entirely was recorded. A good dispersion time of granules was less than five minutes [23].

2.2.6. Larvicidal activity test

The larval instar of *Aedes aegypti* was placed into a tray filled with distilled water up to 100 ml. Furthermore, several amount granules of holy basil leaf ethanol extract were added based on each concentration needed. Positive control was used temephos with a concentration of 0.01%. Replication was done as much as three times for each treatment group. The observation was done after 24 hours by analyzing the percentage of larva mortality rate [24].

2.3. Data analysis

Data were analyzed by applying the ANOVA method with a 95% significance level. Moreover, all statistical analyses were performed to determine the value of the least significant difference (LSD) posthoc test by using software SPSS version 22 (IBM Corp., Armonk, NY., USA). The larva mortality rate was also analyzed with a probit model to determine the LC₅₀ and LC₉₀ values.

3. RESULTS AND DISCUSSION

We have succeeded in formulating granules from the ethanol extract of holy basil leaf into various formulas. Our study showed that the granule of holy basil leaf had a good appearance. The larvicidal activity of holy basil leaf granules could be seen in the LC₅₀ dan LC₉₀ score. The detailed findings related to the physical characteristics and larvicidal activity could be described in the explanation below.

3.1. Organoleptic result of holy basil leaf ethanol extract

The holy basil leaf extract was observed visually in its organoleptic characteristics such as colour, odour, taste, and consistency. The organoleptic test of holy basil leaf extract was deep brownish-green colour, holy basil specific odour, bitter taste, dense and sticky consistency, as seen in Table 2. The chemical substances in the holy basil leaf extract are terpenoids, alkaloids, saponins, flavonoids dan polyphenols, as seen in Table 2. A previous study demonstrated that alkaloid and terpenoid substances could act as larvicidal [25].

Table 2. The chemical substances and organoleptic parameters of holy basil leaf extract

Chemical substance	Description	Result	Organoleptic parameter	
Terpenoids	Dark green colour	+	Colour	Deep brownish green
Alkaloids	White sedimentation	+	Odour	Holy basil leaf
Saponins	Permanent sedimentation	+	Taste	Bitter
Flavonoids	Intensive yellow colour	+	Consistency	Dense, sticky
Polyphenols	Green-blue colour	+		

Note: (+) existence

Table 2 reports that holy basil leaf extract consists of terpenoids, proven by the colour change into dark green. This change was caused by adding a concentrated sulphuric acid to the sample [26]. The extract also consists of alkaloid substances which formed the white sedimentation due to nitrogen reaction in alkaloids with the metal ion K^+ of potassium tetraiodomercurate (II) to produce potassium-alkaloid complex [27]. The saponins in the extract were signified by forming stable foam caused by the glycoside [28]. Saponins, a glycoside, had a steroidal aglycone and triterpenoid, in which the glycosyl group was bound to atom C_3 [29]. The extract also consists of flavonoids proven by the colour change on the filter paper from pale yellow into intensive yellow after being vaporized with ammonia. This colour change was caused by ammonia, causing the hydroxyl group to be ionized and changing the length of wave absorption. Polyphenols in the extract were also identified by the extracted colour changing into the dark green after adding $FeCl_3$. This mechanism was caused by the interaction between the Fe^{3+} hydroxyl group in the polyphenol [30].

3.2. Physical characteristics test of granule holy basil leaf extract

The granule extract of holy basil leaf was evaluated for its specific physical characteristics such as moisture content, flow time, and dispersion time. The physical characteristic of granulated formulation holy basil extract was round and homogenous. Furthermore, the granule colour becomes darker with the increase of extract concentration in the granules. Moisture content had a significant impact on granule flowability. The higher moisture content could result in a decrease in the granule flowability. Furthermore, it could influence the lower quality of granules due to the most significant growth of microbes and fungi. The moisture content results are shown in Table 3.

Table 3. Moisture content test result

Parameter	Placebo	Means±SD (%)			p-value
		F1	F2	F3	
Moisture content	3.09±0.01	3.09±0.01	3.10±0.01	3.10±0.04	0.245

Table 3 reports that the percentage of average moisture content (MC %) in each group as follows: placebo (3.09±0.01), F1 (3.09±0.01), F2 (3.10±0.01), and F3 (3.10±0.04). The statistical analysis with ANOVA shows no significant difference in the moisture content percentage between formulas ($p>0.05$). The granulation process aimed to form a strong bond between particles of the granule to maintain product handling. The primary mechanism of binding formation between granule particles was caused by the cohesion and adhesion force on the surface of the liquid film. These attractive forces could build a solid bridge interlocking due to the water content in the pores of the granule particle [31]. A good humidity should create a thin layer of adsorption film by reducing the distance between particles. Hence, a wider contact region between particulates could produce a strong particle bond. The moisture content of the granule strongly influenced the process of the bonding mechanism. The good moisture content of granules ranged from 2-5% [32]. In this study, the moisture content test of granule had met the standard requirements.

Flow time was a vital parameter requirement of the granules, affecting the volume uniformity in the filling and packaging. A reasonable flow time requirement was less than 10 seconds for 100 g granule or 2.5 seconds for 25 g granule [33]. The flow time test of the holy basil leaf extract granule could be seen in Table 4.

Table 4. The flow time test and LSD posthoc test result

Formula	Means ±SD (second)	Placebo	p-value		
			F1	F2	F3
Placebo	0.50±0.01	-	0.000*	0.000*	0.000*
F1	1.43±0.02	0.000*	-	0.000*	0.000*
F2	1.53±0.02	0.000*	0.000*	-	0.000*
F3	1.57±0.02	0.000*	0.000*	0.030*	-

Note: * p-value less than 0.05

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Table 4 indicates that F3 has a longer flow time compared to F1, F2, and placebo. This result concluded that more extract concentration in the granule would result in a longer flow time [34]. Magnesium stearate was used as a lubricant in the granule formulation process. This lubricant characteristic had cohesive properties and could increase the flow time by preventing dust powder during the filling process. LSD posthoc test shows a significant difference in flow time in each group of formulas ($p < 0.05$). Each physical characteristic of the extract influenced the difference in granule flow time. A dense characteristic of the extract will impact the particle interaction's granule flow forming a liquid bridge. Furthermore, the nucleation of granules could be created as the small wet particle of extract agglomeration to forms a pendular bridge. This pendular bridge will impact the granule's mass, density, porosity, shape, and fragility. Hence, the granule should be spherical (round), uniform, and firm to reduce the friction force between particles to increase the granule's flow time. The dispersion time will influence the amount of chemical compound in the solvent of extract. Shorter dispersion time might result in more active substances in the extract, affecting larvicidal activity. The dispersion time test of granules was shown in Table 5.

Table 5. Dispersion time test and LSD posthoc test result

Parameter	Means \pm SD (%)				p-value
	Negative control	F1	F2	F3	
Dispersion percentage	1.17 \pm 0.06	1.13 \pm 0.06	1.07 \pm 0.06	1.07 \pm 0.12	0.344

Table 5 reports that the increased concentration of holy basil leaf extract in the granule will result in a faster dispersion time. The statistic test result with LSD posthoc indicates no significant differences in dispersion time between each formula ($p > 0.05$). The result concludes that the concentration of holy basil leaf extract does not influence the dispersion time of granules. The disintegrant ability in developing the disintegration force was based on the active ingredients. The granule swelling process was more effective in using ingredients that are not easily dissolved in water. Porosity will increase swiftly in a hydrophilic matrix. The dissolution and disintegration force will decrease due to the disintegrant particle space expands without disturbing the matrix. In this study, we used amylum as the disintegrant. As a disintegrant, amylum could form a hydrogen bond in the granulation to enhance the swelling process. Amylum might also facilitate the liquid transporting into the granule pores to increase the absorption of a liquid substance entering the granule pores. In our study, each formula consisted of an equal amount of amylum which caused the dispersion time to have statistically significant differences.

3.3. Larvicidal activity of holy basil leaf ethanol extract granule

The larvicidal activity [5] of granulated formulation of holy basil leaf ethanol extract was based on the standard of WHO by using the third larval instar of *Aedes aegypti*. The larvicidal activity assessed the value of LC₅₀ dan LC₉₀. The mortality rate of *Aedes aegypti* was observed 24 hours towards various formulas of granules holy basil leaf ethanol extract. The percentage of larva mortality rate could be seen in Table 6.

Table 6. The percentage of larva mortality rate

Formula	Means \pm SD (%)	p-value (LSD posthoc test)				
		Negative control	Positive control	F1	F2	F3
Negative control	0 \pm 0	-	0.025*	0.034*	0.037*	0.034*
Positive control	100 \pm 0	0.025*	-	0.034*	0.037*	0.030*
F1	25.33 \pm 0.57	0.034*	0.034*	-	0.046*	0.043*
F2	50.67 \pm 1.53	0.037*	0.037*	0.046*	-	0.046*
F3	90.67 \pm 0.57	0.034*	0.030*	0.043*	0.046*	-

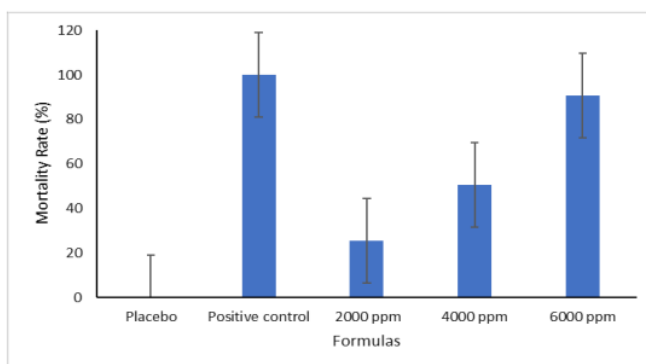
Note: * p-value less than 0.05

Table 6 concludes that the holy basil leaf extract granules had a potent larvicidal activity. This study suggested that a higher concentration of holy basil leaf extract will increase larvicidal activity. Table 6 also reports that the negative control does not have larvicidal activity. Gokulakrishnan *et al.* suggested that the extract of methanol, ethyl acetate, and hexane of *Ocimum sanctum* Linn had larvicidal activity towards the larva *Culex quinquefasciatus* [35]. The LC₅₀ and LC₉₀ probability scores were counted based on the larva mortality rate percentage. The probability score of LC₅₀ and LC₉₀ could be seen in Table 7.

Table 7. Probability score of LC₅₀ dan LC₉₀

Probability	Estimation of concentration (ppm)	Concentration (%)
0.100	3192.240	0.319
0.250	3718.597	0.372
0.500	4405.803	0.441
0.750	5220.006	0.522
0.900	6080.714	0.608
0.990	7907.394	0.791

Table 7 explains the probability value of larva mortality by LC₅₀ of 4405.803 ppm ~ (0.44%) and LC₉₀ of 6080.714 ppm ~ (0.608%). This percentage of larva mortality was caused by the active substances in the holy basil leaf extract. Moreover, Figure 1 shows that the larvicide activity will increase by adding holy basil leaf extract concentration in the granules.

Figure 1. The mortality rate of third larval instar of *Aedes aegypti*

Regarding the 24 hours observation towards the third larval instar of *Aedes aegypti*, it concludes that F3 (6000 ppm) had the highest activity of larvicide with a value of 90.67%, continued by the F2 (4000 ppm) by 50.67% and F3 (2000 ppm) by 25.33%. Table 6 shows a significant difference in the larvicidal activity in each formula ($p < 0.05$). F3 has a higher activity of larvicidal compared to the other formulas. The larvicidal activity of holy basil leaf extract granules was influenced by several active substances such as terpenoids, alkaloids, saponins, and flavonoids dan polyphenols. The mechanism of alkaloids as larvicide was caused by acetylcholinesterase (AChE) inhibition responsible for nerve impulse transmission across a synapse. The terpenoids group could facilitate the transmission into the membrane resulting in paralysis and death to the larva. The mechanism of saponins as larvicide was not clearly understood yet. In addition, the central hypothesis of saponin might act as a repellent and toxic towards cells. The saponin's properties inside water and organic solvent will damage the larva's cuticular membrane [36]. Flavonoids have been reported as a larvicide by hindering acetylcholinesterase enzyme in the mosquito larvae to terminate the food supply and the larva growth [10]. The role of phenolic groups as larvicide has also been suggested that phenolic fraction may influence mosquito growth by reducing adult larva fertility, fecundity, and lifespan [37]. This mechanism was followed by decreasing carbohydrate and fat content in the early stage of larvae instar *Aedes aegypti*. The synergy of whole secondary metabolites in the holy basil leaf ethanolic extract will result in the potent larvicidal activity of granulated pharmaceutical formulation.

4. CONCLUSION

Holy basil leaf ethanolic extract granule (*Ocimum sanctum* Linn) consists of terpenoids, alkaloids, flavonoids, saponins dan polyphenols with a larvicidal activity value of LC₅₀ 4405.803 ppm and LC₉₀ 6080.714 ppm. Therefore, the granulated formulation of holy basil leaf extract could be further developed as a novel larvicide for managing the mosquito vector.

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