Isolation and Identification of Biolarvicide from Soursop (*Annona muricata Linn*) Seeds to Mosquito (*Aedes aegypti*) Larvae

Alfrits Komansilan, Abdul L. Abadi, Bagyo Yanuwiadi, and David A. Kaligis

***Abstract*— Isolation and identification of larvicide bioactive from soursop (*Annona muricata Linn*) seeds against the larvae of Aedes aegypti mosquito, the transmitters of dengue fever has been done. The ethanol extract of soursop seeds was an active larvicide agent with a lethal concentration LC50 = 244.27 ppm. The soursop seed extract contains secondary metabolites compounds of saponin, alkaloid and triterpenoid groups. Test results of the activity fraction of *n*-hexane, ethyl acetate fraction and *n*-butanol fraction towards the Aedes aegypti mosquito's larvae showed that the fraction of *n*-hexane is more effective and has the highest toxicity with the concentration of mortality (LC50) = 73.77 ppm.**

Separation by column chromatography obtained 15 mg of active anti larvicide isolates (F2). Analysis and identification with GC-MS showed peaks of seven compounds, where there were three dominant compounds with a retention time relatively close and have the abundance % that are large enough which are classified as organic fatty acids, namely: methyl palmitate, methyl oleate and methyl stearate with the most dominant compound which is methyl palmitate fatty acid which has a 39.93% abundance.

***Index Terms*—soursop fruit, identification, biolarvicide, *Aedes aegypti*, *Annona muricata Linn*.**

I. Introduction

INDONESIA in general has the risk of dengue fever infection since its cause vector (*Aedes aegypti*) mosquitoes are widespread in residential areas or in public places, except in area over 1000 meters above sea level [1].

The city of Manado is an endemic area of Dengue Hemorrhagic Fever (DHF) disease which in 2010 had an increase in case number. In 2010, 998 cases of DHF were reported in Manado with 25 deaths (CFR = 2.5%). When compared to January - April 2010 with the total of 832 cases, the tendency was sharply decline. However, the occurrence of this disease may change and increase unexpectedly [2].

A. aegypti is a vector of several serious diseases to human, such as malaria, encephalitis, yellow fever, dengue fever, dengue hemorrhagic fever, filariasis, and arbovirus [3]. Dengue Hemorrhagic Fever (DHF) has no medicine or vaccine yet [4]. One way of preventing the spread of DHF is by prevention of Dengue virus infection, by controlling and eradicating the vector to break off the disease transmissions [5]. Methods developed by WHO to combat dengue fever is the same as the method used to combat malaria, which is to eradicate the source of transmission, i.e. mosquito larvae [6]. Eradication of larvae is the key strategy of vector control programs around the world [7].

A. aegypti mosquitoes lay eggs in clear water that is not directly affected by land and prefers containers indoor rather than outdoor due to the indoor temperature is relatively stable. A mosquito can lay eggs 4-5 times during her life with an average number of eggs ranges from 10-100 eggs in a single spawn. Thus the total number of eggs produced by a single female mosquito is between 300-700 eggs [8].

Physical control is conducted by managing the environment to prevent mosquito from breeding. Biological control is performed using predators and pathogenic organisms, while chemical control is carried out by applying synthetic insecticides to kill mosquitoes. Genetic control is done by spreading the sterile males into the ecosystem, and integrated control is performed by combining the various existing control techniques [9]. The most widely used mosquito control is the chemical control. The reason for this selection is the prompt results of this control. However, chemical control using synthetic insecticides actually causes adverse side effects, such as the mosquitoes could become resistant, human and livestock poisoning, contamination of vegetables and fruit gardens, as well as environmental pollution.

The development of new insecticides that are more environmentally friendly and do not pose hazard needs to be done. The use of bioinsecticide looks promising. Bio-insecticide or biological insecticide is an insecticide which is derived from plant material containing chemicals (bioactive) that are toxic to insects but are easily biodegradable in nature. So, it will not pollute the environment and relatively safe for

A. Komansilan is a PhD student at the Graduate School of Agricultural Science, Brawijaya University, Malang 6514 Indonesia and a lecturer at the Dept. of Physics Education, State University at Manado, Tondano 95618, Indonesia (Corresponding author, phone: +62-431-321845; fax: +62-431-321866; e-mail: alfritskomansilan@gmail.com).

A.L. Abadi is with the Faculty of Agriculture, Brawijaya University, Jl. Veteran 2, Malang 6545, Indonesia.

B. Yanuwiadi is with Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Jl. Veteran 2, Malang 6545, Indonesia.

D.A. Kaligis is with the Faculty of Animal Husbandry, Sam Ratulangi University, Jl. Kampus Kleak, Manado 95115, Indonesia.

human. Besides, vegetable insecticides are also selective [10].

Research on bioactive compounds in the Annonaceae family is growing rapidly. Acetogenin compounds from *Annonaceae* type were reported to have toxicity that is effective against insects of several orders such as *Lepidoptera, Coleoptera, Homoptera* and *Diptera* [11, 12]. Other studies reported that *Annonaceae* family contains acetogenin that are larvicidal. Acetogenin also acts as an insecticide, acaricide, antiparasitic and bactericidal [13]. One plant in the *Annonaceae* family that has been assessed of its active compound content is Annona muricata Linn also known as Soursop. This plant can be used as traditional food and insecticides [14]. Soursop seed extract contains annonacin, bullatacin, annonin VI, goniothalamin, and sylvaticin bioactive compounds [15]. Soursop plants contain insecticidal compounds that when synthesized with different procedures will produce different active compounds.

Preliminary test results indicated that the ethanol extract of soursop seeds is an active agent of larvicide. Phytochemical test shows that ethanol soursop seeds extract contains secondary metabolites compounds group of saponin, alkaloids and triterpenoids. These compounds are defense chemical compounds of plant produced in the plant tissue. They are toxic and can also act as the stomach and respiratory poison [16]. This paper will discuss our work in isolating and identifying the bioactive agents of larvicide from the soursop seeds for dengue fever mosquito. We used *A. aegypti* larvae as the bioindicator.

II. Materials and Methods

A. Extract Preparation

We obtained the soursop seeds from restaurants and households in the city of Manado which were fruit juice waste. Prior to blending to make extract, the seeds were cleaned and dried by fan (not under direct sunlight). 2 kg of soursop powder was extracted by maceration using a 15 L ethanol several times for 24 hours until all components were extracted out. The obtained ethanol extract is evaporated to a vacuum rotary evaporator to thicken. The thickened ethanol extract was diluted with 200 mL of ethanol-water, with a ratio of 7:3. The water-ethanol extract was then partitioned with *n*-hexane (3 × 200 mL) to obtain ethanol-water extract and *n*-hexane extract. Ethanol-water extract was evaporated until all the ethanol evaporates and the remaining water extract was partitioned successively with ethyl acetate (3 × 200 mL), and n-butanol (3 × 200 mL). We collected some 190 g ethanol, colored dark brown.

B. Toxicity Test

The derived extracts, e.g. n-hexane, ethyl acetate, and n-butanol extracts, were tested for its biological activity against *A. aegypti* larvae. The most toxic fraction was then purified with a thin layer of chromatography and column chromatography. Separation by column chromatography produced many fractions that were then combined by using the TLC merger. The merged fraction was then tested for its toxicity to *A. aegypti* larvae. The production of media for Aedes aegypti larvae was done by filling a plastic container with water and coating the inner wall with filter paper. Filter paper served as a place to lay the mosquito's eggs. The eggs that were attached to the filter paper was then dried at room temperature and stored in sealed containers. For the hatching of the eggs, the filter paper was dipped into a plastic tray containing water and after 24 hours the eggs would hatch and grow into instar I larvae.

Instar I larvae will develop to instar II, III (4 days) and IV (2 days) larvae. Every two days the larvae were fed with 1-2 g of fish pellets. The water in the larval breeding media was replaced every 2 days. The larvae will grow into pupae for 8 days. The sample used in the study was the instar III/IV larvae.

Larvicide test was performed in 13 vials prepared for testing, 12 vials were for the samples and one vial was for the control. The experiments were conducted in triplicate. 10 mg of each sample was diluted with 10 mL of ethanol. The solution was transferred to the vials as much as 2500 µL, 500 µL, 50 µL, respectively. The solvent was evaporated for 24 hours in desiccators. To each vial was added 5 mL of water, 50 µL of DMSO and 10 *A. aegypti* mosquito larvae, and then the extract solution was added water until the volume reached 10 mL in a concentration of 500, 100 and 10 ppm. For the control, to the vial was added 5 mL of water, 50 µL DMSO and 10 mosquito larvae was then added water until the volume reached 10 mL.

Observations were carried out for 24 hours on the death of the larvae. Data analysis was performed to seek the death concentration (LC50). After the separation and purification process on the most toxic isolate were done, we continued with the identification of larvicide active compounds from the soursop seeds using GC-MS spectrometer.

III. Results and Discussion

A. Toxicity Test

The ethanol extract was diluted with 100 mL of ethanol-water with 7:3 ratio. The mixture was then partitioned successively using the n-hexane, ethyl acetate and *n*-butanol solvents, respectively. The products were 14.7 g thick brown-black *n*-hexane extract, 0.8 g thick yellowish brown ethyl acetate extract, and 4.6 g thick yellowish brown *n*-butanol extract, respectively. The three extracts from the above partition were then tested for their toxicity. The toxicity test of each extract showed the *n*-hexane extract was the most toxic to the *A. aegypti* larvae with LC50 value = 73.77 ppm, while the ethyl acetate extract was toxic to *A. aegypti* larvae with LC50 = 340.71 ppm, and the n-butanol extract with LC50 = 725.18 ppm was the least toxic compound.

B. Isolation and Identification

The most toxic *n*-hexane extract was separated and purified with column chromatography technique. Prior to separation

with column chromatography, eluent selection was performed to find the best eluent that is capable of separating compounds contained in n-hexane extract using thin layer chromatography (TLC). The use of several eluent mixtures with different polarities to separate the chemical components in n-hexane extract has been tried in the TLC. The eluents used were *n*-hexane : ethyl acetate (9:1), chloroform : *n*-hexane (8:2), n-hexane : ethyl acetate (8:2), chloroform : *n*-hexane (7:3), and *n*-hexane : ethyl acetate (7:3). Sample spotting on the TLC plate was done by using a micro pipette in which the dot diameter has to be as small as possible to avoid spreading of the stain or spot-tailed. The *n*-hexane eluent : ethyl acetate (7: 3) was the best eluent since it was able to separate the four compounds contained in the *n*-hexane extract with distance between spots was far enough. Therefore, it can be used in the separation process using column chromatography (CC).

2.0 gram of thick *n*-hexane extract was used for column chromatography separation, using 100 g of silica gel 60, with an eluent mixture motion phase of *n*-hexane : ethyl acetate (7: 3). The motion phase flow rate used was ±1 mL/min. The eluate was drained to vials every 3 mL each and produced 170 vials. They were then brought to the thin layer chromatograph (TLC) again. The eluates were grouped into four groups of fraction. The toxicity test was applied to the groups and the results were summarized in (Table I). The toxicity test revealed that the four fractions from *n*-hexane were all toxic to *A. aegypti* larvae. The F2 fraction was the most toxic with LC50 value = 117, 27 ppm.

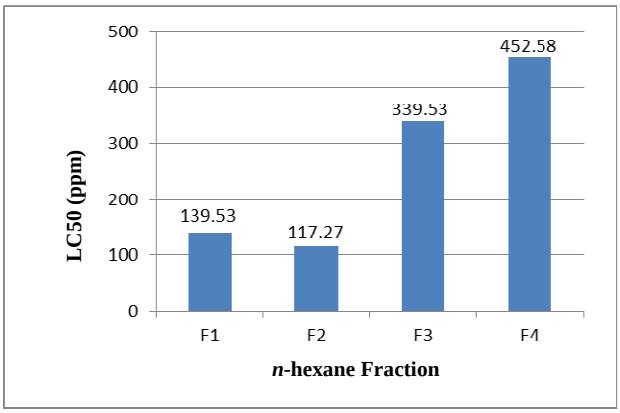

Fig. 1. The lethal concentration LC50 of *n*-hexane fraction to *A. aegypti* larvae after 24 h of experiment.

TABLE I
TOXICITY TEST RESULTS FOR *N*-HEXANE FRACTION OBTAINED FROM SOURSOP SEEDS AFTER 24 H OF TREATMENT

Fraction Type	Concentration (ppm)	Number of Death Larvae (×10 larvae)			Mortality Percentage
		Repeat # 1	Repeat # 2	Repeat # 3	
Control	0	0	0	0	0
Fraction F_1	500	10	10	10	100
	100	0	0	1	3.33
	10	0	0	0	0
Fraction F_2	500	10	10	10	100
	100	1	3	1	16.66
	10	0	0	0	0
Fraction F_3	500	8	10	9	90
	100	0	0	1	3.33
	10	0	0	0	0
Fraction F_4	500	8	10	6	80
	100	0	0	0	0
	10	0	0	0	0

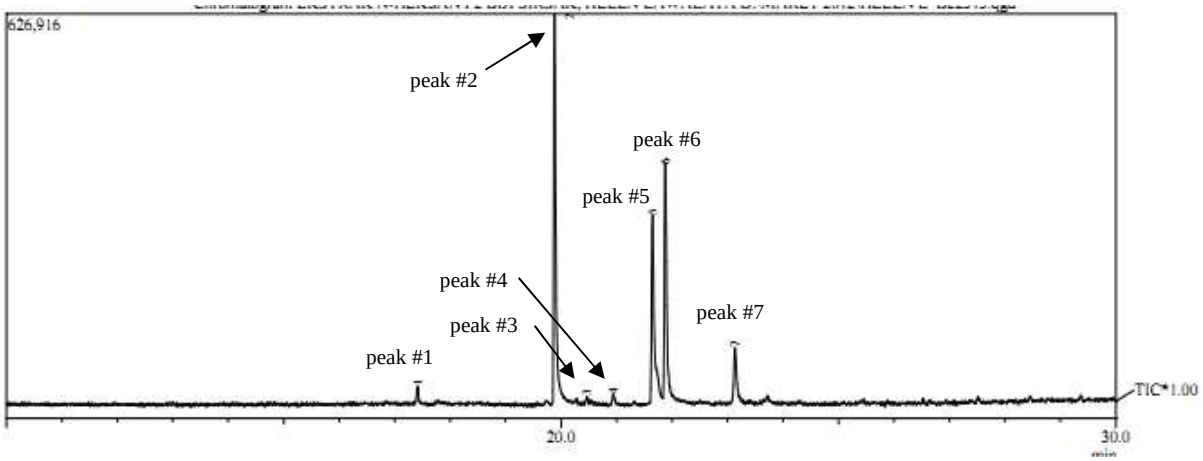

Fig. 2. The GC chromatogram of F2 fraction isolate showing seven peaks.

The isolate terms can be further identified with spectroscopic isolate methods that should be relatively pure in TLC, which has at least one spot/stain. Therefore, the F2 fraction proceeds to the purity test using thin-layer chromatography test with a solvents or eluents of different polarities, such as *n*-hexane : ethyl acetate (7:3); chloroform : *n*-hexane (8:2), and methanol : *n*-hexane (9:1). The test result showed that the F2 fraction contained only one compound that was indicated by only a single spot/stain with various eluent mixtures used. This suggests that isolate of F2 fraction was relatively pure in TLC.

The most toxic F2 isolate fraction was then analyzed using GS-MS spectroscopy to determine its compound content. The GC chromatogram of F2 isolate fraction (Figure 2) shows seven peaks with three dominant peaks. There are three peaks with abundance level <2%, they are compounds 1, 3, and 4 with abundance levels of 1.45, 0.79, and 0.66%, respectively. Compound 7 has an abundance level 6.43% and the remaining three compounds (2, 5 and 6) have abundance levels of 39.93, 25.05, and 25.71%, respectively. The chromatogram reveals that F2 fraction was relatively not pure. The information from the GC-MS of Figure 3 was summarized in Table II.

TABLE II
THE COMPOSITION OF COMPOUNDS CONTAINED IN F2 FRACTION FROM THE GC-MS EXAMINATION

Peak #	Retention Time (min)	Abundance (%)	Possible Compound
1	17.416	1.45	2-cyanoethyl 2-(2-methyl-5-nitrocyclohexyl) acetate
2	19.883	39.93	methyl palmitate
3	20.467	0.79	2-cyanoethyl 3-(iminomethylene)dek-6-enoat
4	20.942	0.66	vinyl 2-(iminomethylene)non-4-enoat
5	21.649	25.05	methyl oleate
6	21.880	25.71	methyl stearate
7	23.135	6.43	10-nonadecanol

It can be seen in Table II that the isolation of F2 fractions of soursop (*Annona muricata Linn*) seeds contained organic fatty acids and alkaloids with the main components of methyl palmitate fatty acid with abundance level of 39.93%. The molecular structure of is methyl palmitate fatty acid is illustrated in Fig. 3.

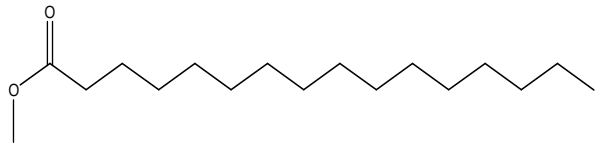

Fig. 3. The structure of methyl palmitate fatty acid.

IV. CONCLUSION

Phytochemical test results showed that the soursop seed extract contained secondary class metabolite compounds of saponins, alkaloids and triterpenoids. We found from preliminary test results that the ethanol extract of active soursop seeds has a potential larvicidal agent with an LC50 = 244.27 ppm. Nevertheless, the biological activity test results revealed that the *n*-hexane extract has the highest toxicity with LC50 value of 73.77 ppm.

Identification of the most active anti larvicide F2 fraction with GC-MS contained three dominant components of the chemical compounds that are organic fatty acids, namely methyl palmitate, methyl oleate and methyl stearate. Presumably these compounds are synergistic anti larvicide active against *Aedes aegypti* mosquito larvae.

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