Uncovering Potential of Neem and Pyrethrum Extract as Biolarvacide of Aedes aegypti for Dengue Control

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Abstract. Currently, several problems raised from the frequent application of chemical insecticide for dengue control such as insecticide resistance and environmental health issues. This fact supporting the need for the alternative active ingredients which are natural and could not develop insecticide resistance. This study aims to find out the potential of Pyrethrum (Chrysanthemum cinerariifolium (Trevir.) Vi) and Neem (Azadirachta indica A. Juss) as biolarvacide against Aedes aegypti. This study used a quasi-experimental study using several combinations of Pyrethrum and Neem extracts. The larvae test used 25 larvae for every combination. Three repetitions applied for each treatment. Larval deaths were observed every hour until 24 hours, and several dead larvae were calculated. Results of this study showed that almost all formulations caused death of larvae in the first hour except formulation 5 (2 ml Pyrethrum + 1 ml DMSO + 24 ml liquid paraffin + 24 ml chitin), formulation 8 (49 ml neem + 1 ml Tween 20) and formulation 11 (49 ml neem + 1 ml DMSO). These results emphasised the potential of a formulation containing Pyrethrum and neem as biolarvacide against Aedes aegypti.… (Introduction, methods, results, discussion, conclusion)

Keywords: dengue, pyrethrum, neem, larvacide

1. INTRODUCTION

Dengue is a mosquito-borne tropical disease caused by dengue virus (DENV) infection. There are commonly four antigenically distinct serotypes of DENV which are serotype-1, 2, 3, 4[1]. In October 2013, the fifth serotype (DENV-5) was found and caused new challenges in dengue control [2]. Dengue usually spread in tropical areas, due to the breeding site of its vector which are Aedes aegypti as primary and Aedes albopictus as secondary vector. This disease is transmitted by the female Aedes sp. mosquito bites which containing DENV [3]. Currently, when dengue vaccine is available, yet dengue prevention and control are still relying on vector control and avoidance from mosquito bites. Several problems raised from frequent application of chemical insecticide for dengue control, such as insecticide resistance and environmental health issues [4][5]. Several previous studies showed the insecticide resistance occurred around the world to several types of mosquitoes insecticide. [6][7] This fact supporting the need for natural alternative active ingredients which could not develop insecticide resistance. Several plants are known to have active ingredients to be used as larvacide, such as Pyrethrum (Chrysanthemum cineraraefolium (Trevir.) Vi) and Neem (Azadirachta indica A. Juss). Both plants contain active ingredients of nerve poisons such as pyrethrin and azadirachtin and have the potential to kill insects [8].

Indonesia has biodiversity sources, including plants that have active ingredients as larvacides. Flowers of Pyrethrum (Chrysanthemum cinerariaefolium (Trevir.) Vis) contain pyrethrin active compounds which could act as insecticides. Several active compounds contained in C. cinerariaefolium are pyrethroids, sesquiterpenoids, flavonoids, coumarins, triterpenoids, steroids, phenolics, purines, lipids, aliphatic compounds and monoterpenoid [9]. Pyrethrum extract is one of the essential natural insecticides extracted from Pyrethrum plants. The biologically active constituents of Pyrethrum extract are the pyrethrins, also known as ‘rethrins’. Previous research confirmed that pyrethrin and its derivatives are extensively used for insecticidal activity [10][11].

Another plant that has the potential to be a natural insecticide is Neem (Azadirachta indica A. Juss). A indica is a fast-growing, long live tree, 12 meters in height with unpleasant smelling wood. It has evergreen pinnate leaves and small fragrant yellow-white flowers, followed by green-yellow berries. Azadirachta indica also known as neem is a tree in the mahogany family Meliaceae. This plant is known as a plant that could be utilised responsibly for the pesticidal,

larvicidal, antifeedant or repellant action on various insects[12]. The active compounds of Neem are Nimbinen, Nimbidine, Meliantriol, Azadirachtin and Salanin. Neem seeds extract can be used to control various types of pests such as Helopeltis sp., Jengkat caterpillar, Aphis sp., Nilarvata sp., and Sitophilus sp. The active ingredients of neem seeds are useful to repel disturbing insects, prevent plant-eating pests, dispel adult larvae and insects, prevent the change of larvae skin, reduce egg production in female insects, and prevent female insects from placing eggs. [12]

Neem seeds contain 60% oil or fat from stearic, palmitic, oleic, linoleic, lauric, uteric and small amounts of essential oils [13]. Other active compounds from neem seeds are phenols, quinones, alkaloids, triterpenoids, and flavonoids. Residues from neem seeds are easily broken down into non-toxic compounds, making them friendly and safe for the environment. [13] A preliminary test was conducted to determine the biological activity of neem seed ethanol extract against mosquito larvae of Aedes aegypti as well as phytochemical preliminary tests. The preliminary test results showed that the neem seed ethanol extract was active as a larvicidal agent with an LC50 value of 282.29 ppm. The phytochemical test results stated that the ethanol extract of neem seeds contained secondary metabolites of the triterpenoid group, flavonoids and alkaloids. [14]

As a single substance, pyrethrin and azadirachtin are known to be very effective in killing mosquito larvae. However, to obtain a single active substance, the cost needed is very expensive, and its effectiveness still needs to be studied again. Various formulations were made to increase the efficacy of extracts against larvae, including by combining active substances with other substances such as chitin. Besides, it showed the effect of the emulsifier used in the effectiveness and extract's effectiveness in killing larvae. The present study aims to examine the potential of various formulations of extracts of Pyrethrum and Neem plants as Aedes aegypti larvacides.

2. MATERIAL AND METHODS

Research design

This study used a quasi-experimental design. Neem (Azadirachta indica) and Pyrethrum (Chrysanthemum cinerariaefolium) were exposed to larvae of Aedes sp.

Extraction of Pyrethrum and Neem

Fresh Neem leaves washed with water, then cut into small pieces. The leaf pieces dried at room temperature, then smoothed to powder. One hundred grams of dry powder macerated using 70% ethanol for 1 x 24 hours. The maceration process repeated for 3 (three) times by replacing ethanol every 24 hours. The extract was evaporated using a vacuum so that the thick extract was obtained. The concentrated extract dissolved with water (7:3) then ethanol was evaporated so that the remaining water extract is left. The water extract is partitioned using n-hexane solvent and evaporated again. The water layer was extracted with chloroform then chloroform evaporated. The remaining water extract was added with ethyl acetate and vaporised again using a vacuum evaporator so that Neem leaf extract was obtained. Formulations were made using solvents and surfactants [15].

Pyrethrum Flower Extract

Pyrethrum flowers were cut into small pieces, then 20 grams of it was wrapped using filter paper and inserted into soxhlet. The flowers were extracted using 96% ethanol for 100 mL at 780C until the solvent returned to its original colour and obtained from the pyretrial flower oil filtrate. The filtrate was purified by extraction using an extractor at a temperature of 780C until pure pyretrial flower oil was obtained. Formulations are made using solvents and surfactants [16].

Larvacidal test

The test was conducted at the Loka LitbangKes Pangandaran, National Institute of Health Research and Development, while Pyrethrum and Neem essential oil were obtained from the Balai Tanaman Obat and Aromatic Bogor (Balitro). The Formulations of Pyrethrum and Neem extracts 100% described in table 1. This study uses three content-based formulations of active ingredients (1.25% pyrethrin, 0,36% azadirachtin, and 0.6% azadirachtin).

Table 1. Formulation of Pyrethrum and Neem Extract

No	Formulation	Content
1	1 ml Pyrethrum + 1 ml Tween 20 + 24 ml palm oil + 24ml chitin	1,25% pyrethrin
2	1 ml Pyrethrum + 1 ml Tween 20 + 48 ml palm oil	1,25% pyrethrin

3	1 ml Pyrethrum + 1 ml Tween 80 + 24 ml palm oil + 24ml chitin	1,25% pyrethrin
4	1 ml Pyrethrum + 1 ml Tween 80 + 48 ml palm oil	1,25% pyrethrin
5	2 ml Pyrethrum + 1 ml DMSO + 24 ml liquid paraffin + 24 ml chitin	1,25% pyrethrin
6	2 ml Pyrethrum + 1 ml DMSO + 24 ml palm oil + 24 ml chitin	1,25% pyrethrin
7	30 ml neem + 1 ml Tween 20 + 20 ml palm oil	0,36% azadirachtin
8	49 ml neem + 1 ml Tween 20	0,6% azadirachtin
9	30 ml neem + 1 ml Tween 80 + 20 ml palm oil	0,36% azadirachtin
10	30 ml neem + 1 ml DMSO + 20 ml palm oil	0,36% azadirachtin
11	49 ml neem + 1 ml DMSO	0,6% azadirachtin

For the larvicidal test, instar 3 of Aedes aegypti larvae prepared in containers containing 25 larvae per cup. 10 mL (concentration of 10%) of formulations added to each cup. Three repetitions applied for each treatment. Larval deaths were observed every hour to 24 hours, and the number of dead larvae was calculated.

Data Analysis

The data were collected and grouped based on their lethal time. The data were categorized into a table to be discussed descriptively.

3. **RESULTS**

This study tested several formulations (Table 1) to Aedes sp. Larvae, then the larval mortality was observed up to 24 hours. The results can be seen in Table 2.

Table 2. Test results of 11 formulations against *Aedes aegypti* for 24 hours

No	Formulation	Number of tested larvae	LT90 (hour)	The average number of active larvae (24h)	The average number of dead larvae (24 h)
1	Control	25	-	25	0
2	Formulation 1	25	1	0	25
3	Formulation 2	25	1	0	25
4	Formulation 3	25	1	0	25
5	Formulation 4	25	1	0	25
6	Formulation 5	25	2	0	25
7	Formulation 6	25	1	0	25
8	Formulation 7	25	1	0	25
9	Formulation 8	25	>24	3	22
10	Formulation 9	25	1	0	25
11	Formulation 10	25	1	0	25
12	Formulation 11	25	>24	11	14

Table 2 showed that almost all formulations caused mortality in the first hour except formulation 5, formulation 8 and formulation 11.

4. **DISCUSSION**

The result of tests carried out on 25 Aedes aegypti larvae showed that the formulation with the main ingredient of Pyrethrin flower extract could cause larval mortality effectively (with most of the lethal time of 90 (LT90) were one hour). The longer LT90 was indicated by formulation five consisted of Pyrethrum flower extract, DMSO, liquid paraffin and chitin (1.25% pyrethrin). Compared to other formulations (formulations 1, 2, 3, 4, and 6), extract number 5 uses additional liquid paraffin in its formulation. Liquid paraffin is a mixture of hydrocarbons obtained from mineral oil, which is a thick liquid, transparent, non-fluorescent, colourless, almost odourless and almost tasteless liquid. Paraffin is not soluble either in water or ethanol.[17]

Liquid paraffin also can maintain microbial viability by preventing drying of microbial culture storage media [18]. The nature of paraffin is predicted as a factor that makes the decrease in the effectiveness of the formulation. The thick properties of liquid paraffin cause the formulation to spread more slowly throughout the surface of the water.

Chrysanthemum extract has been known to have toxic effects on insects. The high pyrethrin content of this plant is widely used in various insect control activities. The effectiveness test of herbal mosquito repellent named 'Morizena', which has the composition of leaves of Passiflora putida (40%), Chrysantemum cinerariaefolium (40%), and essential oils from Cymbopogon nardus (20%), is known to be more effective in killing adult Aedes aegypti than commercial insecticide at the same concentration (LC90:2977 ppm).[19]

Pyrethrin has long been known as a natural insecticide that has a high economic value. Demand for pyrethrin is quite high, yet it is not balanced with the production level of the pyrethrin. Several studies were conducted to improve the productivity of pyrethrin by developing tissue culture or biosynthetic methods with help from microbes or precursors such as ethrel, Chlormequat chloride, Paclobutrazol, and glucose. [20][21][22].

In addition to pyrethrin content, Chrysanthemum cinerariaefolium is also known to have a high flavonoid content. Flavonoids are known as anti-virus, anti-asthma, and have the potential as anti-cancer. [23]. Repellent test results on beetles also show that Chrysanthemum cinerariaefolium flowers are useful to repel adult pulse beetle (Callosobruchus chinensis Linn). [24].

Formulations with Neem ingredients (Formulations 7-12) have diverse effectiveness as well. Formulations 7, 9, and 10 have almost the same efficacy (LT90= 1 hour) while two other formulations (formulations 8 and 12) have LT90 longer than 24 hours. Neem leaves are known to have an antimicrobial activity that can kill pathogenic bacteria. Neem is also widely known as an anti-parasitic and often used for animal food. It has proven to be effective in neutralising snake poisons and insects in arid/desert areas.[25] Neem can also be used for anti-inflammatory as an alternative to dexamethasone.[26]

In addition to leaves and seeds, the neem tree is known to have Limonoid Insect Growth Regulatory (IGRs) which functions to inhibit the growth and development of insects. It can be used as an alternative since azadirachtin that usually has a larvicidal activity, is not present in its stem. [27] Neem secondary metabolites inhibit the biological activity of adult Anopheles

stephensi. The Azadirachtin test in the form of a commercial product has an effect in delaying oviposition, and the analysis shows a slightly toxic effect, it also affects both the follicular cell and the oxytocytes of An. Stephensi.[28] Another advantage of the neem is that it does not affect non-target insects so that the natural predators of the larvae will not be affected even though neem extracts were given in the form of granules. [29] The results of in vivo and in vitro test from the crude extracts of neem leaves are known to have the ability to inhibit dengue virus type two (DENV 2) replication. Minimum doses can inhibit the development of the virus so that it does not cause cytopathic effects. A maximum dose (120-130 mg/ml) can inhibit viral replication. It is confirmed from the absence of clinical symptoms, and RT-PCR examination confirmed the absence of virus-specific amplicons.[30]

Formulations 8 and 11 have lower effectiveness since it is suspected that there are effects of solvents or surfactants used in this formulation. Although the concentration of azadirachtin is 0.6% higher, the mixture of tween 20 and DMSO is considered to reduce toxic effects on larvae. Other formulations added to palm oil showed higher effectiveness for larval mortality even though the azadirachtin concentration in formulations was smaller (0.36%).

Palm oil is known as an antioxidant. An antioxidant is suitable for human well-being because it has carotenoid compounds. The human body can quickly absorb antioxidant [31]. The study predicts that in formulation containing a mixture of palm oil, the larvae rapidly consume the extract into its body. Besides, it is suspected that pyrethrin content is more optimal in oil because of its polarity properties. It was reported that the total pyrethrin content was higher in hexane extract, which happened to be a non-polar substance, compared to pyrethrin content in semi-polar methanol.[32]

This study's formulation might be useful for a researcher or practitioner to be developed further to achieve optimal use from essential oil. Further research is needed to improve the formulation and also to produce larvacide which could be widely implemented in community. Hopefully in the future, essential oil formulation will be the first choice for mosquito control.

The results of this study emphasised the potential of a formulation containing pyrethrum and neem as biolarvacide against Aedes aegypti. We concluded that almost all formulations contain mix of Pyrethrum, tween, DMSO, and Neem, tween, DMSO caused 100% mortality with the lethal time of less than an hour. Except for formulation contains Pyrethrum, DMSO liquid paraffin, and chitin; neem and tween; and neem and DMSO.

5.ACKNOWLEDGMENTS

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