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Research Article

Biolarvicidal Effectivities of Polar and Non-polar Extract Fraction from Kaffir Lime (*Citrus hystrix*) Leaf against 3rd Instar Larvae of *Aedes aegypti*

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Abstract: Indonesia is one of the largest countries in the dengue endemic region and ranked first in ASEAN by the highest number of cases in Dengue Hemorrhagic Fever (DHF). The use of chemical agents such as larvicides causes development of resistance, health, and environmental problem. Plant extracts with larvicidal activity from plants, which are easily available in large quantities and are safe for human needed to replace the chemical larvicides. The aim of this research was to obtain polar (methanol) and non-polar (n-hexane) extract fraction from leaves of kaffir lime (*Citrus hystrix*) which are known to possess several insecticide and an effective biolarvicide. The experiment was designed as a Completely Randomized Design (CRD) for comparative analysis. Polar and non-polar extract fractions of C. hystrix were tested with concentrations of 500 ppm, 1375 ppm, 2250 ppm, 3125 ppm, and 4000 ppm against the 3rd instar larvae of *Aedes aegypti*. The experiment was replicated five times. The number of mosquito larvae mortality was calculated after 24 hours of treatment. The dead larvae were counted and the data was analyzed using probit. The results show that non-polar extract fraction from *C. hystrix* is more toxic and is an effective biolarvicide with LC_{90} = 2,885 ppm compared with polar extract fraction from *C. hystrix* which has an LC_{90} = 3,180 ppm.

INTRODUCTION

Dengue Hemorrhagic Fever (DHF) is one of the global health concerns in tropical area and an endemic disease in more than 110 countries in the world [1]. Approximately 2.5 billion people live in endemic countries of which about 1.8 billion (more than 70%) in Southeast Asia and the Western Pacific Region [2, 3, 4]. Indonesia is one of the largest countries in the dengue endemic region, with a population of 251 million [5]. In 2010, Indonesia was reported having the highest number of cases in DHF and was ranked as number one country in ASEAN with hyperendemic predicate [6].

Dengue virus (DENV) causes DHF and are the most rapidly spreading vector-borne communicable diseases spread by *Aedes aegypti* which is the primary vector of dengue [7]. The main causes of DHF epidemic is uncontrollable reproduction and dispersal of mosquitoes as the disease vector [8]. Controlling of the disease vector is currently prioritized as a means to prevent cases of DHF since the vaccines and effective antiviruses have not been available yet [7, 9]. The most effective and efficient means to control the disease vector is by eradication while it is in larval stage [7].

The chemical mostly used for controlling *A. aegypti* larvae is temephos (organophosphate) [10]. However, recent research suggests that *A. aegypti* larvae has developed resistance to temephos in some countries such as El Salvador [11], Thailand [12], Argentina [13], Indonesia [10, 14], and Colombia [15]. Exposure to synthetic chemical contents of temephos in long term may lead to cancer [16]. Temephos as larvicide is also hazardous for human and environment as it may penetrate the food chain and accumulated within the body of organisms [17]. Fogging with insecticides such as parathyroid and organophosphates can eradicate mature mosquitoes however may cause mutation of VGSC (Voltage Gated Sodium Channel) gene of *A. aegypti*, making them resistant against insecticides [18]. Safer disease vector control measures are essential for controlling human health and environment and alternative ecofriendly insecticides belonging to new or different classes need to be developed to circumvent insecticide resistance, and ecosystem degradation [19]. Secondary metabolite compounds from plants may be potential as biolarvicide for mosquitoes; thereby this topic requires further investigation [20].

Citrus hystrix is one of the plants, which has a high economic value and popular due to its high content of vitamin C and is important in the cuisines of South East Asia countries [21, 22]. The leaf of *C. hystrix* contains secondary metabolites,

which include essential oil, flavonoid, saponin, steroid, and terpenoid [23, 24]. These compounds exhibit insecticidal properties and are toxic to mosquito larvae based on anti-feeding action due to destruction of the function of chemoreceptors, contact or stomach poisoning [19].
The leaves of *C. hystrix* are expected to be future alternative larvicide which is safer for human and environmentally friendly. The aim of this research was to obtain polar (methanol) and non-polar (n-hexane) extract fraction from *C. hystrix* leaf and demonstrate its effectiveness as biolarvicide for 3rd instar larvae of *A. aegypti* through value of lethal concentration 90% (LC_{90}).

MATERIALS AND METHODS

Materials
C. hystrix leaves were used as the plants material. Methanol and n-hexane, as solvent for powdered leaf maceration, distilled water for making extract solution, and Tween 20 solution as homogenizer. *A. aegypti* eggs were provided by Entomology Laboratory, Institute of Tropical Disease Universitas Airlangga. In addition, catfish food for feeding the mosquito larvae and mineral water for mosquito larvae colonization medium were used.

Instruments
Glass jar, Erlenmeyer flask, glass funnel, filter paper, aluminum foil, and rotary evaporator were used for extracting, immersing, filtering filtrates, and evaporating solvents. Plastic pan (sized 30 x 20 x 6 cm) was used for keeping mosquito larvae and colonizing. Plastic pipettes with large end were useful for moving the mosquito larvae. In addition, biological test instrument such as analytical balance for weighing the extract, measuring glass for measuring volume of the extract, plastic glass for biolarvicide testing site, glass stirrer for solution homogenity, and hand counter for counting the number of test mosquito larvae were used.

Methods
This research was conducted in four stages, first citrus leaves were collected, cleaned and powered, second, the leaves extract were made, third, colonization of test mosquito larvae, and fourth was biological testing. The leaves were collected from Surabaya (Indonesia), dried for a month and powdered. The experiment was designed as a Completely Randomized Design (CRD) for comparative analysis. The experiment was carried out at Faculty of Science and Technology Universitas Airlangga. One kilogram of powdered leaves was macerated with methanol and the other was n-hexane. The maceration process was conducted for a week. After a week, the macerates were filtered and evaporated using rotary evaporator such that the extract was acquired. Fixed concentrations were used: 0, 500, 1375, 2250, 3125, and 4000 ppm with each concentration replicated five times. *C. hystrix* leaf extracts were made into solutions according to said concentrations. The acquired condensed extracts were weighed (mg) as needed, and 5 drops of Tween 20 were added before being diluted by distilled water to avoid any debris in the solution. In addition, the control group also used distilled water with 5 drops of Tween 20, such that all extract solutions contained Tween 20 to check if this chemical was responsible for mosquito larvae mortality. The volume of each extracts was measured 100 ml and placed in transparent plastic glasses. Twenty 3rd instar larvae of *A. aegypti* (the amount of samples was accordance with World Health Organization standards for toxicity test) was placed inside each glasses. The exposure was conducted for 24 hours, and the dead mosquito larvae were counted. The mortality data of 3rd instar larvae of *A. aegypti* was analyzed by SPSS 16.0 software using probit analysis for seeking LC_{90} value or extract concentration which is able to exterminate 90% mosquito larvae.

RESULTS AND DISCUSSION

The results of biolarvicide activity testing of polar and non-polar extract fraction of *C. hystrix* leaves were analyzed by probit analysis using SPSS 16.0 software. The lethal concentration 90% (LC_{90}) from non-polar extract fraction was found to be more toxic and effective as biolarvicide for 3rd instar larvae of *A. aegypti* with LC_{90} = 2,885 ppm compared to polar extract fraction of *C. hystrix* leaves which had an LC_{90} = 3,180 ppm.
The LC_{90} value of polar extract fraction of *C. hystrix* leaves is between 2,858 to 3,617 ppm intervals, while the LC_{90} value of non-polar extract fraction of *C. hystrix* leaves is 2,434 to 3,758 ppm interval (Table 1). The extract solution made between those concentrations interval is effective and would be able to kill 90% 3rd instar larvae of *A. aegypti*.

Table 1. Interval of LC from *Citrus hystrix* polar and non-polar extract fraction

Citrus hystrix	LC_{90} (ppm)	Lower Bound	Upper Bound
Polar Extract Fraction	3,180	2,858	3,617
Non-polar Extract Fraction	2,885	2,434	3,758

Mosquito larvae mortality as a result of 24 hours exposure to polar and non-polar extract fraction of *C. hystrix* leaves is the effect of biolarvicidal compound present in the extract. Besides, the graphs indicate that the higher extract concentration, the higher the larval mortality. In Figure 1, the non-polar extract fraction of *C. hystrix* leaves at concentration of 3,125 ppm and 4,000 ppm caused 96% mortality rate of *A. aegypti* larvae. In Figure 2, the polar extract fraction of *C. hystrix* leaves at concentration 4,000 ppm caused 99% mortality rate of *A. aegypti* larvae.
Steroid compounds have been identified in n-hexane solvent fraction [25] and are non-polar compounds which can be attracted by n-hexane solvent [26]. Steroid, terpenoid, and alkaloid compounds found in n-hexane solvent fraction [27, 28]. Saponin found in *Citrus aurantifolia* which is non-polar and can be attracted by non-polar solvent fraction [29]. Methanol extract of *Citrus* spp., Chemical compounds of flavonoid, terpenoid, saponin, and essential oil are identified [30].
The saponin compound ingested by *A. aegypti* larvae can irritate the mucous of tractus digestivus, damaging its cell membranes, decreasing their appetite leading to starvation and death of mosquito larvae [31]. Steroid compounds affect mosquito larvae growth and simultaneously cause irritation to its digestion system, causing anxiety symptoms and death [30]. Terpenoid compounds are potential as antifeedant to insects, larvacidal, and insect repellent. Terpenoid compounds of group limonoid can cause loss of

organ coordination in *A. aegypti* larvae [31]. Alkaloid compounds may work as stomach poisoning. Therefore, alkaloid ingestion by mosquito larvae can impair its digestion system [32]. Essential oil functions as respiratory poison [30] and flavonoid can penetrate the cuticula of the larvae which can damage their cell membranes [31].

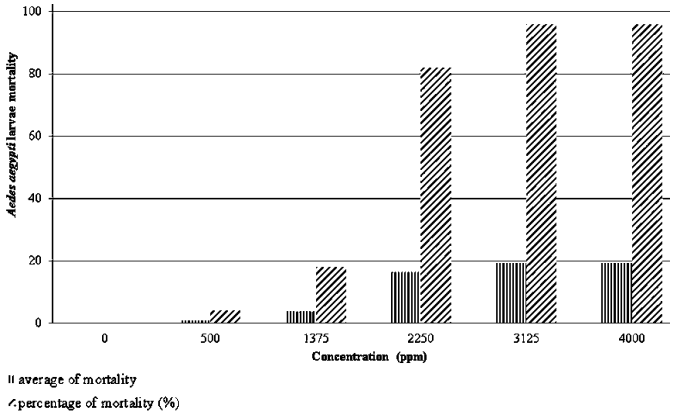

Figure 1. Number of mortality for 3rd instar larvae of *A. aegypti* given non-polar extract fraction of *C. hystrix* leaf.

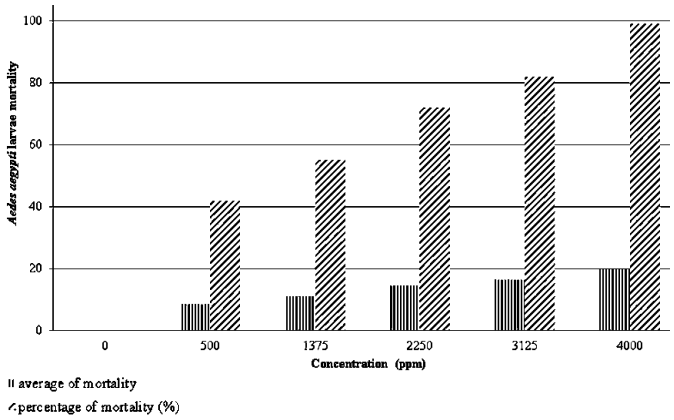

Figure 2. Number of mortality for 3rd instar larvae of *A. aegypti* given polar extract fraction of *C. hystrix* leaf.

CONCLUSION

Chemical larvicides, which are commonly used for the controlling mosquitoes larvae, are not safe for human being. Hence, there is a need to develop future alternative larvicide which is safer for human and environmentally friendly. *A. aegypti* is a vector for transmitting disease such as dengue hemorrhagic fever (DHF). *A. aegypti* are commonly found in tropical and subtropical regions. Controlling of the disease vector is currently prioritized as a means to prevent cases of DHF. Kaffir lime (*Citrus hystrix*) are commonly found in South East Asia, include Indonesia. Polar and non-polar extract fraction from leaves of *C. hystrix* which are known to possess several insecticides and an effective biolarvicide. Polar and non-polar extract from leaves of *C. hystrix* were tested with concentration of 500 ppm, 1375 ppm, 2250 ppm, 3125 ppm, and 4000 ppm against the 3rd instar larvae of *A. aegypti*. The experiment was replicated five times. The number of mosquito larvae mortality was calculated after 24 hours of treatment. The dead larvae were counted and the data was analyzed using probit. The results show that non-polar extract fraction from *C. hystrix* is more toxic and is an effective biolarvicide with LC_{90} = 2,885 ppm compared with polar extract fraction from *C. hystrix* which has an LC_{90} = 3,180 ppm.

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COMPETING INTERESTS: The author has declared that no competing interests exist.

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