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Research Article

Larvicidal Activities of Extract Flower *Averrhoa bilimbi* L. Towards Important Species Mosquito, *Anopheles barbirostris* (Diptera: Culicidae)

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Abstract

The extract flower of *Averrhoa bilimbi* was studied for its larvicidal and chemical composition against malaria vector mosquito, *Anopheles barbirostris*. The larvicidal assay was conducted to record the LC_{50} and LC_{84} values. The larva mortality was observed after 24, 48 and 72 h of exposure period. The LC_{50} value of extract were 8.892 ppm after 24 h exposure, 4.015 ppm after 48 h exposure and 540 ppm after 72 h exposure whereas the LC_{84} value of extract were 66.881 ppm after 24 h exposure, 27.836 ppm after 48 h exposure and 2.084 ppm after 72 h exposure. Totally 22 compounds were identified by GC-MS. The major chemical compounds were cycloeicosane followed by benzenedicarboxylic acid and benzenepropanoic acid. The results of this study showed that extract of *Averrhoa bilimbi* is inexpensive, eco-friendly and save for human sources of natural mosquito larvicidal agent to control and reduce population of malaria vector mosquito.

Key words: *Averrhoa bilimbi*, larvicidal, chemical composition, *Anopheles barbirostris*

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The malaria has been a major killer disease in many countries of Africa and Asia, where it affects approximately 300-500 million people annually, most of them are children (Garcia, 2010). Malaria is a major public health problem in most tropical countries, including Indonesia. Recent models estimate that over 105 million of Indonesia's 239 million populations are at risk for malaria infection, with transmission varying widely across this most populous entirely tropical country (The Global Health Group, 2011). Though formally reported deaths are less than 1,000 annually with 6 million clinical cases and 700 deaths each year. The number of deaths caused by malaria is not known. The most recent WHO estimate of the number of deaths due to malaria is approximately 3,000 per year in Indonesia (WHO., 2011a).

This effort is in line with the recent rise of malaria elimination in the global public health agenda, signaled by the commitment of the 60th World Health Assembly in 2007 that all countries should commit to eliminate malaria by 2050 (WHO., 2007) and Indonesia aims to eliminate malaria by 2030 (Ministry of Health Indonesia, 2009).

Insect vectors, especially mosquitoes are responsible for spreading serious human diseases like malaria, Japanese encephalitis, yellow fever, dengue and filariasis. The various synthetic products and devices designed to combat such vectors are not successful because of increased resistance developed by various mosquito species. Most of the mosquito control programmes target the larval stage in their breeding sites with larvicides, because adulticides may only reduce the adult population temporarily (Ghosh *et al*., 2012).

Various chemical compounds are being used to control larval and adult of mosquito. Insect growth regulators (methoprene, novaluron and pyriproxifen) and the organophosphate, temephos, which has low mammalian toxicity, low odor and is available in long-lasting formulations, are applied in different strategies to mosquito breeding sites to reduce larval populations (WHO., 2011b; Ranson *et al*., 2010; Vontas *et al*., 2012). One recent approach proposes the auto-dissemination by adult females attracted to resting spots containing the insecticides and subsequent spread to new breeding sites (Caputo *et al*., 2012).

Increased use of insecticides for agricultural pest control, for direct control of mosquito or for control of sympatric vectors (such as other *Anophelinae* and *Culicinae* species) has imposed selection pressures on mosquito populations for increased resistance. Resistance to larvicides, primarily temephos, is documented in Asia (China, Pakistan, Malaysia and Thailand), Central and South America (Caribbean islands and Brazil) and Europe (Italy and Greece) programs (Ranson *et al*., 2010; Vontas *et al*., 2012). Resistance to DDT and pyrethroids is recognized in mosquito populations native to Asia, including China, Pakistan, Malaysia and Thailand and emerging in Africa (Cameroon) (Ranson *et al*., 2010; Vontas *et al*., 2012; Kamgang *et al*., 2011; Hafiz *et al*., 2011; Wan-Norafikah *et al*., 2013; Chuaycharoensuk *et al*., 2011). The monitoring of insecticide resistance, adoption of standardized procedure for resistance assessment, publication of results through a centralized database and characterization of biomarkers for understanding resistance would be of great benefit to rational design of control programs (Ranson *et al*., 2010; Vontas *et al*., 2012). The selective pressure of conventional insecticides, such as organochlorines, carbamates, pyrethroids and Organophosphorus Compound (OP), is enhancing resistance of mosquito populations at an alarming rate, resulting in widespread resurgence, undesirable effects on non-target organisms and environmental and human health concerns.

Because of the high cost of developing new drugs and vaccines, development of drug resistance and concerns over drug residues associated with the continuous use of chemicals, there is a renewed interest in the use of botanicals for the safe, effective and cheap control of pests of agriculture and public health importance (Yildirim *et al*., 2012). Scientists all over the world are now actively engaged in research into the use of plants and plant-derived products to fight against dengue vector. Botanical products are effective, have no harmful effects on environment and non-targeted organisms, easily biodegradable, inexpensive and readily available in many areas of the world. Plants and plant derived products are rich in natural phytochemicals (Fan *et al*., 2011), which make them effective against different microbes and pests. Some of these chemicals have also been used successfully for controlling mosquito because of their larvicidal, ovicidal and skin repellent effects.

One of the most effective alternative approaches under the biological control programme is to explore the floral biodiversity and enter the field of using safer insecticides of botanical origin as a simple and sustainable method of mosquito control. Further, unlike conventional insecticides which are based on a single active ingredient, plant derived insecticides comprise botanical blends of chemical compounds which act concertedly on both behavioral and physiological processes. Thus there is very little chance of pests developing resistance to such substances. Identifying bio-insecticides that are efficient, as well as being suitable and adaptive to ecological conditions, is imperative for continued effective vector control management. Botanicals have widespread insecticidal properties and will obviously work as a new weapon in the arsenal of synthetic insecticides and in

future may act as suitable alternative product to fight against mosquito borne diseases (Ghosh *et al*., 2012).

Biologically active plant extracts have been, therefore studied for their potential efficacy to minimize the extent of population and reduce the cost (Liu *et al*., 2013). Use of plant extracts is one of the possible methods of pollution free method in insect control. Promising results have been achieved towards attaining this goal by treating eggs, nymphs and adult insects with extract of whole plants, leaves, roots fruits and seeds of various species of plants (Panneerselvam and Murugan, 2013).

There is no report of insecticidal activity in *Averrhoa bilimbi* flower and this study shows that it has a good potential to be used as a biopesticide for malaria vector mosquito and at the same time it safeguards for human health and environment.

MATERIALS AND METHODS

The flowers of *Averrhoa bilimbi* was collected from South Jakarta District (06°14'55''S 106°46'06.6''E). The flowers dried 3 days in the shade at the environmental temperatures, 25-36°C then were powdered mechanically using commercial electrical stainless steel blender and extracted with methanol 95% in a soxhlet apparatus (boiling point range 60-80°C) for 2 days. The extract was concentrated under reduced pressure 22-26 mm Hg at 32°C using rotary evaporator and the crude extract obtained was stored at 4°C. One gram of crude extract was first dissolved in 100 mL of acetone (stock solution). From the stock solution, 1,000, 5,000, 10,000, 15,000 and 20,000 ppm were prepared with dechlorinated tap water.

Mosquito culture: *Anopheles barbirostris* larvae were collected from Medical Research Institute, to start the colony and larvae were kept in plastic and enamel trays containing tap water. They were maintained at 27±2°C and 75-85% relative humidity under 14:10 light and dark cycles. Larvae were fed a diet of yeast, biscuits and algae collected from ponds in a ratio of 3:1:1, respectively. Pupae were transferred from the trays to a cup containing tap water and were maintained in our insectary (45×45×40 cm) where adults emerged. Adults were maintained in glass cages and continuously provided with 10% sucrose solution in a jar with a cotton wick.

Larvicidal bioassay: Bioassays were conducted essentially following the standard (WHO., 1981) larval susceptibility test method at laboratory. Test were carried out in ten repilate with 10 larvae in each petridish. Result were obtained after 24, 48 and 72 h after continuous exposure and were expressed as percent mortality. Pure water and methanol without flower extract served as control. The mortality was calculated by POLO's formulae and LC_{50}, LC_{90} of 95% confidence were calculated by using probit analysis (Robertson *et al*., 2007).

The numbers of dead larvae were counted after 24, 48 and 72 h of exposure and the percentage mortality was reported from the average of ten replicates. However, at the end of 72 h the selected test samples turned out to be equal in their toxic potential.

Gass chromatography mass spectrometry analysis: GC-17A gas chromatograph equipped with a DB-5 column (30×0.25 mm id, 0.25 µm film thickness, J and W) and a Hitchi M-7200 mass selective detector were used for chemical analysis. Helium was used as the carrier gas at a column head pressure of 100 kPa. GC was set for splitless injection (splitter open after 1 min). The temperature program of the column oven was isotherm 5 min at 35°C, 4°C/min gradient to 200°C, isotherm 16 min at 200°C, 10°C/min gradient to 300°C and isotherm 5 min at 300°C. The injector and detector temperature were 220 and 150°C, respectively.

RESULTS

Larvicidal toxicity: The result of larvicidal toxicity towards extract flower of *Averrhoa bilimbi* is summarized in Table 1. It shows that the minimum dosage as low as 1,000 ppm caused mortality 24 h after application while 20,000 ppm has an effect on the mortality of larvae significantly compared to other concentrations.

Table 1: Mortality larva of *Anopheles barbirostris* towards extract flower *Averrhoa bilimbi*

	Hours after application (h)		
Concentrations (ppm)	24	48	72
1,000	2.1^{b}	3.6^{b}	7.9^{b}
5,000	2.9^{b}	4.4^{b}	9.7^{c}
10,000	6.5^{c}	7.8^{c}	10^{c}
15,000	6.9^{cd}	7.9^{c}	10^{c}
20,000	7.4^{d}	9.1^{d}	10^{c}
Control	0.0^{a}	0.2^{a}	0.4^{a}

Numbers followed by the same character on the same column shown not significant with Turkey HSD^{a} Test 5%

Table 2: Probit analysis extract flower of *Averrhoa bilimbi* towards *Anopheles barbirostris* larvae

	Hours after exposure (ppm)			
Lethal concentrations	24	48	72	Significance level
LC_{16}	3.072	579.25	579.25	
LC_{50}	8,892.64	4,015.55	540.34	0.05
LC_{84}	66,881.38	27,836.68	2,084.13	

The increasing concentration 1,000-5,000 ppm and to 10,000 ppm after 24 and 48 h after application caused increased mortality larvae. Larva of *Anopheles barbirostris* mortality achieved more than 50% after 24 h in concentration 10,000 and 15,000 ppm whereas on 20,000 ppm mortality was achieved more than 74% after 24 h after application. Control did not have any influence at all on the larvae.

In the toxicity study, a total of 5 different concentrations were investigated and their respective toxicity data are given in Table 2. The 50% lethal concentration or LC_{50} value is commonly accepted as the basis of comparison to investigate relative toxicity among compounds. The LC_{50} value is defined as the concentration that will kill half the population of the test insect, in this case, *Anopheles barbirostris* larva. In quantal response experiment using the probit method, with the data well dispersed over a wide range of concentrations, the LC_{50} concentration is more precisely determined than any other concentrations.

The data for each of the eight experiments were analyzed individually by the same method. As expected, the means of the eight values of the LC_{50} with the values for the pooled data. The LC_{50} from 24-48 h after exposure shows declining shape with the minimum concentration 8,892.64 ppm (S.E Log_{10} (LC_{50}) = 3.94 $p<0.05$). The shape reached the minimum concentration 540.34 ppm (S.E Log_{10} (LC_{50}) = 3.60; $p<0.05$) 72 h after exposure.

Gas chromatography mass spectrometry (GC-MS) analysis: By using GC-MS with authentic standard, components were eventually recognized in the flower crude extract of *Averrhoa bilimbi*. Comparing retention times with those compounds that coincided in retention checked the nature of the compounds. The compounds are listed in Table 3.

Twelve compounds were found at flower crude extract of *Averrhoa bilimbi*. Crude extract of *Averrhoa bilimbi* chemical analysis revealed 3,5-bis(1,1-dimethylethyl), $C_{14}H_{22}O$ (Retention time: 14.71 min = 92%) followed by 9-Octadecene; $C_{12}H_{26}O$ (Retention time: 15.97 min = 94%), Methyl tetradecanoate; $C_{15}H_{30}O_2$ (Retention time: 17.89 min = 81%), Cycloeicosane; $C_{20}H_{40}$ (Retention time: 18.72 min = 95%), 6,10,14-Trimethyl-2-pentadecanone; $C_{18}H_{36}O$ (Retention time: 19.37 min = 91%), Hexadecanoic acid; $C_{18}H_{36}O_2$ (Retention time: 20.27 min = 94%), Benzenepropanoic acid; $C_{18}H_{28}O_3$ (Retention time: 20.56 min = 78%), 1-Octadecene; $C_{18}H_{36}$ (Retention time: 20.96 min = 91%), 9,12-Octadecadienoic acid; $C_{18}H_{32}O_2$ (Retention time: 22.01 min = 93%), 9-Octadecenoic acid; $C_{19}H_{36}O_2$ (Retention time: 22.15 min = 92%), Methyl stearate; $C_{19}H_{38}O_2$ (Retention time: 22.34 min = 94%) and Hexadecane; $C_{20}H_{42}$ (Retention time: 26.58 min = 86%).

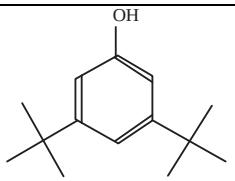
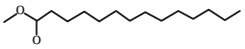
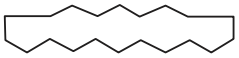
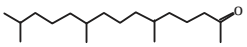
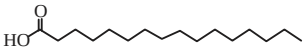
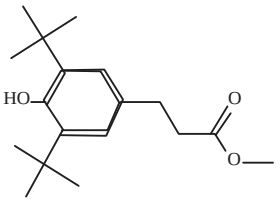
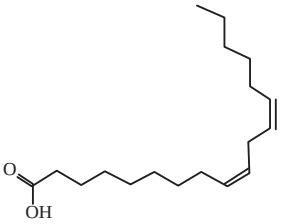
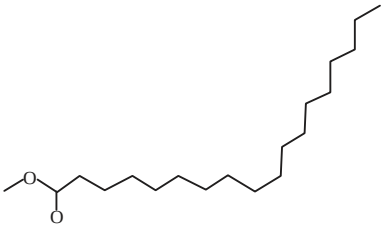
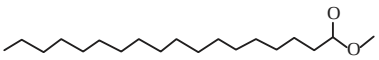
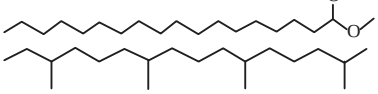
DISCUSSION

The important results obtained in this study confirmed that flower extract of *Averrhoa bilimbi* has insecticidal activity against malaria mosquito, *Anopheles barbirostris*. Since the best concentration were the minimum dosage as low as 1,000 ppm caused mortality 24 h after application while 20,000 ppm has an effect on the mortality of larvae significantly compared to other concentrations. The LC_{50} value of extract were 8.892 ppm after 24 h exposure, 4.015 ppm after 48 h exposure and 540 ppm after 72 h exposure whereas the LC_{84} value of extract were 66.881 ppm after 24 h exposure, 27.836 ppm after 48 h exposure and 2.084 ppm after 72 h exposure. Similar result was recorded by Kovendan *et al.* (2012) that the highest larval mortality on leaf extract of *Acalypha alnifolia* was methanolic extract to control three mosquito vectors. The early fourth-instar larvae of *A. stephensi* had values of LC_{50} = 197.37, 178.75, 164.34, 149.90 and 125.73 ppm and LC_{90} = 477.60, 459.21, 435.07, 416.20 and 395.50 ppm, respectively. The *A. aegypti* had values of LC_{50} = 202.15, 182.58, 160.35, 146.07 and 128.55 ppm and LC_{90} = 476.57, 460.83, 440.78, 415.38 and 381.67 ppm, respectively. The *Culex quinquefasciatus* had values of LC_{50} = 198.79, 172.48, 151.06, 140.69 and 127.98 ppm and LC_{90} = 458.73, 430.66, 418.78, 408.83 and 386.26 ppm, respectively.

The results of the leaf extract of *A. alnifloia* are promising as good larvicidal activity against the mosquito vector, *A. stephensi*, *A. aegypti*, *C. quinquefasciatus*. Nurhayati (2013) also reported that leaf extract *Averrhoa bilimbi* had effectiveness larvicidal on *Aedes aegypti* since *Averrhoa bilimbi* leaf extract had significance correlation based on statistical test between concentration elevation and count of larva mortality at 24 h test.

The major chemical compounds of extract flower of *Averrhoa bilimbi* were cycloeicosane followed by benzenedicarboxylic acid and benzenepropanoic acid. Lalitharani *et al.* (2009) reported that using GC-MS analysis of

Table 3: Compound found at flower crude extract of *Averrhoa bilimbi*

Compound names	Retention time (min)	Percentage	Molecular weight	Molecular formula	Structural formula
3,5-bis (1,1-dimethylethyl)	14.71	92	206	$C_{14}H_{22}O$	
9-Octadecene	15.97	94	186	$C_{12}H_{26}O$	
Methyl tetradecanoate	17.89	81	242	$C_{15}H_{30}O_2$	
Cycloeicosane	18.72	95	280	$C_{20}H_{40}$	
6,10,14-Trimethyl-2-pentadecanone	19.37	91	268	$C_{18}H_{36}O$	
Hexadecanoic acid	20.27	94	284	$C_{18}H_{36}O_2$	
Benzenepropanoic acid	20.56	78	292	$C_{18}H_{28}O_3$	
1-Octadecene	20.96	91	252	$C_{18}H_{36}$	
9,12-Octadecadienoic acid	22.01	93	280	$C_{18}H_{32}O_2$	
9-Octadecenoic acid	22.15	92	296	$C_{19}H_{36}O_2$	
Methyl stearate	22.34	94	298	$C_{19}H_{38}O_2$	
Hexadecane	26.58	86	282	$C_{20}H_{42}$	

Pothos scandens leaf revealed the presence of dodecanoic acid, tetradecanoic acid, 3, 7, 11, 15- Tetramethyl-2-hexadecan 1 ol, n-Hexadecanoic acid, phytol, 9, 12, Octadecaadienoic acid (Z,Z)-, 9,12,15, octadecatrienoic acid (Z,Z,Z)-1,2-benzenedicarboxylic acid, diisooctylester. Among the identified phytochemicals, dodecanoic acid, tetradecanoic acid and n-Hexadecanoic acid have the property of antioxidant activity. 9,12, octadecadienoic acid (Z,Z)-and 9, 12, 15-octadecatrienoic acid (Z,Z,Z)-have the property of anti-inflammatory and antiarthritic. The presence of phenols, flavonoids and tannins in the plants is likely to be responsible for the free radical scavenging effects observed. Flavonoids and tannins are phenolic compounds and plant phenolics are a major group of com-pounds that act as primary antioxidants or free radical scavengers (Iqbal, 2012; Saeed *et al*., 2012).

Since no previous record of flower extract of *Averrhoa bilimbi*, only (Shahreen *et al*., 2012) reported that fruit extract has ability to exhibit antibacterial activity against some of the

bacteria suggested the presence of hydrophilic and hydrophobic antibacterial compounds. It can be considered that the gram negative bacteria were more susceptible to the extracts when compared to the Gram-negative bacteria. Based on previous studies, the antibacterial activity of *A. bilimbi* could be associated with the presence of bioactive compounds of flavonoids type like luteolin and apigenin. Das *et al.* (2011) also reported that *A. bilimbi* methanolic fruit extract has antibacterial activity against some bacteria and also has potent cytotoxic activity. In comparison with Vincristine sulfate, the cytotoxicity exhibited by the methanolic extracts of leaf and fruit of *A. bilimbi* were highly significant. A comparison of cytotoxic activity of methanolic extracts of fruit and leaf. Fruit fraction was more cytotoxic than leaf portion. This clearly indicates the presence of potent bioactive principles in these extracts, which might be very useful as antiproliferative, antitumor, pesticidal and other bioactive agents.

Environmental safety is considered to be of paramount importance. An insecticide does not need to cause high mortality on target organisms in order to be acceptable but should be eco-friendly in nature. Phytochemicals may serve as these are relatively safe, inexpensive and readily available in many parts of the world. Several plants are used in traditional medicines for the mosquito larvicidal activities in many parts of the world. The screening of locally available medicinal plants for mosquito control would generate local employment, reduce dependence on expensive and imported products and stimulate local efforts to enhance the public health system. The ethno-pharmacological approaches used in the search of new bioactive toxins from plants appear to be predictive compared to the random screening approach. Ghosh *et al.* (2012) reported that recently developed new isolation techniques and chemical characterization through different types of spectroscopy and chromatography together with new pharmacological testing have led to an interest in plants as the source of new larvicidal compounds. Synergic approaches such as application of mosquito with botanical blends and microbial pesticides will provide a better effect in reducing the vector population and the magnitude of epidemiology.

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