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Citrus reticulata extract as biocides to control *Aedes aegypti*, the vector of dengue

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This study aimed to explore the effective compounds from local plants as a safe mean of control by evaluating the anti-larval activity of *Citrus reticulata* extract (CRE) against the *Aedes aegypti* mosquito which is the dengue vector in Indonesia. The study conducted in a completely randomized design (CRD). Non-polar fraction of CRE tested with concentrations of 0 ppm, 500 ppm, 1375 ppm, 2250 ppm, 3125 ppm, and 4000 ppm against the third instar larvae of *Aedes aegypti*. Every concentration was replicated five times. The larvae number of *Aedes aegypti* mortality was calculated after 24 hours of treatment. Then, the data of dead larvae was analyzed by probit. Significantly, the study result found that non-polar fraction of CRE has anti-larval activity against the third instar larvae of *Aedes aegypti* mosquito with LC_{90} = 4,810 ppm. In sum, this study had identified non-polar fraction of CRE with lethal effect against *Aedes aegypti* larvae and illustrated the poisoning of the mosquito larvae by the phytochemicals of *Citrus reticulata*.

Keywords: ***Aedes aegypti* –*Citrus reticulata* – dengue – insecticide –plant extract**

INTRODUCTION

Dengue is among the most effluent mosquito-borne viral diseases in the world (Benelli and Mehlhorn, 2016). In the recent years, over one hundred endemic countries with around 2.5 billion people were infected by dengue virus (World Health Organization, 2014). Indonesia is one of the largest countries in the dengue endemic region, with a population of 251 million (Karyanti et al., 2014). Historically, Indonesia has been experiencing epidemic cycles of dengue since its first introduction in the country in 1968 in Jakarta and Surabaya (Sumarmo, 1987). The country suffered the highest economic burden of dengue in Southeast Asia region (Shepard et al., 2013). The frequent dengue cases are often followed by increasing numbers of infections and severity which affected all 34 provinces in the country (Sumarmo, 1987; Setiati et al., 2006). Moreover, Dengue Haemorrhagic Fever (DHF) is triggered by the different types of four dengue viral serotypes (DEN 1, 2, 3 and 4) which are closely linked to antigenically. The rates of transmission and spread vary greatly among the four dengue serotypes (Christofferson, 2015). In addition, Mustafa et al. (2015) led the discovery of the new serotype of dengue virus, DENV-5. World Health Organization (2012) mentioned that dengue viruses are spread in metropolitan cities especially in tropical and subtropical areas by the disease mosquito vector of *Aedes aegypti*, a species closely linked with human residences. Unfortunately, Dias and Moraes (2014) stated that there are no effective medicines against dengue

virus infections.

Indonesia has a vast biodiversity with a high number of potential medicinal plants (Husen et al., 2017; Wahyuni et al., 2017; Ansori et al., 2018b).Citrus is one of Magnoliopsida which belongs to Rutaceae family and has ahigh economical value because of its vitamin C. *Citrus* fruit is consumed as fruit and juice; while the leaves can be added to dishes as flavoring and fragrances. Citrus has many secondary metabolites which have the potential to be anti-bacterial, anti-inflammatory, anti-cancer, antioxidants, and can maintain our health, thus it can be considered as a medicinal plant (Akram et al., 2010; Adrianto et al., 2014; Hamidah and Adrianto, 2017; Adrianto et al., 2018; Ansori et al., 2018a).The leaf of Citrus contains some chemical compounds which have role as the secondary metabolites such as essential oil, flavonoid, saponin, steroid, and terpenoid (Adrianto et al., 2014; Ansori et al., 2015a; Adrianto et al., 2018; Ansori et al., 2018a). These compounds are toxic to mosquito larvae (Adrianto et al., 2018; Ansori et al., 2018a).

One of the most efficient techniques to control mosquitoes is to prevent mosquitoe's breeding by using easily biodegradable insecticides (Adrianto et al., 2018; Ansori et al., 2018a). Many researcheswhich are designed against *Aedes aegypti* mosquitoes have used the by-products of plants that are already recognized as medicinal plants (Adrianto et al., 2014). The use of plant origin insecticides hasincreased over the last decades and they have been proven to not leave any residues which can damage the environment, therefore, it is suitable for integrated vector management programs (IVMP).The IVMP is based on two approaches; the chemical control (uses the temephos as a larvicide and the organophosphates and pyrethroids as adulticides) (Benelli, 2016). However, recent research suggests that Aedes aegypti larvae have developed aresistance to temephos in several countries in the world (Lazcano et al., 2009; Sornperg et al., 2009; Llinás et al., 2010; Grisales et al., 2013), including Indonesia (Mulyatno et al., 2012). Furthermore, the exposure to synthetic chemical contents of temephos in the long term may lead to cancer. Temephos as larvicide is also hazardous to human and environment as it may penetrate the food chain and accumulated within the body of organisms (Adrianto et al., 2014; Ansori et al., 2015b). Based on the precedent, the study aimed to scientifically evaluate the larvicidal activity of CRE against Aedes aegypti as the main vector which is responsible for the spread of dengue in Indonesia.

MATERIALS AND METHODS

Citrus reticulate leaves were used as the material. n-Hexane (Sigma) was applied as the solvent for powdered leaf maceration, distilled water was for making extract solution, and Tween® 20 (Sigma) was utilized as the homogenizer. Aedes aegypti eggs (Surabaya strain, purchased from Entomology Laboratory, Institute of Tropical Disease Universitas Airlangga). In addition, a mixture of yeast, milk (skimmed-milk powder) and baked bread (at equal ratios) were employed to feed these larvae, and also mineral water was for mosquito larvae colonization medium.

Instruments

Glass jar, Erlenmeyer flask, glass funnel, filter paper, aluminum foil, and Rotavapor® R-300 (Buchi) were used for extracting, filtering filtrates, and evaporating solvents. Plastic pan (sized 30 x 20 x 6 cm) was applied to keep and colonize the mosquito larvae. Plastic pipettes were used for moving the mosquito larvae. Furthermore, some biological test instruments such as analytical balance for weighing the extract, measuring glass for measuring the volume of the extract, plastic glass for larvicide testing site, glass stirrer for solution homogeneity, and hand counter for counting the number of test mosquito larvae were employed.

Methods

The study was conducted in a completely randomized design (CRD). Fresh leaves of *Citrus reticulata* were collected from Indonesian *Citrus* and Subtropical Fruits Research Institute (ICSFRI) in Batu, East Java, Indonesia. The leaves were identified by taxonomist at Universitas Airlangga, Surabaya, East Java, Indonesia. The leaves were taken to the laboratory and left to completely dry at the room temperature (25-30 °C) after washing with tap water. The leaves were grounded into powder using the electric grinder. The active ingredients which were found in the leaves' powder were extracted using n-hexane at room temperature. The maceration process was conducted for a week. After a week, the macerates were filtered and evaporated using a rotary evaporator thus the extract was acquired. All experiments were run in 200 mL plastic cups containing 100 mL tap water. Different concentrations of the plant extract were prepared

with variation of 0, 500, 1375, 2250, 3125, and 4000 ppm. The third instar larvae (20/replicate) were put immediately into the plastic cups which contained different concentrations of extracts (the number of samples was in accordance with World Health Organization standards for toxicity test). Five replicates were usually used for each tested extract concentration. The larvae were provided with food during the test. The experiments were run under controlled condition temperature (27 °C). The larval control group received only five drops of Tween® 20 without any extract in the same amount of water (100 mL). Mortality rate was recorded after 24 hours. The mortality data of third instar larvae of Aedes aegypti was analyzed by the probit analysis for seeking the LC_{90} value (Adrianto et al., 2018; Ansori et al., 2018a).

RESULTSAND DISCUSSION

The larvicidal effect was observed at 24 hours post exposure which illustrated the poisoning of the mosquito larvae by the phytochemicals of Citrus reticulata. The lethal concentrations which demonstrated the 90% (LC_{90}) larval mortality for Aedes aegypti larvae presented in Table 1. Non-polar fraction of CRE caused mosquito larval mortality with LC_{90} value of 4,810 ppm**.**

Table1. Interval of LC from Non-Polar Fraction of CRE

LC_{90} (ppm)	Lower Bound	Upper Bound
4,810	4,526	5,232

The mosquito larvicidal action of the *Citrus* leaf extract was supported the studies conducted by other researchers. The previous study revealed that the larvicidal potential of n-hexane extract of *Citrus hystrix* against larvae of Aedes aegypti (LC_{90} 2,885 ppm). Furthermore, the n-hexane extract of *Citrus hystrix* was more effective than the methanol extract (LC_{90} 3,180 ppm) to *Aedes aegypti* larvae (Ansori et al., 2015a). Ansori et al., (2015b) stated that the larvicidal potential of n-hexane extract of *Citrus aurantifolia* against larvae of *Aedes aegypti* (LC_{90} 1,511 ppm). Also, Ansori et al., (2018a) revealed that the methanol extract of *Citrus hystrix* and *Citrus aurantifolia* have biolarvicidal activity against larvae of Culex *quinquefasciatus* with LC_{90} 1,653 ppm and 2,797 ppm, respectively. In addition, Hamidah and Adrianto (2017) examined the potential of *Citrus mitis* and *Citrus maxima* against larvae of *Aedes aegypti.* On the other hand, Akram et al., (2010) examined seed extracts of Citrus aurantium, *Citrus grandis*, *Citrus pseudolimon*, *Citrus paradisi, Citrus* limon, *Citrus sinensis*, and *Citrus jambhiri.*

Adrianto et al., (2014) mentioned that Citrus extracts are not only effective and potent but also provide eco-friendly medium when evaluated against *Aedes aegypti*. Therefore, there is an urgent need to develop new biocides for controlling mosquito vectors which are more environmentally friendly, biodegradable, non-toxic effects on human and domestic animals. In recent years, indigenous plant-based oils were used in combination of many solvents (methanol, ethyl acetate, petroleum ether, benzene, chloroform absolute alcohol, and acetone) to discover lethal level for the reduction of mosquitoes (Kamaraj et al., 2010). But in this study, we used only one solvent (n-hexane) to identify lethal concentration 90%.

Citrus is one of Magnoliopsida which belongs to Rutaceae family and has a high economical value because of its vitamin C. Citrus fruit is consumed as fruit and juice; while the leaves can be added to dishes as flavorings and fragrances. Citrus has many secondary metabolites which have the potential to be anti-bacterial, anti-inflammatory, anti-cancer, antioxidants, and maintain our health, so that it becomes medicinal plant (Akram et al., 2010; Adrianto et al., 2014; Hamidah and Adrianto, 2017; Adrianto et al., 2018; Ansori et al., 2018a). The leaf of Citrus contains chemical compounds which are considered as the secondary metabolites such as essential oil, flavonoid, saponin, steroid, and terpenoid (Adrianto et al., 2014; Ansori et al., 2015a; Adrianto et al., 2018; Ansori et al., 2018a). Secondary metabolites of plants which many of them produced by the plant for its protection against microorganisms and predator insects are natural candidates for the discovery of new products to combat *Aedes aegypti* (Adrianto et al., 2018; Ansori et al., 2018a).

Saponin compound ingested by *Aedes aegypti* larvae can irritate the mucous of digestive system by damaging its cell membranes, decreasing their appetite leading to starvation and finally death of mosquito larvae. While, the steroid compounds affect the mosquito larvae's growth and can cause anxiety symptoms, terpenoid compounds are potential as an anti-feedant to insects, larvacidal, and insect repellent. Terpenoid compounds of limonoid group can cause loss of organ coordination in Aedes aegypti larvae. Alkaloid compounds can perform as a stomach poison. Therefore, alkaloid ingestion by mosquito

larvae can impair its digestive system. Essential oil functions as a respiratory poison and flavonoid can damage their cell membranes (Adrianto et al., 2014; Ansori et al., 2015a; Ansori et al., 2015b; Ansori et al., 2018a).

CONCLUSION

In sum, this study had identified non-polar fraction of CRE with lethal effect against Aedes aegypti larvae with LC_{90} = 4,810 ppm. Today, environmental safety is considered to be paramount important. A larvicide does not need to cause high mortality on target organisms in order to be acceptable but should be eco-friendly in nature.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHOR CONTRIBUTIONS

ANMA worked out a major part of the technical details, designed, and performed the experiments, also took the lead in writing the manuscript in consultation with HA. MKJK, HI, NP, AF, AP, SR, IK, AKA, analyzing and verifying the results. All authors read and approved the final manuscript.

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