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ito Subject: [IJPHS] Editor Decision

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07- Dear Prof/Dr/Mr/Mrs: Azis Ikhsanudin,

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07:

00 We have reached a decision regarding your submission entitled "Biolarvacide Potential of  
A Granulated Pharmaceutical Products Using Indonesian Holy Basil Leaf Extract" to  
M International Journal of Public Health Science (IJPHS), a peer-reviewed and an OPEN  
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I look forward for hearing from you

Thank you

Best Regards,  
Dr. Lina Handayani  
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08- Dear Prof/Dr/Mr/Mrs: Azis Ikhsanudin,

04

09:  
04 It is my great pleasure to inform you that your paper entitled "Biolarvacide Potential of  
A Granulated Pharmaceutical Products Using Indonesian Holy Basil Leaf Extract" is  
M ACCEPTED and will be published on the International Journal of Public Health Science  
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# Larvicidal Activity of Granulated Pharmaceutical Products Using Indonesian Holy Basil Leaf Extract

Azis Ikhsanudin<sup>1</sup>; Lolita Lolita<sup>1</sup> and Zayyana Septya Ramadani<sup>1</sup>

<sup>1</sup>Faculty of Pharmacy Universitas Ahmad Dahlan, Special District of Yogyakarta Province, Indonesia

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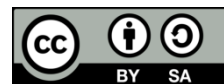
Granules

Larvicide.

## 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 had a larvicidal LC<sub>50</sub> of 4405.803 ppm and LC<sub>90</sub> 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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## Corresponding Author:

Azis Ikhsanudin,

Faculty of Pharmacy

Universitas Ahmad Dahlan

Jl. Prof. Dr. Soepomo, SH, Janturan Umbulharjo, Yogyakarta Province, Indonesia.

Email: [azis.ikhsanudin@pharm.uad.ac.id](mailto:azis.ikhsanudin@pharm.uad.ac.id)

## 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 LC<sub>50</sub> value of 1290.39 ppm and an LC<sub>90</sub> 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 developed 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 METHODS

### 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 extracts

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 3 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 FeCl<sub>3</sub> 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 concentration 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 consist of 50% active substances and 50% excipients, as seen in Table 1.

Table 1. The formula of holy basil extract 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 Granule

##### a. Moisture content

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 granule is 3-5% [21].

##### b. Flow time

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].

##### c. Dispersion time

400 mg of granules were poured into a glass, and then approximately adding 1000mL of water. The time needed for the granule to be dispersed entirely was recorded. A good dispersion time of granules was less than 5 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 concentration of 0,01%. Replication was done as much as three times for each treatment group. Observation was done after 24 hours by analyzing the percentage of larva mortality rate [24].

### 2.3. DATA ANALYSIS

Data was analyzed by applying the ANOVA method with a 95% significance level. Moreover, all statistical analyses were performed to determine the value of the 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 LC<sub>50</sub> and LC<sub>90</sub> values.

## 3. RESULTS AND DISCUSSIONS

We have succeed 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 larvicide activity of holy basil leaf granules extract could be seen in the LC<sub>50</sub> dan LC<sub>90</sub> score. The detailed findings related to the physical characteristics and larvicide 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 terms of 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 were terpenoids, alkaloids, saponins, flavonoids dan polyphenols, as seen in Table 2. A previous study demonstrated that alkaloid and terpenoid substances could act as larvicide [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
Alkaloids	White sedimentation	+	Odour
Saponins	Permanent sedimentation	+	Taste
Flavonoids	Intensive yellow colour	+	Consistency
Polyphenols	Green-blue colour	+	Dense; Sticky

Note: (+) existence

Table 2 reported that holy basil leaf extract consists of terpenoids, proven by the colour change into dark green. These change was caused by adding a concentrated sulphuric acid in 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<sup>+</sup> of potassium tetraiodomercurate (II) to produce potassium-alkaloid complex [27]. The existence of 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 C3 [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 dark green after adding FeCl<sub>3</sub>. This mechanism was caused by the interaction between the Fe<sup>3+</sup> 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 in terms of 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 has 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 test result could be seen in Table 3.

Table 3. Moisture content test result

Parameter	Means ±SD (%)				p-value
	Placebo	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 reported 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 showed 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 in order to maintaining 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 influences the process of the bonding mechanism. The good moisture content of granules ranges from 2-5% [32]. In this study, the moisture content test of granule has met the standard requirements.

Flow time is a vital parameter requirement of the granules, affecting the volume uniformity in the filling and packaging process. A reasonable flow time requirement is 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)	p-value			
		Placebo	F1	F2	F3
Placebo	0.50 ± 0.0058	-	0.000*	0.000*	0.000*
F1	1.43 ± 0.0170	0.000*	-	0.000*	0.000*
F2	1.53 ± 0.017	0.000*	0.000*	-	0.000*
F3	1.57 ± 0.023	0.000*	0.000*	0.030*	-

Note: \* p value less than 0.05

Table 4 indicated that F3 has a longer flow time compared to F1, F2, and placebo. This result concluded that more extract concentration in the granule will result in a longer flow time [34]. Magnesium stearate was used as a lubricant in the granule formulation process. This lubricant characteristic has cohesive properties and could increase the flow time by preventing dust powder during the filling process. LSD post hoc test showed a significant difference in flow time in each group of formulas ( $p < 0.01$ ). Each physical characteristic of the extract influences 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 may result in a higher amount of active substance in the extract, affecting larvicide activity. The dispersion time test of granules could be seen 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 $\pm$ 0.0573	1.13 $\pm$ 0.0576	1.07 $\pm$ 0.0583	1.07 $\pm$ 0.115	0,344

Table 5 reported that the increase of holy basil leaf extract concentration in the granule will result in a faster dispersion time. The statistic test result with LSD post hoc indicated no significant differences in dispersion time between each formula ( $p > 0.005$ ). It concluded 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 an equal amount of amylum which causes the dispersion time was not statistically significant differences.

### 3.3 Larvicidal Activity of Holy Basil Leaf Ethanol Extract Granule

The larvicidal activity test of granulated formulation of holy basil leaf ethanol extract was based on the standard of WHO by using third larval instar of *Aedes aegypti*. The larvicidal activity assessed the value of  $LC_{50}$  dan  $LC_{90}$ . The mortality rate of *Aedes aegypti* was observed 24 hours towards various formulas of granules holy basil leaf ethanol extract. The positive and negative control could be seen in Table 6 below.

Table 6. The percentage of larva mortality rate

Formula	Means $\pm$ SD (%)	p-value (LSD post-hoc 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*	-

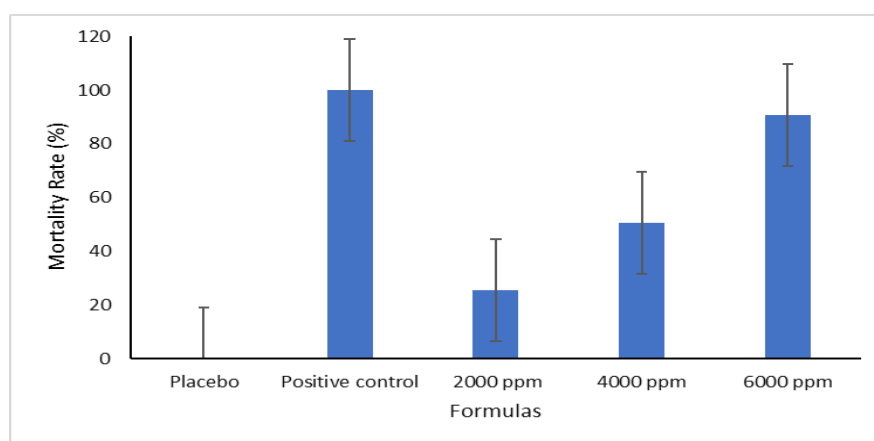
Note: \* p value less than 0.05

Table 6 concluded 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 reported that the negative control does not have larvicidal activity. Gokulakrishnan *et al.*, suggest that the extract of methanol, ethyl acetate, and hexane of *Ocimum sanctum* Linn. has larvicide activity towards the larva *Culex quinquefasciatus* [35]. The  $LC_{50}$  and  $LC_{90}$  probability score was counted based on the larva mortality rate percentage. The probability score of  $LC_{50}$  and  $LC_{90}$  could be seen in Table 7.

Table 7. Probability score of LC<sub>50</sub> dan LC<sub>90</sub>

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 reported the probability value of larva mortality by LC<sub>50</sub> of 4405,803 ppm ~ (0.44%) and LC<sub>90</sub> 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 2 showed that the larvicide activity will increase by adding holy basil leaf extract concentration in the granules.

Figure 2. The mortality rate of third larval instar of *Aedes aegypti*

Regarding the 24 hours observation towards the third larval instar of *Aedes aegypti*, concluded 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 4 showed a significant difference in the larvicidal activity in each formula ( $p < 0.01$ ). F3 had 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, flavonoids dan polyphenols. The mechanism of alkaloids as larvicide was caused by inhibition of acetylcholinesterase (AChE) 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 saponins 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 in order 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 the mosquito growth by reducing the fertility, fecundity, and lifespan of adult larva [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.

## CONCLUSION

Holy basil leaf ethanolic extract granule (*Ocimum sanctum*, Linn) consists of terpenoids, alkaloids, flavonoids, saponins dan polyphenols with a larvicidal activity value of LC<sub>50</sub> 4405,803 ppm and LC<sub>90</sub> 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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Dear Editor-in-Chief

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We would like to appreciate the time and effort that the reviewers dedicated to providing feedback of accepted with revision on our manuscript and are grateful for the insightful comments on and valuable improvements to our paper. We have attached a revised manuscript of **“Larvicidal Activity of Granulated Pharmaceutical Products Using Indonesian Holy Basil Leaf Extract” with Reference ID Number : 21004.**

No conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication. We would like to declare on behalf of my co-authors that the work described was original research which has not been published previously, and not under considerations for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

In this work, we have revised our final manuscript comprehensively in yellow color highlighted within the manuscript. We hope this revision is suitable for “International Journal of Public Health Science”.

We deeply appreciate your consideration of our manuscript. If you have any queries, please don't hesitate to contact us at the address below.

Yours Sincerely,

Corresponding author:

Best regards!

Azis Ikhsanudin, M.Sc., Apt

Assistant Professor, Department of Pharmaceutics,

Universitas Ahmad Dahlan

Prof Dr Soepomo Warungboto Umbulharjo, Yogyakarta, Indonesia

## Biolarvacide Potential of Granulated Pharmaceutical Products Using Indonesian Holy Basil Leaf Extract

Azis Ikhsanudin<sup>1</sup>; Lolita Lolita<sup>1</sup> and Zayyana Septya Ramadani<sup>1</sup>

<sup>1</sup>Faculty of Pharmacy Universitas Ahmad Dahlan, Special District of Yogyakarta Province, Indonesia

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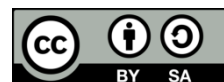
Granules

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### ABSTRACT

*Ocimum sanctum*, Linn, known as holy basil, is characterized as biolarvacide, which relatively safe than synthetic insecticides. This study aims to examine the granule formulation of Indonesian holy basil leaf extract as biolarvacidal activity toward the third larva instar of *Aedes aegypti*. The extract of holy basil leaves is obtained by maceration process with ethanol 96%. The granule was formulated with various concentrations of holy basil leaf extract, including F1 (2000 ppm), F2 (4000 ppm), and F3 (6000 ppm), Analysis of variance (ANOVA) followed by the least significant difference (LSD) methods were performed with SPSS version 22. The chemical compound of holy basil leaf extract contains terpenoid, alkaloid, saponin, flavonoid, and polyphenol. The extract granule has moisture content up to 3.01%, flowability up to 1.51 second, and dispersion time up to 1.09 second. The percentage of mosquito mortality rate in each formulation group was significantly different towards positive control with the value of F1 (25,33%), F2 (50.67%), and F3 (90.67%) ( $p < 0,05$ ). In conclusion, the granulated formulation of holy basil leaf extract has a biolarvacidal activity value of LC50 4405,83 ppm and LC90 6080,714 ppm. Therefore, a granulated pharmaceutical product from holy basil leaf extract could be developed as a potent biolarvacide in controlling dengue fever.

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### Corresponding Author:

Azis Ikhsanudin,

Faculty of Pharmacy

Universitas Ahmad Dahlan

Jl. Prof. Dr. Soepomo, SH, Janturan Umbulharjo, Yogyakarta Province, Indonesia.

Email: [azis.ikhsanudin@pharm.uad.ac.id](mailto:azis.ikhsanudin@pharm.uad.ac.id)

## 1. INTRODUCTION

Dengue fever, a viral infection, has rapidly spread by the *Aedes aegypti* mosquito and infected nearly 2.5 billion people worldwide. Currently, WHO reported that there are 50 million infection cases of dengue fever annually. The tropical climate in Indonesia becomes a significant factor in the mosquito vector reproduction in dengue virus (DENVs) species such as *Aedes aegypti* and *Aedes albopictus*. Most of the regions in Indonesia are DENVs endemic which a disease mortality rate reaches up to 1% [1], [2].

Vaccinations and antiviral can be used as strategic therapeutic management in dengue fever patients. However, these strategic development are still under appraisal and yet to be applied in larger human populations. Mosquito vector control becomes a major alternative in reducing dengue fever incidence. The controlling of mosquito vectors is carried out to terminate the chain of infection by preventing mosquito bites to humans and eradicating a big scale of mosquito larvae [3], [4]. A long time using synthetic larvicide might lead to vector resistance and environmental damage [5]. Furthermore, the use of organophosphorus larvicide may induce various diseases in humans, such as



cardiovascular illness, male impotence, nervous system disorders, dementia, non-Hodgkin lymphoma, disorders of pregnancy duration, and neurological problem in children [6]. Hence, controlling the vector by utilizing natural and environmentally safe products is very urgent to develop [7].

A potential Indonesian plant that can be developed as biolarvicide is the holy basil plant. Holy basil leaf has several chemical compounds such as flavonoid, saponin, tannin, and eugenol, which are toxic for larva [8][9]. Kartika (2014) states that ethanolic extract of holy basil leaf has larvicidal activity against the 3rd larva instar of *Aedes aegypti* with LC50 value of 1290,39 ppm and LC90 value of 3173,53 ppm [10], [11]. In this study, the biolarvacidal activity test was still using the crude extract of holy basil leaf. To the best of our knowledge, a pharmaceutical product development based on larvicidal activity from holy basil leaf has not been widely studied before. Therefore, this research aims to formulate the granule product of holy basil extract and evaluate its potential of biolarvicide towards the 3rd larva instar of *Aedes aegypti*. The granulated formulation has a good characteristic in improving surface texture, porosity, wettability to increase disintegration and solubility properties of active substances [12]. Furthermore, the granulation process can also produce large particles from the active substance that may increase the flowability and accuracy of dosage forms [13], [14]. Hence, this study is expected to develop the Indonesian natural medicinal product as a potent biolarvicide with high activity and practical use by the public.

## 2. RESEARCH METHOD

### 2.1 MATERIALS

Holy basil leaves (*Ocimum sanctum*, Linn) are collected from Kulonprogo District, Yogyakarta, Indonesia. The plant sample is identified in the Laboratory of Plant Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences, Ahmad Dahlan University. The 3rd larvae instar of *Aedes aegypti* are collected from the Laboratory of Entomology, Faculty of Public Health, Ahmad Dahlan University. The granule is composed of lactose, amylum manihot, magnesium stearate, polyvinylpyrrolidone (PVP) which are obtained from PT. Brataco Indonesia. Furthermore, temephos is obtained from PT. BASF Indonesia. All of the ingredients have a standard analytical level.

### 2.2 METHODS

#### 2.2.1 Preparation of extracts

Sample of holy basil is dried under shady place. The dried holy basil leaf is ground into a soft powder. Holy basil leaf powder is dried once more under a shady place at room temperature (27-30°C) in the afternoon. The holy basil leaf extraction is processed by implementing the maceration technique to proper ethanol solvent. As much as 650 g of holy basil leaf dried powder is poured into a 500 mL glass beaker while being mixed by using a mixer (IKA RW 20®) for three days with constant speed at 200 rpm for 180 minutes per day. The mixing process is done at a temperature around 25-28°C. Finally, the solvent is evaporated by 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 is identified visually in terms of color, odor, taste, and consistency. The evaluation of extract consistency is done by using a density parameter by pressing the extracted sample between the thumb and the index finger.

#### 2.2.3 Identification of chemical compound of extract

Screening of chemical compounds is done towards the extract of *Ocimum sanctum*, Linn, to determine the plant's active substance. The evaluation is carried out towards ethanol, terpenoid, alkaloid, saponin, flavonoid, and polyphenol [15], [16]. The holy basil leaf extract is poured into a test tube in each experiment. The identification of extract chemical compound to determine the active substance in holy basil leaf are as follows:

##### a. Identification of ethanolic

5 ml extract is added by 1 ml NaOH 1N slowly. After 3 minutes, add 0,1 ml of PVP. If the extract contains ethanol, iodoform odor will spread, and yellow sediments will be visible within 30 minutes [17].

##### b. Identification of terpenoid

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

##### c. Identification of alkaloid

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



**d. Identification of saponin**

0,2 g of the extract is dissolved into 5 ml of distilled water and heat it until boiling. Filter the solution after cooled, and add 3 ml of distilled water to the filtrate while being shaken. If stable foam is formed, saponin can be identified in the extract [19].

**e. Identification of polyphenol**

200 mg of holy basil leaf extract is heated with 10 ml of water for 20 minutes in a water bath. Then, filter it. When the filtrate cool, add three drops of FeCl<sub>3</sub> into the solution. Polyphenol is shown with the change of color into bluish-green [20].

**2.2.4. Granulated Formulation from Holy Basil Extract**

In our study, the treatment group is allocated based on the variation of concentration of the holy basil leaf extract by F1 (2000 ppm), F2 (4000 ppm), and F3 (6000 ppm). The negative control group uses a placebo without holy basil extract. A positive control group contains active substances of temephos. The additional ingredients needed for formulating the granule consist of filler and binder substances. Lactose is used as the filler, whereas amyllum, magnesium stearate, and PVP are the binder. The composition of granule forming consists of 50% active substance and 50% excipient, as seen in Table 1.

Table 1. The formula of holy basil extract granule

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

**2.2.5 Physical Characteristic Test of Holy Basil Leaf Extract Granule****a. Moisture content**

Moisture content test of granule is carried out by applying the Moisture Balance Toledo. One gram granule of holy basil leaf ethanolic extract is put into the apparatus with a temperature set at 105 for 15 minutes. Then, record the result shown by the Moisture Balance. A good percentage of moisture granule is 3-5% [21]

**b. Flow time**

As much as 25 g granule is put into a funnel with a valve at the bottom of the apparatus. Then, the bottom valve is opened so the granule can flow down and can be contained in a glass beaker. Flow time test is done by recording the time needed for the granule to flow [22].

**c. Dispersion time**

400 mg of granule is poured into a glass, and then approximately 1 liter of water is added. After that, it must be stirred. The time needed for the granule to be completely dispersed is recorded. A good dispersion time of granule is less than 5 minutes [23].

**2.2.6 Larvicidal Activity Test**

The larvae instar of *Aedes aegypti* are placed into a tray filled with distilled water up to 100 ml. Then, some amount of granule holy basil leaf ethanol extract is added based on each concentration needed. Positive control was used temephos with concentration up to 0,01%. Replication is done as much as three times for each treatment group. Observation is done after 24 hours by analyzing the percentage of larva mortality rate [24].

**2.3. DATA ANALYSIS**

Data are analyzed by applying the ANOVA (Analysis of Variance) method with a 95% level of significance. Moreover, the statistical analysis continued to determine the value of the LSD post-hoc test by using SPSS 22. The larva mortality rate is also analyzed with a probit model to determine the LC<sub>50</sub> and LC<sub>90</sub> values.

**3. RESULTS AND DISCUSSIONS**

We succeed in formulating granule from the ethanol extract of holy basil leaf into some formulas. Our study shows that the granule of holy basil leaf has a good appearance. The larvicide activity of granule holy basil leaf extract can be seen in the score of LC<sub>50</sub> dan LC<sub>90</sub>. Detailed findings related to the physical characteristics and larvicide activity can be seen in the explanation below.

**3.1 Organoleptic Result of Holy Basil Leaf Ethanol Extract**

The holy basil leaf extract is observed visually in terms of its organoleptic characteristics such as color, odor, taste, and consistency. The organoleptic test of holy basil leaf extract shows deep brownish-green color, holy basil specific

odor, bitter taste, dense and sticky consistency, as seen in Table 2. The chemical substance in the holy basil leaf extract includes terpenoid, alkaloid, saponin, flavonoid dan polyphenol, as seen in Table 2. A previous study demonstrates that alkaloid and terpenoid substances could act as a larvicide [25].

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

Chemical substance	Description	Result	Organoleptic parameter	
Terpenoid	Dark green colour	+	Colour	Deep brownish green
Alkaloid	White sedimentation	+	Odour	Holy basil leaf
Saponin	Permanent sedimentation	+	Taste	Bitter
Flavonoid	Intensive yellow colour	+	Consistency	Dense; Sticky
Polyphenol	Green blue colour	+		

Note: (+) existence

Table 2 shows that holy basil leaf extract consists of terpenoid, which is proven by the color change into dark green. The color change is caused by the addition of concentrated sulphuric acid in the sample [26]. The extract also consists of an alkaloid substance that forms the white sedimentation due to nitrogen reaction in alkaloid with the metal ion  $K^+$  of potassium tetraiodomercurate (II) to produce potassium-alkaloid complex [27]. Besides, the existence of saponin in the extract is signified by forming permanent foam caused by the glycoside in the water [28]. Saponin, a glycoside, has a steroidal aglycone and triterpenoid, which glycosyl group is bound to atom C3 [29]. The extract also consists of flavonoids proven by the color change on the filter paper from pale yellow into intensive yellow after being vaporized with ammonia. This color change is caused by ammonia which causing the hydroxyl group to be ionized and changing the length of wave absorption. Polyphenol in the extract is also identified by the extract color changing into the dark green after adding  $FeCl_3$ . This is 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 is evaluated in terms of its specific physical characteristics such as moisture content, flow time, and dispersion time. The physical characteristic of granulated formulation holy basil extract is round and homogenous. Furthermore, the granule color becomes darker with the increase of extract concentration in the granule. The granulated formulation of holy basil leaf ethanolic extract could be seen in Figure 1.



Figure 1. The physical characteristic appearance of granule in (a) placebo; (b) F1 (2000 ppm); (c) F2 (4000 ppm); (d) F3 (6000 ppm)

Moisture content has 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 granule due to the most significant growth of microbes and fungi. The moisture content test result can be seen in Table 3.

Table 3. Moisture content test result

Parameter	Means $\pm$ SD (%)				<i>p</i> -value
	Placebo	F1	F2	F3	
Moisture content	3.09 $\pm$ 0.01	3.09 $\pm$ 0.01	3.10 $\pm$ 0.01	3.10 $\pm$ 0.04	0.245

Table 3 shows that the percentage of average moisture content (MC%) in each group as follows placebo (3.09  $\pm$  0.01), FI (3.09  $\pm$  0.01), F2 (3.10  $\pm$  0.01), and F3 (3.10  $\pm$  0.04). The statistical analysis with ANOVA shows no significant difference in the moisture content percentage between formulas ( $p > 0.05$ ). The granulation process aims to form a strong bond between particles of the granule to maintain product handling. The primary mechanism of binding formation between granule particles is caused by the cohesion and adhesion force in 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 be able to 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 influences the process of the bonding mechanism. The good moisture content of granules ranges from 2-5% [32]. In this study, the moisture content test of granule has met the standard requirements.

Flow time becomes a major parameter requirement of the granule, affecting the volume uniformity in the filling and packaging process. A good flow time requirement is 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 can be seen in Table 4.

Table 4. The flow time test and LSD post hoc test result

Formula	Means $\pm$ SD (second)	p-value			
		Placebo	F1	F2	F3
Placebo	0.50 $\pm$ 0.0058	-	0.000*	0.000*	0.000*
F1	1.43 $\pm$ 0.0170	0.000*	-	0.000*	0.000*
F2	1.53 $\pm$ 0.017	0.000*	0.000*	-	0.000*
F3	1.57 $\pm$ 0.023	0.000*	0.000*	0.030*	-

Note: \* p value less than 0.05

Table 4 indicates that F3 has a longer flow time compared to F1, F2, and placebo. This result concludes that more extract concentration in the granule will result in a longer flow time [34]. Magnesium stearate is used as a lubricant in the granule formulation process. This lubricant characteristic has cohesive properties and could increase the flow time by preventing dust powder during the filling process. LSD post hoc test shows a significant difference in flow time in each group of formulas ( $p < 0.01$ ). The physical characteristic of extract influences the difference in granule flow time. An extract with a dense characteristic will impact the particle interaction's granule flow forming a liquid bridge. 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 mass, density, porosity, shape, and fragility of the granule. 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 can result in a higher amount of active substance in the extract, affecting biolarvicide activity. The dispersion time test of granule can be seen in Table 5.

Table 5. Dispersion time test and LSD post hoc test result

Parameter	Means $\pm$ SD (%)				p-value
	Negative control	F1	F2	F3	
Dispersion percentage	1.17 $\pm$ 0.0573	1.13 $\pm$ 0.0576	1.07 $\pm$ 0.0583	1.07 $\pm$ 0.115	0,344

Table 5 reports that the increase of holy basil leaf extract concentration in the granule will result in a faster dispersion time. The statistic test result with LSD post hoc indicates no significant differences in dispersion time between each formula ( $p > 0.005$ ). It concludes that the concentration of holy basil leaf extract does not influence the dispersion time of granule. The disintegrant ability in developing the disintegration force is based on the active ingredients. The granule swelling process is reported to be more effective using ingredients which is 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 use amylum as the disintegrant. As a disintegrant, amylum could form a hydrogen bond in the granulation to enhance the swelling process. Amylum can 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 contains an equal amount of amylum which causes the dispersion time to be not statistically significant differences.

### 3.3 Biolarvacidal Activity of Holy Basil Leaf Ethanol Extract Granule

In this study, the biolarvacidal activity test of granulated formulation of holy basil leaf ethanol extract is based on the standard of WHO by using the 3rd larva instar of *Aedes aegypti*. The biolarvacidal activity assesses the value of LC50 dan LC90. The mortality rate of *Aedes aegypti* is observed 24 hours towards various formulas of granule holy basil leaf ethanol extract. The positive and negative control can be seen in Table 6 below

Table 6. The percentage of larva mortality rate

Formula	Means $\pm$ SD (%)	p-value (LSD post-hoc 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 granule has a potent biolarvicidal activity. This study suggests that a higher concentration of holy basil leaf extract will increase biolarvicidal activity. Table 6 also reports that the negative control does not have biolarvicidal activity. Gokulakrishnan *et al.*, 2015) suggest that the extract of methanol, ethyl acetate, and hexane of *Ocimum sanctum* Linn. has larvicide activity towards the larva *Culex quinquefasciatus* [35]. Furthermore, the LC50% and LC90% probability score is count based on the percentage of larva mortality rate. The probability score of LC50 and LC90 can 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 reports the probability value of larva mortality by LC50 4405,83 ppm ~ (0.44%) and LC90 6080,714 ppm ~ (0.608%). This percentage of larva mortality is caused by the active substances in the holy basil leaf extract. Moreover, Figure 2 shows that the biolarvicide activity will increase by the addition of holy basil leaf extract concentration in the granule.

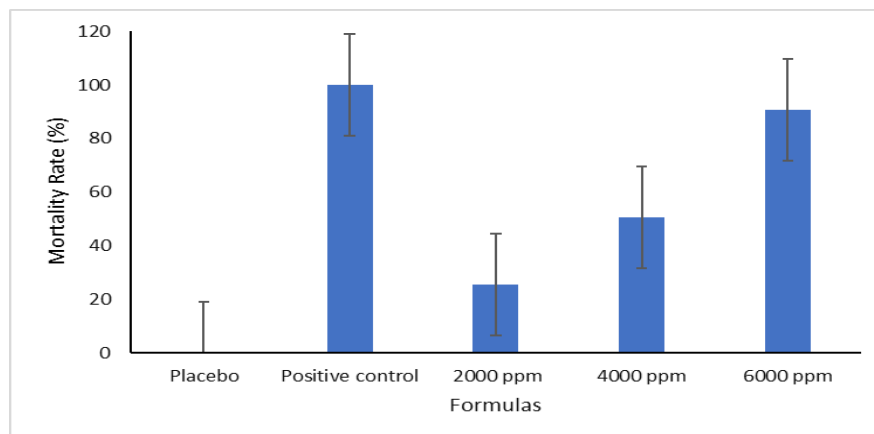


Figure 2. The mortality rate of 3<sup>rd</sup> larva instar of *Aedes aegypti*

Regarding the 24 hours observation towards the 3rd larva instar of *Aedes aegypti*, conclude that F3 (6000 ppm) has the highest activity of biolarvicide with a value of 90,67%, continued by the F2 (4000 ppm) by 50,67% and F3 (2000 ppm) by 25,33%. Table 4 shows a significant difference in the biolarvicidal activity in each formula ( $p < 0.01$ ). F3 has a higher activity of biolarvicidal compared to the other formulas. The biolarvicidal activity of holy basil leaf extract granule is influenced by several active substances such as terpenoid, alkaloid, saponin, flavonoid dan polyphenol. The mechanism of alkaloid as larvicide is caused by inhibition of acetylcholinesterase (AChE) which is responsible for nerve impulse transmission across a synapse. Terpenoid group could facilitate the transmission into membrane resulting in paralysis and death to the larva. The mechanism of saponin as larvicide is yet to be clearly understood. In addition, the main hypothesis of saponin may act as a repellent and toxic towards cells. The saponin properties inside water and organic solvent will damage the larva's cuticular membrane [36]. Flavonoid is also reported as larvicide by hindering acetylcholinesterase enzyme in the mosquito larvae to terminate the food supply and the larva growth [10]. The role of phenolic group as larvicide has also been suggested that phenolic fraction may influence the mosquito growth by reducing the fertility, fecundity, and lifespan of adult larva [37]. This is 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 biolarvicidal activity of granulated pharmaceutical formulation.

## CONCLUSION

Holy basil leaf ethanolic extract granule (*Ocimum sanctum*, Linn) consists of terpenoid, alkaloid, flavonoid, saponin dan polyphenol has a biolarvicidal activity with a value of LC50 4405,83 ppm and LC90 6080,714 ppm. Therefore, the granulated formulation of holy basil leaf extract could be further developed as a novel biolarvicide for managing the mosquito vector.

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## BIOGRAPHIES OF AUTHORS (10 PT)

	<p>Azis Iksanudin, is a lecturer in Faculty of Pharmacy, Ahmad Dahlan University, Yogyakarta Indonesia. His research interests are in the field of pharmaceutical technology, natural product, biomaterial, and drug delivery. He has many experience in conduct a researched granted from Higher Education of Indonesia and published some articles in Indonesian and scopus indexed International Journal.</p>
	<p>Lolita is a Assistant Professor of Ahmad Dahlan University, Yogyakarta, Indonesia. She is also a Ph.D candidate from Nanjing Medical University, P.R. China. She has published some scientific articles related to social pharmacy, community health in accredited national journal of Indonesia. Her research interest in the topic of clinical pharmacy, pharmacogenomics, community health, and epidemiology. She is also a reviewer in scopus indexed international journals.</p>
	<p>Zayyana Septya Ramadani is an Indonesian Pharmacist. He graduated from the Bachelor program at the Faculty of Pharmacy, Ahmad Dahlan University. Now, she works as Quality Assurance Officer at PT Phapros Tbk, a pharmaceutical company in Semarang Indonesia.</p>



# Larvicidal Activity of Granulated Pharmaceutical Products Using Indonesian Holy Basil Leaf Extract

Azis Ikhsanudin<sup>1</sup>; Lolita Lolita<sup>1</sup> and Zayyana Septya Ramadani<sup>1</sup>

<sup>1</sup>Faculty of Pharmacy Universitas Ahmad Dahlan, Special District of Yogyakarta Province, Indonesia

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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 had a larvicidal LC<sub>50</sub> of 4405.803 ppm and LC<sub>90</sub> 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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## Corresponding Author:

Azis Ikhsanudin,

Faculty of Pharmacy

Universitas Ahmad Dahlan

Jl. Prof. Dr. Soepomo, SH, Janturan Umbulharjo, Yogyakarta Province, Indonesia.

Email: [azis.ikhsanudin@pharm.uad.ac.id](mailto:azis.ikhsanudin@pharm.uad.ac.id)

## 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 LC<sub>50</sub> value of 1290.39 ppm and an LC<sub>90</sub> 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 developed 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 METHODS

### 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 extracts

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 3 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 FeCl<sub>3</sub> 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 concentration 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 amyllum, magnesium stearate, and PVP were the binder. The composition of granules forming consist of 50% active substances and 50% excipients, as seen in Table 1.

Table 1. The formula of holy basil extract granules

Ingredients	Placebo	F1	F2	F3
Extract ethanol of holy basil leaf (ppm)	0	2000	4000	6000
Lactose	45%	45%	45%	45%
Amyllum	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 Granule

##### a. Moisture content

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 granule is 3-5% [21].

##### b. Flow time

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].

##### c. Dispersion time

400 mg of granules were poured into a glass, and then approximately adding 1000mL of water. The time needed for the granule to be dispersed entirely was recorded. A good dispersion time of granules was less than 5 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 concentration of 0,01%. Replication was done as much as three times for each treatment group. Observation was done after 24 hours by analyzing the percentage of larva mortality rate [24].

### 2.3. DATA ANALYSIS

Data was analyzed by applying the ANOVA method with a 95% significance level. Moreover, all statistical analyses were performed to determine the value of the 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 LC<sub>50</sub> and LC<sub>90</sub> values.

## 3. RESULTS AND DISCUSSIONS

We have succeed 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 larvicide activity of holy basil leaf granules extract could be seen in the LC<sub>50</sub> dan LC<sub>90</sub> score. The detailed findings related to the physical characteristics and larvicide 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 terms of 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 were terpenoids, alkaloids, saponins, flavonoids dan polyphenols, as seen in Table 2. A previous study demonstrated that alkaloid and terpenoid substances could act as larvicide [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 reported that holy basil leaf extract consists of terpenoids, proven by the colour change into dark green. These change was caused by adding a concentrated sulphuric acid in 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<sup>+</sup> of potassium tetraiodomercurate (II) to produce potassium-alkaloid complex [27]. The existence of 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 C3 [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 dark green after adding FeCl<sub>3</sub>. This mechanism was caused by the interaction between the Fe<sup>3+</sup> 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 in terms of 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 has 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 test result could be seen in Table 3.

Table 3. Moisture content test result

Parameter	Means ±SD (%)				p-value
	Placebo	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 reported 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 showed 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 in order to maintaining 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 influences the process of the bonding mechanism. The good moisture content of granules ranges from 2-5% [32]. In this study, the moisture content test of granule has met the standard requirements.

Flow time is a vital parameter requirement of the granules, affecting the volume uniformity in the filling and packaging process. A reasonable flow time requirement is 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)	p-value			
		Placebo	F1	F2	F3
Placebo	0.50 ± 0.0058	-	0.000*	0.000*	0.000*
F1	1.43 ± 0.0170	0.000*	-	0.000*	0.000*
F2	1.53 ± 0.017	0.000*	0.000*	-	0.000*
F3	1.57 ± 0.023	0.000*	0.000*	0.030*	-

Note: \* p value less than 0.05

Table 4 indicated that F3 has a longer flow time compared to F1, F2, and placebo. This result concluded that more extract concentration in the granule will result in a longer flow time [34]. Magnesium stearate was used as a lubricant in the granule formulation process. This lubricant characteristic has cohesive properties and could increase the flow time by preventing dust powder during the filling process. LSD post hoc test showed a significant difference in flow time in each group of formulas ( $p < 0.01$ ). Each physical characteristic of the extract influences 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 may result in a higher amount of active substance in the extract, affecting larvicide activity. The dispersion time test of granules could be seen 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 $\pm$ 0.0573	1.13 $\pm$ 0.0576	1.07 $\pm$ 0.0583	1.07 $\pm$ 0.115	0,344

Table 5 reported that the increase of holy basil leaf extract concentration in the granule will result in a faster dispersion time. The statistic test result with LSD post hoc indicated no significant differences in dispersion time between each formula ( $p > 0.005$ ). It concluded 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 an equal amount of amylum which causes the dispersion time was not statistically significant differences.

### 3.3 Larvicidal Activity of Holy Basil Leaf Ethanol Extract Granule

The larvicidal activity test of granulated formulation of holy basil leaf ethanol extract was based on the standard of WHO by using third larval instar of *Aedes aegypti*. The larvicidal activity assessed the value of  $LC_{50}$  dan  $LC_{90}$ . The mortality rate of *Aedes aegypti* was observed 24 hours towards various formulas of granules holy basil leaf ethanol extract. The positive and negative control could be seen in Table 6 below.

Table 6. The percentage of larva mortality rate

Formula	Means $\pm$ SD (%)	p-value (LSD post-hoc 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*	-

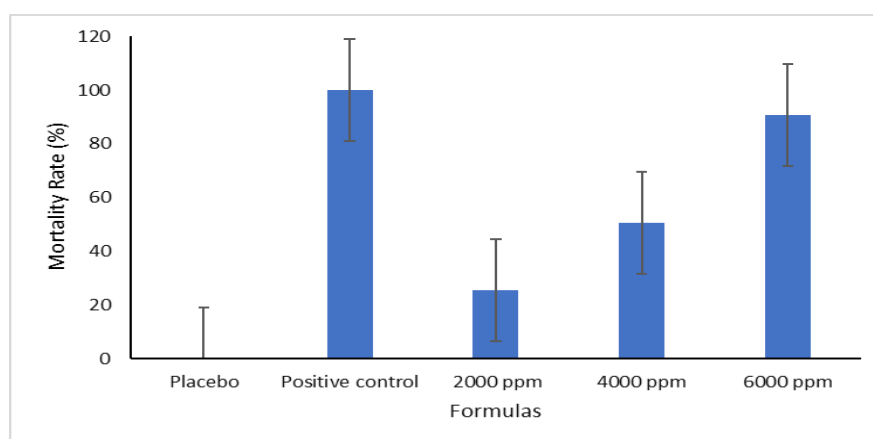
Note: \* p value less than 0.05

Table 6 concluded 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 reported that the negative control does not have larvicidal activity. Gokulakrishnan *et al.*, suggest that the extract of methanol, ethyl acetate, and hexane of *Ocimum sanctum* Linn. has larvicide activity towards the larva *Culex quinquefasciatus* [35]. The  $LC_{50}$  and  $LC_{90}$  probability score was counted based on the larva mortality rate percentage. The probability score of  $LC_{50}$  and  $LC_{90}$  could be seen in Table 7.

Table 7. Probability score of LC<sub>50</sub> dan LC<sub>90</sub>

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 reported the probability value of larva mortality by LC<sub>50</sub> of 4405,803 ppm ~ (0.44%) and LC<sub>90</sub> 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 2 showed that the larvicide activity will increase by adding holy basil leaf extract concentration in the granules.

Figure 2. The mortality rate of third larval instar of *Aedes aegypti*

Regarding the 24 hours observation towards the third larval instar of *Aedes aegypti*, concluded 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 4 showed a significant difference in the larvicidal activity in each formula ( $p < 0.01$ ). F3 had 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, flavonoids dan polyphenols. The mechanism of alkaloids as larvicide was caused by inhibition of acetylcholinesterase (AChE) 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 saponins 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 in order 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 the mosquito growth by reducing the fertility, fecundity, and lifespan of adult larva [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.

## CONCLUSION

Holy basil leaf ethanolic extract granule (*Ocimum sanctum*, Linn) consists of terpenoids, alkaloids, flavonoids, saponins dan polyphenols with a larvicidal activity value of LC<sub>50</sub> 4405,803 ppm and LC<sub>90</sub> 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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