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PHYSICAL TEST AND LETHAL CONCENTRATION 95 (LC_{95}) ASSAY OF KAFFIR LIME SKIN GRANULE EXTRACT (*Citrus hystrix* DC.) ON *Aedes aegypti* MOSQUITO LARVAE AS A NEW CANDIDATE BIOINSECTICIDE

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ABSTRACT

Indonesia needs to be encouraged by the discovery of new bioinsecticides substituting synthetic insecticides, which have posed a danger to human survival. One of the plants that have the potential to become bioinsecticides is kaffir lime (*Citrus hystrix* DC). On the skin's kaffir lime contains chemical compounds such as secondary metabolites and essential oils. The most considerable and largest content of kaffir lime essential oil is limonene or limonoid. This compound is a member of monoterpenes which has vigorous insecticide activity. Limonene/limonoid act as stomach poisons of mosquito larvae. Limonene/limonoid enters the body of the larvae through immersion of the consumed extract concentration. This research aims to determine the right formulation and specifications through physical properties test and determine the lethal concentration 95 (LC_{95}) of kaffir lime granule extract (*Citrus hystrix* DC) against *Aedes aegypti* mosquito larvae so that it meets the requirements as a new bioinsecticide. Physical tests carried out include tests of water content, flow time test, moisture content test, indexing, and fragility test. Based on the results of formulations and specifications through physical properties test, the water content of granules is 0.80-1.40%, the flow time is 7.00, the moisture content is 3.40, the index is 7.11, and the fragility is 0.2. While the results of probit analysis obtained the value of LC_{95} of kaffir lime skin extracts (*Citrus hystrix* DC.) for 24 hours of bran is 7915.85 ppm with a lower limit of 6802.33 ppm and an upper limit of 9718.21 ppm.

Keywords: *Aedes aegypti*; granules; lethal concentration; lime; physical test.

INTRODUCTION

Vector infectious disease becomes Indonesian common health problem, especially when infected by mosquitoes [1]. One of these dreadful diseases is dengue hemorrhagic fever (DHF), which is vectored by *Aedes aegypti* mosquitoes. From 1968 to 2009, Indonesia recorded as a country with the highest DHF cases in Southeast Asia. At present, *Aedes aegypti* mosquitos causing dengue fever is a challenge that must be immediately addressed strategically, nationally, and comprehensively by all elements of Indonesian. Dengue prevention and eradication programs can only reduce mortality rates but not morbidity rates. The number of DHF sufferers from year to year tends to increase, so does its spread [2].

Nowadays, synthetic insecticide is still the primary choice in efforts to eradicate mosquito nests because it is considered more effective and relatively short in term [3]. The insecticide that popularly used in Indonesia is abate. Abate has been used in Indonesia since 1976, and in 1980 abate or temephos 1% was established as part of the *Aedes aegypti* mosquito eradication program [4]. The insecticide used lately to eradicate *Aedes aegypti* that have been caused a wider spread of DHF and more death as its result. Indonesia needs to be spurred on by the discovery of new bioinsecticide substitutes to synthetic insecticides which pose a horrible danger to human survival. Therefore, an effort is needed to obtain more environmentally-friendly natural larvicides.

One of the plants that potentially become natural larvicide is kaffir lime [5]. The kaffir lime peel consists of active compounds whose essential oils are in it [6]. Its essential oil contents of limonene, linalool, terpinene-4-ol, gamma-terpinene, and isopulegol. The largest content of it is limonene or limonoid [7]. Limonene or limonoid is a group of monoterpenes that have strong insecticide activity and can inhibit Acetylcholinesterase (AChE) activity. Inhibition of AChE activity are a big cause of death and paralysis against insects due to the blocking of nerve signal transduction [8]. The content of limonene/limonoid in kaffir lime essential oil acts as poison-like to abdomen and gets into the body of the larvae through an immersion of inedible extract concentration. Limonene/limonoid caused disintegration to the digestive tract at the abdomen and made mosquito lack energy to live [9].

This research aimed to determine the right formulation, specification and granule lethal concentration 95 (LC_{95}) of kaffir lime fruit extract against *Aedes aegypti* mosquito larvae to meet the requirements as a new bioinsecticide. In detail, the purpose of this study is to ensure the results of various tests including water content test, flow time test, moisture content test, tapping index, friability test, and granule lethal concentration 95 (LC_{95}) of kaffir lime fruit extract against *Aedes aegypti* mosquito larvae so that it is perfectly established as a new natural bioinsecticide.

MATERIALS AND METHODS

This type of research is an experimental laboratory using a completely randomized design. There are two tests conducted in this study, the physical properties test of the granules and lethal concentration 95 (LC_{95}) test. The testing methods of physical properties granules extract of lime (*Citrus hystrix* DC.) are as follows:

Granular Flow Speed

Twenty five grams of granules are put into a funnel whose underneath has been covered previously. We pulled the lid to close the bottom of the funnel while turning on the stopwatch. Also, we took note of the duration for all the granules to move downward [10].

Water Absorption

Absorption test equipment is connected to an electrical scale with an ampoule filled by water on it. Then, the surface is flushed using water in the absorption test tube (following the U tube principle). The ampoule position is arranged in such a way in the order it is in contact with the capillaries connected to the material. Then the filter paper is placed on the surface of the absorption test tube while the remaining water around it is cleaned. The material to be tested is placed on the filter paper and the scale is set at zero [11].

The decrease of air in the ampoule on the scale indicates the amount of air absorbed by the

material. The observations were carried out for 15 minutes with 300 mg test material which results determined as the air velocity produced per time unit. The wet granules obtained were weighed every 20 grams for six times and dried in the petri dish for 15, 30, 45, 60, 90 minutes and 3 days. After granules dried, they removed from the drying cupboard and allowed it to cool. Dry granules and cups are weighed again to find out dehydrated granules' weight and calculate the dry shrinkage of the granules at each drying time.

Granule Fragility

Thirty grams of granules put into a multilevel sieve with the top sieve number 30 is Mesh and the lowest is pan. A sieving machine did a sifting. The engine starts at 50 amplitudes for 30 minutes. The formula to calculates the fragility of the granules bellow:

$$Fragility = \frac{\text{first weight} - \text{remain weight}}{\text{first weight}} \; x \; 100\%$$

The Average Diameter of Granules

25 grams of granules are weighed and put in a stratified sieve arranged from rough to smooth, i.e., number 14, 16, 20, 30, 50 Mesh and pans. Sifting was carried out by a filter machine which was run 50 amplitudes for 15 minutes. The granules left in each sieve are weighed and the percentage is calculated, the formula calculates the average diameter ($\bar{r}$) of granules bellow:

$$\bar{r} = \frac{\text{the hole large everages x \% left}}{100}$$

Determination of the Optimum Formula

The optimum formula is obtained by input the results of the flow velocity, absorption, humidity and granule tapping index as a response into the Design Expert using a factorial design method with a numeric approach. The contour plot is made to obtain the value of β_0, β_a, β_b, β_{ab} based on the equation of each parameter. All contour plots put together (superimposed) so that the optimum area finally obtained by wondered the physical properties of the granules.

$$Y = \beta_0 + \beta_a X_a + \beta_b X_b + \beta_{ab} X_{ab}$$

The test procedure of granules lethal concentration 95 of kaffir lime extract (*Citrus hystrix* DC.) begins by pointing on the research variables. The independent variable of this study is any serial granule concentrations of kaffir lime peel (*Citrus hystrix* DC.) 250 ppm, 1500 ppm, 2750 ppm, 4000 ppm, 5500 ppm, and 7000 ppm. While the bounded variable is the mortality of *Aedes aegypti* larvae for 24 hours. The control variables in this research were larvae, aquades, and laboratory conditions. The tested larvae used were final instar III larvae to the initial instar IV larvae. This research was conducted in Toxicology Sub Lab of Faculty of Teacher Training and Education, Jember University.

The test is carried out in 2 stages, a preliminary test and a final test. The preliminary test aims to obtain concentrations that can kill 5% and 95% of tested larvae. The preliminary test is carried out without repetition. The results of this preliminary test will be used to determine the serial concentration that will be used in the final test. While 4 repetitions performed of each in the final test. Mortality percentage of *Aedes aegypti* mosquito larvae due to the toxicity of kaffir lime (*Citrus hystrix* DC.) extract calculated using the Abbot formula. Then proceed with a normality test and homogeneity test using the SPSS application. While the determination of LC_{95} value from the concentration of kaffir lime peel extract (*Citrus hystrix* DC.) for 24 hours using Probit analysis. The software used is Minitab 18 for Windows.

RESULTS AND DISCUSSION

Granule Moisture Content

The granules of kaffir lime peel extract (*Citrus hystrix* DC.) are amorphous of shape, brownish of color and 40 mesh of size showed in Fig. 1. The drying process carried out at 40°C for 12 hours. Granule water content is 0.80-1.40% showed at Fig. 2. A number of granules are put into a tilted measuring cup and then enforced. The granules added to a volume of 100 ml, recorded as V_0. A measuring cup is attached to the instrument then, the rotor ignited. Note the volume change in minutes 5, 10, 25, 50 and 100, recorded as V_t. If a constant volume has not reached then continue until a constant volume obtained, recorded as V_c.

T% is calculated (after obtaining a constant volume) and the weight of the used granule is recorded [12] The index can be calculated using the formula bellow:

$$i = \frac{V_0 - V_t}{V_0} \; x \; 100\%$$

Drying on the granule is intended to reduce the water content in the ingredients. High water content in a material can encourage enzymatic reactions that cause chemical changes. The changes in chemical composition, especially in active compounds can reduce effective toxicity. Besides, high water content is best growth medium for microorganisms or fungi that can contaminate the material [13,14].

Fig. 1. Kaffir lime peel (*Citrus hystrix* DC.) granule extract

The granule flow time is visible by looking at the number of granules flowing every second. Granules which have uniform weights and consistent active ingredients are the characteristic of a good flow granule. A good granule flow time is less than 10 seconds for 100 grams granules [15]. Particle shape, particle size, surface conditions, and moisture of the granule affect the granule flow time. A larger granule usually has better flow times because the cohesive force will be smaller and not be lumpy easily [16]. The research results showed in Fig. 3 that flow time values in the three formulas met the requirements of good flow time criteria. These results explain that a low heating temperature will respond to a longer granule flow time and vice versa [17].

The situation is related to the moisture contained in the granules. A low temperature exposure will cause the water content in the granules is less evaporated than a higher temperature exposure. High temperatures will create more evaporation effects on the water contained in the granules. The greater granules moisture content will provide a longer response because the bond among granule particles is stronger. However, the stronger cohesiveness of granule makes it more difficult to flow [17,18].

The factors that can affect the flow time of granules are 1) particle shape and texture, for equidimensional particles (round, cube) the larger its diameter the better its flow characteristics. At the same time, the results can be different for anisomeric particles. The best flow properties occur at the particles optimum diameter (200-500 µm). The particle less than 100 µm will be more cohesive. Particles or granules that have a small friction force will more easily flow, whereas the bigger the friction force the more its particles or granules is difficult to flow; 2) Porosity, granules with a large porosity will have better flow time because the contact among granule particles will be smaller; 3) Moist content, large contact surface area of granule particles will increase if the moisture content of granules is high which causes bonds of particles is stronger. It causes the granules will be more difficult to flow because the tensile force between granule particles is getting stronger [19]. Suitable granules criteria of time flow could gain many things to improve the granular flow velocity. The things that can we do are adding gluten, anti-adherent, and lubricant.

Glidant is a substance used to improve granules' flow from the hopper to the feeder and even into the mold hole, so that the mass can fill the die in a uniform amount. Several mechanisms of glidant action are the dispersion of electrostatic charge on the surface of the granule, the distribution of the glidant during granulation, the adsorption of a gas on the gliding opposite to the granulation, minimizing the van der Waals force by separating the granules, reducing friction between particles and reducing the rough surface of the granules by attaching glidant during granulation [20]. Amylum is the most popular glidant because it can be functioned as glidant as well as disintegrant, with concentrations up to 10% [21].

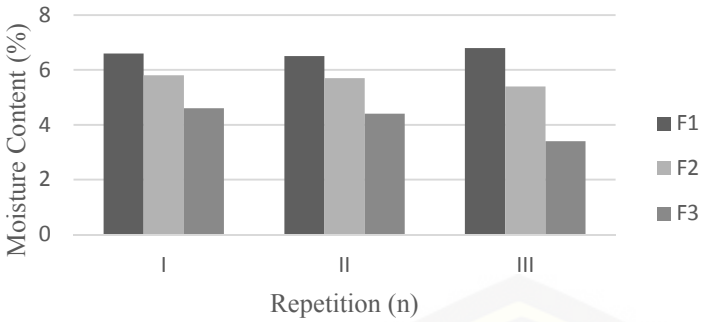

Fig. 2. Granule moisture content

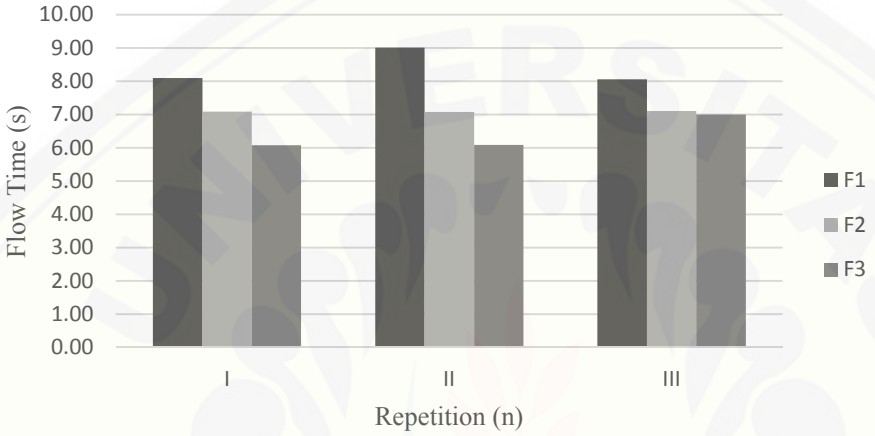

Fig. 3. Granule flow time

Anti-adherent is a substance that is used to avoid granular mass pasting on punch and to reduce sticking to the mold wall. The indispensable ingredients of substances that are easily affixed is vitamin E. Magnesium stearate and corn starch are two examples of good anti-adherent ingredient. Lubricant is a substance used to reduce friction between the granules and mold wall. It can be used in two operations such as fluid lubrication and boundary lubrication. Liquid lubrication works by using both granule and wall surfaces. While boundary lubrication works because of the sticking of a molecular part that has a long carbon chain to the metal surface of the molded wall. Since its appearance looks stronger, this displacement is better than fluid lubrication. In general, 200 mesh or finer is added, and is able to pass through 100 mesh filter (from nylon) before being added to the granulation wall [22].

Lethal Concentration 95 (LC_{95}) Test

Based on our preliminary test results showed in Fig. 4, only obtained concentration that can kill 5% and 95% of tested larvae for 24 hours is 250 ppm and 7000 ppm. At a concentration of 250 ppm, one larva died, while at a concentration of 7000 ppm, 19 from total of 20 tested larva died. The results of this preliminary test serve as a reference in determining the concentration series that will be used in the final analysis.

Based on our previous finding, we decided to use the serial concentrations used in the final analysis are 250 ppm, 1500 ppm, 2750 ppm, 4000 ppm, 5500 ppm and 7000 ppm. In the final analysis, 4 repetitions were performed for each treatment so that our finding more valid.

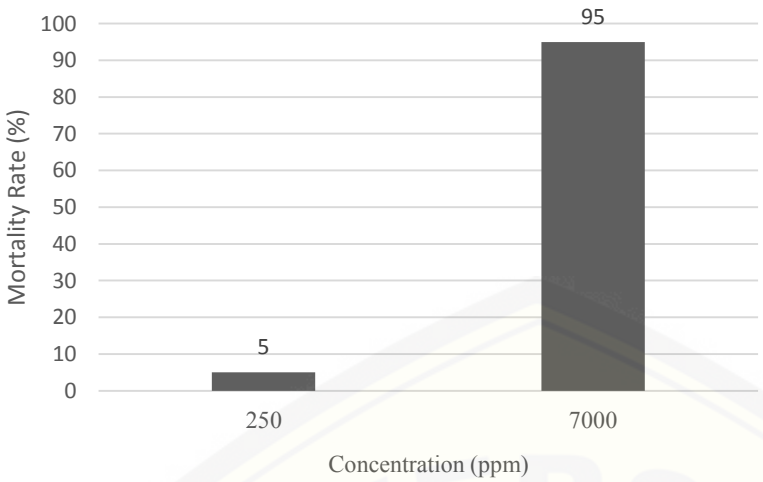

Fig. 4. Mortality (%) of *Aedes aegypti* mosquito larva in the preliminary test using granule extract of kaffir lime peel (*Citrus hystrix* DC.) for 24 hours

Fig. 5 showed us the higher granule concentration of kaffir lime extract (*Citrus hystrix* DC.) increases the average mortality rate of *Aedes aegypti* larvae. The lowest *Aedes aegypti* larvae mortality was at 250 ppm with a mean and standard deviation of mortality 7.5 ± 2.89. In comparison, the highest *Aedes aegypti* larvae mortality was at a 7000 ppm with a mean and mortality standard deviation 96.25 ± 2.50. At concentrations of 1500 ppm, 2750 ppm, 4000 ppm and 5500 ppm, respectively the mean and standard deviation of mortality are 30 ± 4.08, 30 ± 4.08, 43.75 ± 4.79, 70 ± 9.13, 85 ± 4.08, and 96.25 ± 2.50.

We also conducted probit analysis, it showed the LC_{95} value of kaffir lime peel (*Citus hystrix* DC.) extract for 24 hours was 7915.85 ppm with lower limit 6802.33 ppm and upper limit 9718.21 ppm (Table 1). To find out the value of LC_{95} granule extract of kaffir lime skin, the tested larva were put into a container that had been poured granule of kaffir lime fruit extract with a particular concentration for 24 hours. The toxic effect

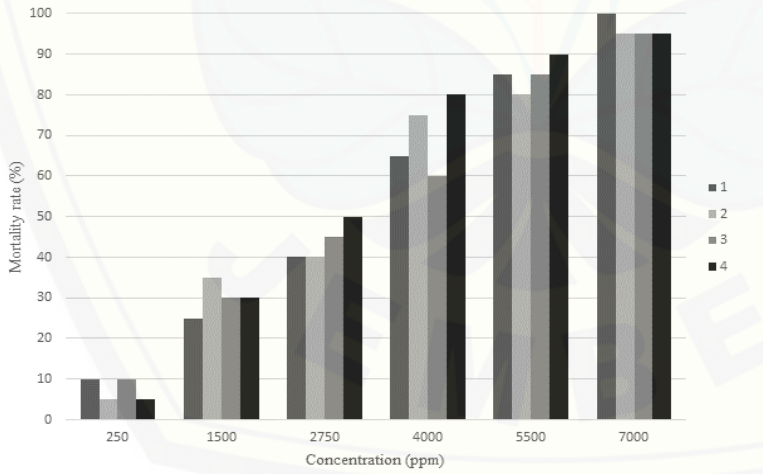

Fig. 5. Mortality (%) of *Aedes aegypti* mosquito larva in the final test using granule extract of kaffir lime peel (*Citrus hystrix* DC.) for 24 hours

Table 1. The probit analysis of kaffir lime peel (LC_{95}) granule extract against *Aedes aegypti* mosquito larvae

Lethal concentration LC_{95}	LC_{95} (ppm)	Upper limit (ppm)	Lower limit (ppm)
Granules of kaffir lime peel (*Citus hystrix* DC.) extract	7915,85	9718,21	6802,33

caused by a substance depends on the concentration of the substance and the length of exposure time. Our finding slightly differs to other research conduct at Surabaya, Indonesia. They used non-polar and polar extraction for leaf kaffir extract. Leaf kaffir polar extract fraction showed LC_{90} against *Aedes aegypti* at 3180 ppm [23]. This finding showed more than half the amount less than our findings. We analyzed this vary because they use different extraction approaches and different parts of kaffir lime. Based on this finding, we evaluate that kaffir lime extract (*Citrus hystrix* DC.) is toxic to *Aedes aegypti* mosquito larvae.

Kaffir lime peel contains of essential oil such as limonene, linalool, terpinene-4-ol, gamma-terpinene, citronellyl propionate and isopulegol. These essential oils potentially become a great repellent [7]. These compounds can affect the physical condition and metabolism of mosquito larvae, also can be used to kill Aedes aegypti mosquito larvae. The largest content of kaffir lime essential oil is limonene. The compound is a part of monoterpenes which has strong insecticide activity. In addition, limonene has AChE inhibiting activity such the mechanism of nerve signal transduction blocking that can cause of paralysis and even death against insects [3].

CONCLUSION

Our finding concludes kaffir lime peel extract (*Citrus hystrix* DC.) had effective active compounds to eradicate *Aedes aegypti* larva. The best concentration for killing mosquito larva is 7914.85 ppm. Based on this result, we suggest to conduct further research by isolating and identifying toxic chemical compounds from *Citrus hystrix* DC skin extract granules against *Aedes aegypti* larvae as a vector of Dengue Hemorrhagic Fever. So that it can be known what the toxic compounds are. Further research to discover toxic chemical compounds and can be mass-produced easily, cheap with high efficiency also does not damage the environment.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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