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Larvicidal and pupicidal activities from *Citrus hystrix* against *Aedes aegypti* mosquitoes

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ABSTRACT

Aedes aegypti is a dengue vector that is known to be very threatening to human's life. Dengue virus can cause dengue haemorrhagic fever (DHF) disease that are known to kill can human beings. Control is done during this using abate. However, abate is synthetic insecticide that is not environmentally friendly. Therefore, the control of mosquitoes by using natural materials such as essential oil of *Citrus hystrix*is one of the wise choice to mosquito control. This study aims to analyse the content of compounds in the essential oil of *Citrus hystrix* and analyse the mortality of larvae and pupa due to the provision of essential oil of kaffir lime based on nano emulsion technology. Essential oil is produced through the process of extracting *Citrus hystrix* using steam distillation, to determine the chemical compound of kaffir lime peel oil GC-MS was used, to make nano emulsionsonicator was used. Nano emulsion is then tested on larvae and pupa of *Aedes aegypti*. The identified major compounds were *beta-pinene* and *limonene*. The particle size identified from nano emulsion was ± 129.45 nm and ± 109.15 nm. The highest larvicidal activity was 21.6 ± 1.67% (LC_{50} = 546.592 ppm) and pupicidal activity 36 ± 1.67% (LC_{50} = 3045.323 ppm). Nano emulsion of essential oil of *Citrus hystrix* showed larvicidal and pupicidal activity on larva and pupa of *Aedes aegypti* and it is more environmentally friendly than abate. The results of this study can be recommended for control systems on *Aedes aegypti* mosquitoes.

Key words: *Aedes aegypti, Essential oil, Citrus hystrix, Larvicidal, Pupicidal*

Introduction

Aedes aegypti mosquito is the main vector of dengue virus. Dengue virus is very dangerous for humans. The virus can cause Dengue Haemorrhagic Fever (DHF), filariasis, yellow fever, malaria and various types of encephalitis (Waritkoo *et al.,* 2011). By 2016, the number of dengue cases in Indonesia is reported to reach 160.000 with the death of 10.000 (Ministry of Health of the Republic of Indonesia, 2020).

DHF efforts have been carried out by the Ministry of Health of the Republic of Indonesia in 2011 which includes: prevention, discovery, relief and reporting, epidemiological investigation and observation of dengue fever and counselling. However, due to lack of awareness and ignorance of people about dengue fever, dengue virus cannot be stopped. Therefore, *Aedes aegypti* mosquito control is required.

Generally, the mosquito control system is done using chemical pesticides. Chemical pesticides are pesticides that are not environmentally friendly and can harm other organisms (Nugroho, 2011). Therefore, it needs controlling technology from natural materials that are environmentally friendly and do not harm other organisms and are effective in controlling mosquitoes.

Potential natural ingredients for mosquito control are kaffir lime peel (*Citrus hystrix*). Peel of Kaffir lime is underutilized. Characteristic from leave oil dominant are essential (-)-(S)-citronellal (80%), and citronellol (10%), neroland limonene. *Citrus hystrix*is another citrus enantiomerdominant (+)-(R)-citronellal. Some of the chemical compounds in the essential oil of kaffir lime peel are the *limonene* and β-*pinene* compounds (Kasuan *et al.,* 2011). The *limonene* and *â-pinene* compounds are known to have larvicidal activity (Akono *et al.,* 2015).

Essential oils have volatile and unstable properties (Noveriza *et al.,* 2017). Therefore, the essential oil of kaffir lime peel needs to be formulated in a more stable form, such as in the form of nano emulsion. In addition, the reduction of particle size in the form of nano emulsion of essential oil can also be effective as a larvicidal agent (Anjali *et al.* 2011). Nano emulsion has average droplet size is between 100 to 500 nm (Shah *et al.,* 2010). This latest technology becomes one of the solutions for economic problems and also environmental friendliness (El-khal *et al.,* 2015). Small size particles are expected to improve the absorption of active ingredients quickly and deeply. The nanoemulsion formulation which is widely used is ultrasonic emulsification method because of its ease of use and economics (Sugumar *et al.,* 2014). The use of nanotechnology as a pesticide will reduce the toxic effects from synthetic pesticides.

Recently, control of vector-borne diseases was performed using nano emulsion from essential oil and was a good alternative against *Aedes aegypti* (Duarte *et al.,* 2015). This study was conducted to analyse the larvicidal and pupacid activity of nano emulsion of *Citrus hystrix* oil against larvae and pupa mosquitoes. Instar III larvae and *Aedes aegypti* mosquito pupa were exposed for 24 hours at various concentrations. The small particle size of nano emulsion will make it the ideal larvicide and pupaside against *Aedes aegypti*.

Methodology

Collection and Processing of Plants (Othman *et al.,* 2016)

The material used in this study is the peel of *Citrus hystrix* obtained from Citrus Plantation Semarang, Indonesia. Kaffir lime was chosen based on diameter, and color of the lime peel. Diameter of *Citrus hystrix* kaffir used is 5.0-7.5 cm.

Mosquito Rearing (Kumar *et al.,* 2010)

Mosquitoes were reared by inserting albino mice into mosquito coils. Female mosquitoes will suck the blood of albino rats for egg maturation. The breeding of *Aedes aegypt* mosquitoes was conducted at Biology Laboratory of Universitas Negeri Semarang at 28 ± 1°C and humidity 80 ± 5% RH. While for larvicide and pupicide, the eggs produced from rearing mosquitoes will be hatched and allowed to grow into instar larvae III and pupa.

Extraction of Essential Oils (Khandagle *et al.,* 2011)

The steam distillation process began by inserting 4 kg of dried lime peel of *Cytrus hystrix* into the extractor container. Then, the process was followed by heating the extractor by using steam generated from water heating. Furthermore, the essential oil was carried through the steam generated from the heating process then it went to condensation process. The essential oil produced was 54 mL. The essential oil was then stored in a small bottle at 4 °C.

Gas Chromatography – Mass Spectroscopy (*GC-MS*) (Loh *et al.,* 2011)

Content analysis of the compound in the essential oil of kaffir lime peel used GC-MS. The GC-MS process method using Shimadzu QP 5050 which is a mass spectrometer system equipped with a capillary column of BPX 5 (film trapping 30 m x 250 m, 0.25). Helium is used as gas carrier. MS operating conditions are: ionization induced by electron (EI) at 70 eV, ion source 250 °C. This compound is identified by performing a library search using Shimadzu NIST/ EPA/ NIH + mass spectral databases.

Nanoemulsion Preparation (Sugumar *et al.,* 2014)

Crude emulsion was prepared in proportion 16.66% oil: 16.66% tween 20: 66.68% water in (v/v) was made by stirring it using magnetic stirrer at 250 rpm for 10 minutes. The next process was ultrasonic emulsification by using 20 kHz sonicator (Ultrasonics, USA). The sonicator with a 13 mm probe diameter was immersed in a coarse emulsion. The temperature difference between the initial crude emulsions for the final nanoemulsion should be less than 10 °C. The heat was generated during the high-energy ultrasonic emulsification process. This heat is reduced by the storage of emulsion samples in a beaker glass containing relatively larger ice.

Analysis of Nanoemulsion Size (Anjali *et al.*, 2011)

Size and morphology of nano emulsion particles of essential oil of kaffir lime peel (*Citrus hystrix*) can be determined by using an transmission electron-microscopy (TEM) device. For the use of this TEM tool, a drop of nano emulsion was placed on the copper grid and allowed to dry in a vacuum. This electron micrograph transmission used Tecnai G-10 (Philips) instrument, 80 kVTEM with W-search and a piece of ultrahigh-resolution pillar with point-to-point resolution 1.9Å.

Larvacidal Test (WHO, 2005)

Nano emulsions of *Cytrushystrix*were prepared at concentrations of 50, 100, 150, 200 and 250 ppm. Nano emulsions of kaffir lime peel with various concentrations respectively were tested on 25 instar larvae III of *Aedes aegypti*. The controls in this study were 250 mL of water. The rate of larvicidal mortality was assessed after 24 hours. Larvae are considered dead if the larvae cannot move and cannot transfer the siphon to the water surface.

Pupicidal Test (Krishnappa *et al.*, 2012)

Pupacide test by using experimental protocol was performed according to. Ten pupas of *Aedes aegypti* were taken into 500 mL nano emulsion solution of *Citrus hystrix* kaffir lime peel oil and abate with various concentration. The control was 500 mL of water. Bioassay at test concentrations of 62.5, 125, 250, 500, 1000 ppm (WHO, 2005). The study room is preferably at temperature (28 ± 2) °C with general photoperiode (12: 12h/ L: D). Pupa was considered dead when the pupa cannot move when it is touched for many times with a soft brush. Mortality of each pupa was recorded after 24 hours of exposure to the selected concentration of chemical compositions following the Abbott formula.

Statistical analysis

The data were calculated by using the software statistical package of social science (SPSS) version 23.0 for windows. The significance level was set at $P<0.05$. Probit analysis SPSS 23.0 was applied to determine lethal concentrations causing 50% (LC_{50}) mortality of larvae and pupae 24h after treatment application.

Results and Discussion

Chemical Compounds of Essential Oil of *Citrus hystrix*

Chemical compounds in essential oil of kaffir lime peel can be determined by Chromatography Gas - Mass Spectroscopy (GC-MS). There are 18 compounds identified. The highest peak of GC-MS chromatogram is peak 4 (*β-pinene*) and peak 9(*limonene*). The *β-pinene* compound was seen at retention time of 8.142 minutes and by molecular weight 136. The presence of *β-pinene* compound in the essential oil of *Citrus hystrix*was 38.60%. The *limonene* compound was seen at retention time of 9.750 minutes and with molecular weight of 136. The presence of *limonene* compound in the *Citrus hystrix* oil was 15.46%. *Limonene* compounds are the second highest compound after *β-pinene* in the essential oil of *Citrus hystrix*. Both these compounds give a distinctive aroma. The *limonene* compound gives a strong orange scent while *β-pinene* gives the woody-green scent in the essential oil of the kaffir lime peel (Kasuan *et al.*, 2013). The identified compounds can be seen in Table 1.

Table 1 shows that the major compounds in the essential oil of *Citrus hystrix* are *β-pinene* (38.6%) and *limonene* (15.46%). This is confirmed by research

Table 1. Chemical compound of essential oil of *Cytrus hystrix*

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

from Kasuan *et al.* (2013) which shown that *limonene* compounds (17.2%), *β-pinene* (13.5%) are the main compounds in essential oil of kaffir lime *Cytrus hystrix*. However, the presence of *β-pinene* compounds from the results of this study is greater. *β-pinene* and *limonene* compounds are compounds that have larvicidal activity (Akono *et al.*, 2015).

Morphology and Size of Nanoemulsion

Transmissions Electron Microscopy (TEM) analysis aims to find out particle size and morphology of nanoemulsion of *Citrus hystrix* oil. The TEM test results revealed that by looking the nanoemulsion of *Citrus hystrix* oil by microscope at 500 nm magnification, showed 2 nanoemulsion particles. Both particles of nanoemulsion of essential oil of *Citrus hystrix* skim has a size of ± 129.45 nm and ± 109.15 nm. According to Shah *et al.* (2010), the average nanoemulsion size ranges from 20-200 nm. The visible nanoemulsion morphology is spheric in shape. Similar results were obtained from Anjali *et al.* (2011) which stated that the nanoemulsion results of Neem oil nanoemulsion are spheric in shape.

Larvicidal Activity of Nanoemulsion of *Citrus hystrix* Oil

Nanoemulsion of essential oil of *Citrus hystrix* was then tested on *Aedes aegypti* larvae. The larvicidal activity of nanoemulsion of kaffir lime peel oil and abate can be seen from larvae mortality calculated after 24 hours of treatment. The result of observation of the effect of nanoemulsion of essential oil of *Citrus hystrix* and abate to mortality of larvae *Aedes aegypti* is presented in Table 2.

Based on Table 2, it can be seen that the highest percentage of mortality in nanoemulsion of essential oil of *Cytrus hystrix* is 21.6 ± 1.67% and abate has mortality 100 ± 0.00%. No mortality was observed in the control groups. ANOVA test indicated statistical differences in mortality between tested concentrations after 24 hours (F value = 17.733; $P = 0.000$). According to Balasubramani *et al.* (2017), mortality of larvae of nanoemulsion *Vitex negundo* reach 80.66 ± 0.66% for 24 hours of application. However, concentration of *Vitex negundo* nanoemulsion used is higher at 400 ppm. This condition resulted in higher mortality. Therefore, if the nanoemulsion concentration is increased then the mortality of the *Aedes aegypti* larvae will also increase. In addition, the results of study by Duarte *et al.* (2015) also confirm that the length of time of exposure may affect larval mortality. The mortality results from the essential oil of nanoemulsion of *Rosmarinus officinalis* reached mortality > 75%. The results are quite high because of the length of application from *Rosmarinus officinalis* which is up to 48 hours. Based on this fact, if this experiment is conducted for 48 hours, the mortality of the larvae will also increase.

Table 2. Larvicidal activity of nanoemulsion of *Cytrus hystrix* oil and abate against larvae of *Aedes aegypti*

Concentration (ppm)	Average Mortality of larvae (%)*	
	Nanoemulsion	Abate
0	0 ± 0.00[a]	0 ± 0.00[a]
50	0 ± 0.00[a]	100 ± 0.00[f]
100	8 ± 1.58[b]	100 ± 0.00[f]
150	15.2 ± 1.09[c]	100 ± 0.00[f]
200	16.8 ± 1.48[d]	100 ± 0.00[f]
250	21.6 ± 1.67[e]	100 ± 0.00[f]

*Values are means ± standard deviations from five replication

The toxic potential of essential oils of *Cytrus hystrix* and their compounds against Aedes aegypti larvae may vary significantly according to extrinsic and intrinsic factors. Plant species, age of the plant, chemotypes, plant parts, and geographic conditions (such as season, humidity percentage, temperature, sunlight, rainfall, and altitude) in which the plant was collected, methods used and larval source, in general, induce different larval responses (Subekti *et al.*, 2018).

Additionally, the different ecological niches of mosquito larvae can influence their susceptibility to toxic compounds. It was hypothesized that the field collected larvae are more resistant to chemicals than those reared in laboratory because the former are better adapted to adjust to environmental variations and have a higher genetic variability.

Pupicidal Activity Nanoemulsion Essential Oil of *Cytrus hystrix*

Nanoemulsion of essential oil of *Cytrus hystrix* was then tested on *Aedes aegypti* pupae. The pupicidal activity of nanoemulsion essential oil of *Cytrus hystrix* and abate can be seen from larvae mortality calculated after 24 hours of treatment. The result of observation of the effect of nanoemulsion of essential oil of *Cytrus hystrix* and abate to mortality of pupae *Aedes aegypti* is presented in Table 3.

The result of the pupaside test in Table 3 shows

Table 3. Pupicidal activity of nanoemulsion essential oil of *Cytrus hystrix* and abate against pupa of *Aedes aegypti*

Concentration (ppm)	Pupae Mortality ± SD		% Mortality ± SD	
	Nanoemulsion	Abate	Nanoemulsion	Abate
Control	0 ± 0.55	0 ± 00	4 ± 0.55	0 ± 00
62.5	14.58 ± 1.30	2 ± 0.44	18 ± 1.30	2 ± 0.44
125	16.67 ± 1.87	8 ± 0.84	20 ± 1.87	8 ± 0.84
250	16.67 ± 2.34	18 ± 1.48	20 ± 2.34	18 ± 1.48
500	25 ± 1.09	42 ± 2.68	28 ± 1.09	42 ± 2.68
1000	33.34 ± 1.67	50 ± 1.87	36 ± 1.67	50 ± 1.87

that the highest mortality in the essential oil of *Citrus hystrix*is 36 ± 1.67% and abate 50 ± 1.67%. The mortality observed in the control groups was 4 ± 0.55%. ANOVA test indicated statistical differences in mortality between tested concentrations after 24 h (F value = 2.296; P = 0.077). According to study of Priyadarshini *et al*. (2012) which was aimed at investigating the potential of silver nanoparticles synthesized from *Euphorbia hirta* to *Anopheles stephensi* showed the highest mortality 55.8% at 250 ppm.

Another study on mosquito control was reported by Lame *et al*. (2015) stating that the chloroform fraction could kill 36% larvae at 2500 ppm concentrations. The concentration used in the study was quite high, but the mortality of larvae was only 36%. When compared with the results of this study, nanoemulsion of essential oil of kaffir lime peel has a higher mortality.

Mortality in larvae and pupa of mosquito caused by nanoemulsion of essential oil of *Citrus hystrix* is caused by the active compound content in essential oil of *Cytrus hystrix*. According to Akono *et al.* (2015), larval mortality is caused by the presence of *β-pinene* and *limonene* compounds *in Citrus limon, Citrus sinensis* and *Citrus reticulata*. In this experiment, *β-pinene* and *limonene* compounds are the main compounds with the highest content in the essential oil of kaffir lime peel. Deaths of larvae and pupa of *Aedes aegypti* are most likely due to *β-pinene* and *limonene* compounds in the essential oil of *Citrus hystrix*.

The use of essential oil as an insecticide material is a good alternative to reduce the impact of synthetic insecticide used in the environment. The results showed that nanoemulsion of essential oil of kaffir lime peel has the ability to kill larvae and pupa of mosquito *Aedes aegypti* L. The larvicidal activity of nanoemulsion of essential oil of *Citrus hystrix* and abate is higher than the activity of its pupicidal activity. The mortality of larvae and pupa because of the presence of abate is higher than that of the presence of nanoemulsion of essential oil of *Citrus hystrix*. However, nanoemulsion of essential oil of *Citrus hystrix* is more environmentally friendly than that of abate.

Conclusion

The essential oil of *Citrus hystrix* could be promoted as an efficient repellent larvicidal and pupacidal agent. The major chemical compounds were *beta-pinene* and *limonene,* both as active components that can kill the larvae and pupae. The particle size from nanoemulsion was ±129.45 nm and ±109.15 nm. The larvicidal activity was 21.6 ± 1.67% (LC_{50} = 546.592 ppm) and pupicidal activity 36 ± 1.67% (LC_{50} = 3045.323 ppm). Nanoemulsion essential oil of *Citrus hystrix* is more environmentally friendly than abate.

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