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Ovicidal, Larvicidal and Pupicidal Activities of Essential oils of *Citrus hystrix* againts *Aedes aegypti* Based on Nanoemulsion Technology

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Abstract

Aedes aegypti mosquito is a *dengue* virus vector that is known to be very dangerous to humans. *Dengue* virus is a global health problem because this attack caused various diseases and caused as many as 100,347 DHF patients with the number of deaths 907 people. Various efforts to control these mosquitoes have been done, but the effort is not effective because of lack of awareness and ignorance of the community, In addition, the chemicals used so far are not environmentally friendly. Therefore, the control of mosquitoes using natural ingredients such as essential oil of lime peel is a wise choice in mosquito control. This study aims to analyze the content of *linalool* compounds in essential oil of lime peel and the activity of *linalool* compound from essential oil of kaffir lime based on nanoemulsi technology to eggs, larvae and pupa *Aedes aegypti.* The method used in this research is the extraction of essential oil with steam distillation, *GC-MS*, nano technology with sonication process, ovicidal test, larvicidal and pupacidal. The results showed that the highest ovicidal test results were at 1000 ppm (92.8±0.83), larvicidal test at 250 ppm (21.6±1.67) and the highest pupacid test at 1000 ppm (33.33±1.67). The results show that nanoemulsi essential oil of kaffir lime peel has activity of ovicidal, larvacidal and pupicidal. Nanoemulsi essential oil of kaffir lime is more environmentally friendly compared to abate. The results of this study can be recommended for control systems only on *Aedes aegypti* mosquitoes.

Keywords: technology · nanoemulsion · larvacidal · *Aedes aegypti*

1. Introduction

Dengue virus is one of the dangerous viruses that can cause dengue fever, yellow fever, malaria, filariasis, and various types of encephalitis.[1] The virus can be transmitted to humans through *Aedes aegypti* mosquitoes. According to Nugroho[2] DHF can spread to all places contained *Aedes aegypti* mosquitoes. This easy spread causes dengue disease to be very high.

One of the causes of the high number of people with DHF is the lack of awareness of the community in preventing the spread of this *dengue* virus. In 2014 the number of dengue fever patients reported as many as 100,347 cases with the number of deaths as many as 907 people.[3] Therefore, the prevention of DHF is required by controlling the breeding of mosquitoes carrying this dengue virus.

DHF efforts have been undertaken, including: prevention, discovery, relief and reporting, epidemiological investigations and observations of dengue hemorrhagic fever, countermeasures as necessary, other mitigation and extension.[4] However, due to lack of awareness from the community and public ignorance about DHF disease so that mosquito prevention is less than the maximum. Therefore, the need for a control that is easy to understand and powerful in controlling the *Aedes aegypti* mosquitoes.

Mosquito control systems have been done using chemicals. Chemicals are not environmentally friendly and may harm other living things. Therefore, it needs environmentally friendly control technology. Essential oils are volatile substances that have bioactive compounds and potentially fight against insects in terms of fecundity, male sterility, larvacidal, ovicidal, and oviposition activity.[5] Kaffir lime is one of the plants that contain *linalool* compounds, especially on the peel of the fruit.

The process of making essential oil used a steam distillation apparatus. To make the evaporation process run quickly, the peel of kaffir lime was chopped first before it was inserted in a steam distillation apparatus. Essential oil of kaffir lime peel produced will be made nanoemulsion. Essential oil from citrus plant is very environmentally friendly and not toxic for non target animals.[6]

Nanoemulsion is one of the technological developments that utilize the properties of nano-sized molecules with emulsion form. Nanotechnology is one solution to economic problems and also environmentally friendly.[7] Nanoemulsion has droplets of various sizes ranging from 100 - 600 nm.[8]

The small particle size of hope can have faster, deeper and more effective absorption. The transfer of nanoparticles has received great attention in recent years.[9] The making of nanoemulsion from the essential oil of kaffir lime peel is done by using the oil method in water from Sugurmar *et al.*[10]

Material and Methods

Material

Kaffir lime obtained from Ungaran and identified in Biology Laboratory State University of Semarang. Rearing of mosquitoes is done to meet the needs of testing eggs, larvae and pupae. Eggs used are fresh eggs, larvae used are instar larvae III and pupa used is fresh pupa.

Mosquito Rearing

Rearing of Aedes aegypti mosquitoes is done by the method of Kumar *et al*.[11] Mosquitoes were reared by inserting albino mice into mosquito coils. Female mosquitoes will suck the blood of albino rats for egg maturation. Rearing of *Aedes aegypti* mosquitoes was done at Biology Laboratory of Semarang State University at temperature of 28 ± 1 ° C, humidity 80 ± 5% RH, and lighting 14: 10 L / D photoperiod. Mosquitoes were reared by inserting albino mice into mosquito coils. Female mosquitoes will suck the blood of albino rats for egg maturation. Cup was inserted into the cage as a place to spawn female mosquitoes. This cup was used as an ovitrap. Ovitrap for *Aedes aegypti* mosquito L. was given what filter paper on its side so that the egg will be attached to *whatman* filter paper The resulting eggs were sterilized in containers containing de-chlorinated water. Hatching eggs will become larvae Larvae was fed until these larvae grow into third instar and larvae pupae.

Ekstraction of Essential Oils

Kaffir lime peel was washed with aquades water and dried in the shade place at room temperature. Kaffir lime peel, which had been dried was distilled steam. The resulting volatile oil was stored at 4°C.[12]

Gas Chromatography – Mass Spectroscopy

GC-MS used Shimadzu QP 5050 which was a mass spectrometer system equipped with capillary column BPX 5 (film trapping 30 m x 250 m, 0.25). Helium was used as carrier gas. MS operating conditions were: ionization induced by electron (EI) at 70 eV, ion source 250°C. These compounds were identified by conducting library searches using Shimadzu NIST / EPA / NIH + mass spectral databases.[13]

Nanoemulsion Preparation

The making of nanoemulsion of essential oil of lime peel used method of oil in water.[10] Crude emulsion was prepared at 16.66% oil proportion : 16.66% of tween 20 : 66.68% of water in (v / v) made by stirring it using a magnetic stirrer at 250 rpm for 10 minutes. The next process was ultrasonic emulsification using 20 kHz sonicator (Ultrasonics, USA). The sonicator with a 13mm probe diameter was immersed in a crude emulsion. The temperature difference between the initial crude emulsion for the final nanoemulsion should be less than 10°C. The final nanoemulsion was stored in a glass beaker containing ice to reduce the heat generated during the ultrasonic emulsification process.

Analysis of Nanoemulsion Size

Particle size of essential oil of lime peel nanoemulsion can be determined by using electronmicroscopy transmission (TEM). A drop of nanoemulsion was placed on a copper grid and was allowed to dry in a vacuum. Transmission of electron micrograph used Tecnai G-10 (Philips) instrument, 80 kVTEM with W-search and ultrahigh-resolution piece pillar with point-to-point resolution of 1.9 Å[14]

Ovicidal Test

Experimental protocol was performed according to Elango *et al.* [15] With modifications from Kamakshi *et al.*[16] Nanoemulsion of essential oil of kaffir lime peel and abate was prepared with various concentrations (62,5, 125, 250, 500 and 1000 ppm). A total of 25 eggs were inserted into the cup containing nanoemulsion solutions of the essential oil of kaffir lime peel and abate. The controls in this study were water without treatment.

The egg hatchability was assessed 96 hours after treatment. The experiment was done three times and each treatment was repeated five times. Ovicidal activity was assessed based on the mortality rate of eggs (EMR) using the formula below.

$$\text{EMR} = \frac{jumlah\ telur\ yang\ tidak\ menetas}{total\ telur\ yang\ diletakan}\ x\ 100$$

Data were analyzed using one way anova and followed by Duncan's multiple range test (DMRT). DMRT was performed to determine the difference in egg mortality rate between concentrations.

Larvacidal Test

The experimental protocol was conducted according to WHO.[17] Nanoemulsion kaffir lime peel and abate prepared at concentrations of 50, 100, 150, 200, and 250 ppm. Next, nanoemulsion and abate were tested on 25 instar larvae III of *Aedes aegypti*. The control in this study was 250 ml of water.

The mortality rate was recorded after 24 exposures. The larva was considered dead if the larvae cannot move and move the shipon on the surface of the water. The experiment was done in fivefold. The data obtained were analyzed statistically and significantly when showing differences with the control. analyzed using DMRT (Duncan's Multiple Range Test) ($P < 0.05$).

Pupicidal Test

A total of 10 pupa of *Aedes aegypti* L. were inserted into nanoemulsion solution of essential oil of lime peel and abate with various concentration of 500 ml each. The control of this study was 500 ml of water. Pupa was considered dead when it cannot move when shoveled many times with a soft brush. The mortality of each pupa was calculated after 24 hours. The data were analyzed using the Abbott formula[18] and analyzed using DMRT (Duncan's Multiple Range Test) ($P < 0.05$).

RESULT AND DISCUSSION

Essential oil is a volatile compound containing various chemical compounds. Some of the chemical compounds contained therein are compounds that have insecticidal activity. In addition, chemical compounds in natural ingredients such as essential oil of kaffir lime skin are environmentally friendly or toxic for target and non-toxic animals for non target animals. Chemical compounds in essential oil of kaffir lime peel can be known through Gas Chromatography - Mass Spectroscopy (GC-MS) test. The results of GC-MS essential oil of lime peel can be seen Figure 1.

Based on the results of the chromatogram in Figure 1. It shows that there are 18 peak points which means that in the essential oil of orange peel there are 18 kinds of chemical compounds. A total of 18 compounds are then compared with data library Wiley and NIST (*National Institute of Standart and Technology*). Chemical compounds present in the chromatogram ie the first peak to 18 can be seen in Table 1.

Based on Table 1, it is known that the essential oil compound of kaffir lime peel consisted of 18 compounds. Senyawa kimia utama dalam minyak atsiri kulit jeruk purut diantaranya *beta-pinene, limonene, 3-Cyclohexen* dan *6-Octenal,3,7-dimethyl.* Subsequently followed by the compound *Gamma-terpinene, Alpha-terpinene, Alpha-pinene, Cis-linalol oxide* dan *(+)-2-Carene* dan sisanya yaitu senyawa *Napthalene, Beta-myrcene, Benzene, Copaene, Bicyclo 3.1.0 hex-2-ene, Caryophyllene, Alpha-phellandrene, Champhene* dan *9-Octadecenoid acid.*

The pattern of fragmentation of peak compound 11 (Cis-linalol oxide) can be seen in Figure 2. which shows that the 11th compound shows the peak of M-11 (m / z 59). The *cis- linalool-oxide* compound ($C_{10}H_18_{O2}$) was seen at retention time of 11.142 minutes and with molecular weight of 170. The presence of *cis-linalol oxide* compounds in volatile oil was 2.53%. The cis-linalol-oxide compound was one of the compounds to be utilized as the active ingredient of *Aedes aegypti's* control.

In this study, the essential oil of kaffir lime skin was made into nanoemulsion. TEM test results showed that nanoemulsion of essential oil of kaffir lime peel had a size of ± 203,75nm. According to Solans *et al.*[8] the essential oil nanoemulsion size ranges from 100nm - 600nm. Based on this, nanoemulsion of essential oil made from kaffir lime peel was successfully synthesized into nanoemulsion which will be applied to control *Aedes aegypti* mosquito.

The effect of nanoemulsion of essential oil of kaffir lime peel and abate with variation of concentration is showed in table 2. Based on table 2, it is known that nanoemulsion of lime peel oil at

the concentration of 1000 ppm showed the percentage of mortality of 92,8 ± 0,83. The lowest mortality at a concentration of 62,5 ppm was 85,6 ± 1,14. The higher the concentration, the higher the mortality.

This ovicidation test showed no difference between the concentration and between the solutions. This can happen by several factors. According to Subekti,[19] environmental factors affect the life of insects, such as temperature, humidity, light intensity and altitude. In addition, the pH of water also affects the development of eggs, larvae and pupa mosquitoes.[19]

Water temperature and less than optimal space can affect the development of eggs. Water and space temperatures at the time of the tests were 24 ° C and 29-31 ° C, respectively, while the optimal water and space temperature ranged from 27-32 ° C[20] dan 20-30°C.[21] The humidity of the test chamber is about 68% while the optimum humidity is at 80-90.5%.[22] Then for the pH of water is still in optimal condition that is 8 because the optimal pH of water ranges from 6-8.[20]

In addition to environmental factors, another factor that can affect is the quality of the eggs tested. The eggs in the test were taken randomly so the quality of the eggs was unknown, while the eggs issued by mosquitoes were not all of good quality. *Aedes aegypti* eggs can hatch about 80% of all total eggs released.[23]

The effect of nanoemulsion of essential oil of kaffir lime peel and abate with variation of concentration tested was presented in table 3. From table 3 it can be seen that the percentage of mortality of nanoemulsion of essential oil of kaffir lime peel was at the concentration of 250 ppm that was 21,6 ± 1,67. Menurut Balasubramani *et al.*[24] the larval mortality reached 80.66 ± 0.66 at the concentration of nanoemulsion Vitex negundo 400ppm. The concentration in this study was too small so the nanoemulsion concentration should be added to obtain optimal mortality.

According to Duarte *et al*.[25] nanoemulsion from *Rosmarinus officinalis* essential oil achieved mortality >75% within 48 hours. Based on this, if this experiment was conducted for 48 hours, then the mortality of the larvae will also increase. When compared to abate, the results were quite different because at a concentration of 50 ppm, this abate already has a percentage mortality of 100%.

The effect of nanoemulsion of essential oil of kaffir lime peel and abate with variation of concentration tested on *Aedes aegypti* L. pupa is presented in Table 4. Table 4 shows that the highest percentage mortality at 1000 ppm is 33.33±%. The larvacidal activity of nanoemulsion of essential oil of kaffir lime peel began to be seen at the smallest concentration of 62.5 ppm with the percentage of mortality by 25 ± 1,09.

The result of mortality percentage at concentration 62,5 ppm is known higher than with abate at the same concentration that is 8 ± 0.83%. The ability of nanoemulsion of essential oil of kaffir lime peel as a pupicide is not much different from abate. It can be seen from the results of the test of the pupicide where the highest concentration of 1000 ppm with mortality in nanoemulsion and abate respectively are 33,33±0,67% dan 50±1,87% respectively.

Based on the research of Puckerd and soonwera,[26] the pupicidal activity of C. *xanthorrhiza* oil has a mortality of 15.2 ± 14.0%. In addition, the results of research conducted by Ramar *et al.*[27] has a pupa mortality up to 100% at a concentration of 1000 ppm. From these results, it can be shown that nanoemulsion of *Citrus hystrix* and abate is not better. This is because nanoemulsi essential oil of lime peel is a natural material that is safe for the environment and not toxic for non target animals.

Mortality of pupa is influenced by environmental conditions. Laboratory environmental conditions are less than optimal. It can be known from room temperature which is 30°C and optimally 32-34 °C, has a water temperature of about 24 ° C while the optimum temperature is 25-30 ° C, the pH ranges from 8 while optimal at pH 7 and with moisture 68 % While the optimum is 70%.[28]

The ovicidal, larvicidal and pupicidal activities were caused by chemical compound of essential oil of kaffir lime peel. Based on the GC-MS results, it is known that the essential oil of kaffir lime peel contains of active compounds that have insecticidal activity such as *linalool*, which is seen as cis-linalool-oxide, limonene and citronella. The mortality of the larvae is caused by the *linalool* and *limonene* compounds.[6,29] The use of essential oil as an insecticide material is a good alternative to

reduce the impact of the use of chemical insecticides in the environment. The essential oil of Citrus plant is very environmentally friendly and can be used to control mosquitoes.[6]

Based on the results of the research and data analysis, it is concluded that nanoemulsion of essential oil of kaffir lime peel has the ability to kill eggs, larvae and pupa of *Aedes aegypti* L. mosquitos. In this study, abate have better mortality. This is because abate is a chemical, in which the abate chemical compound is intended to kill eggs, larvae and pupa mosquitoes. The concentration of nanoemulsion of essential oil of kaffir lime peel and abate have an effect on mortality. The nanoemulsion test of the highest concentrated kaffir lime peel oil has the highest percentage of mortality as well. The results were different in the larvicidal test with abate wherein at the lowest concentration (50ppm), the abate had a 100% mortality.

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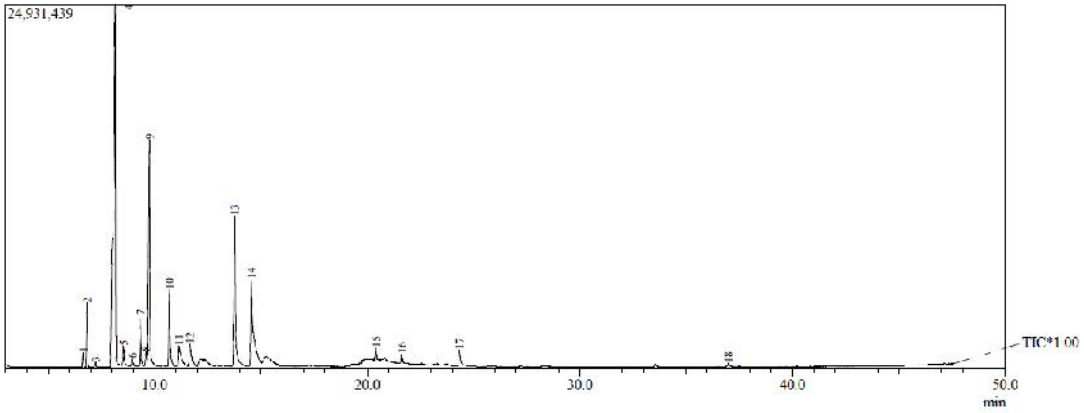

Figure 1: Chromatogram essential oil of lime peel

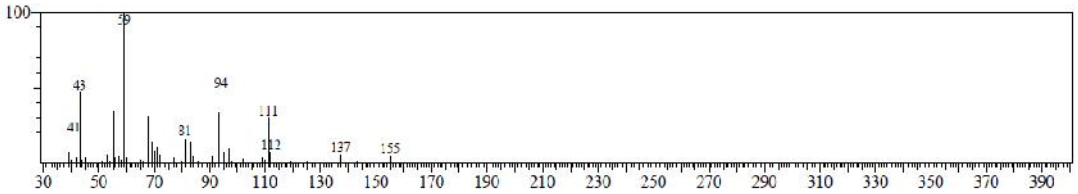

Figure 2. Spectrogram mass fractionation of compound peak 11 (*Cis-linalool oxide*)

Table 1. Chemical compounds essential oil of lime peel

Peak	Compound	Retention Time	% of Essential Total
1	Bicyclo 3.1.0 hex-2-ene	6.617	0.7
2	Alpha-pinene	6.817	3.05
3	Champhene	7.233	0.22
4	Beta-pinene	8.142	38.6
5	Beta-myrcene	8.525	1.26
6	Alpha-phellandrene	8.933	0.41
7	Alpha-terpinene	9.325	3.18
8	Benzene	9.595	0.88
9	Limonene	9.75	15.46
10	Gamma-terpinene	10.692	5.12
11	Cis- Linalol oxide	11.142	2.53
12	(+)-2-Carene	11.658	2.4
13	6-Octenal, 3, 7-dimethyl	13.767	11.85
14	3-Cyclohexen	14.567	11.38
15	Copaene	20.4	0.84
16	Caryophyllene	21.625	0.63
17	Napthalene	24.342	1.33
18	9-Octadecenoid acid	36.975	0.15

Table 2. Activity of nanoemulsi ovisida essential oil of kaffir lime skin and abate against eggs of *Aedes aegypti.*

Concentration (ppm)	Egg mortality rate (%)	
	Nanoemulsion	Abate

control	80±1.58	88 ± 2.68
62,5	85,6±1.14	97,6 ± 0.89
125	85,6±1.14	89,6 ± 2.50
250	87,2±0.44	95,2 ± 0.89
500	88,8±0.83	92,8 ± 1.09
1000	92,8±0.83	88,8± 1.58

Table 3. Larvicidal activity of nanoemulsion of kaffir lime peel essential oil and abate to instar larvae III of *Aedes aegypti*.

concentration (ppm)	**% mortality**	
	Nanoemulsi	**Abate**
Control	0 ± 0	100 ± 0
50	0 ± 0	100 ± 0
100	16,8 ± 1,48	100 ± 0
150	8 ± 1,58	100 ± 0
200	15,2 ± 1,09	100 ± 0
250	21,6 ± 1,67	100 ± 0

Table 4. Activity of nanoemulsion pupisida essential oil of kaffir lime and abate on pupae of *Aedes aegypti.*

Concentration (ppm)	**% Mortality**	
	Nanoemulsi	**Abate**
62,5	25 ± 1,09	8 ± 0,83
125	16,66 ± 1,87	2 ± 0,44
250	14,58 ± 1,30	18 ± 1,48
500	16,66 ± 2,34	42 ± 2,68
1000	33,33 ± 1,67	50 ± 1,87
control	0 ± 0, 54	0 ± 0