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Research Article
Evaluation of Toxicity in Four Extract Types of Tuba Root against Dengue Vector, *Aedes aegypti* (Diptera: Culicidae) Larvae

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Abstract

Background and Objective: Since the Dengue virus spreads rapidly and the vector becomes resistant to insecticides and larvicides, exploration of new compounds that overcome resistance problems, are easily degraded and do not lead to bioaccumulation, is needed. This study evaluated four extract types of *Derris elliptica* represented the polar, semi-polar and nonpolar extract against the 3rd-instar larvae of *Ae. aegypti* and determined the effective concentration among the extracts. **Materials and Methods:** The crude extract was obtained from the maceration of root powder of the plant with methanol and subsequently evaporated. The crude extract was diluted in distilled water and partitioned sequentially with ethyl-acetate, n-hexane and water to obtain their fractions. All the fractions were evaporated to obtain their extract types. Initial bioassay test of the extracts with concentration ranges of 50, 100, 500 and 1,000 mg L^{-1} against *Ae. aegypti* larvae and resulted in 86-100% larval mortality rates at concentrations of 50 and 100 mg L^{-1}, except for water extract. The lower concentration range of 3, 5, 10, 25, 50 and 100 mg L^{-1} of three extract types were tested. **Results:** Larval mortality rates of 18.4-100, 1.6-99.2 and 0.8-98.4% with LC_{50} of 4.088, 14.066 and 21.063 mg L^{-1}, respectively for n-hexane, methanol and ethyl-acetate. FTIR analysis indicated nine lead compounds in which rotenone and ceramides were observed in all extract types. **Conclusion:** The n-hexane extract showed the highest larvicidal toxicity and its specific compounds are necessarily isolated to obtain pure bioactive ingredients.

Key words: Larvicidal potency, *Derris elliptica*, *Aedes aegypti*, tuba root extract, n-hexane

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Since the Dengue virus spread rapidly in the past five decades from nine countries in 1970 to 128 countries in the tropics and subtropics recently[1,2], community attention and involvement in Dengue endemic areas in controlling *Aedes aegypti* mosquitoes have increased[3,4]. Aedes mosquito larvae become a strategic target in the Dengue vector control, where temephos rely upon larvicides. The campaign to use temephos is done seriously following and combining with the other methods to control the adult and larval stage of Aedes mosquitoes. This phenomenon occurs widely and intensively in Dengue endemic areas throughout the world for a long time[5] and results in the emergence of resistant strains of *Ae. aegypti* larvae against temephos, which have been reported in many countries[6], including in Indonesia[7,8].

The development of *Ae. aegypti* larvae resistance to temephos has hampered the Dengue vector control program. This condition triggers the researcher to find the new chemical compounds that are effective, biodegradable and do not cause bioaccumulation in environment[9]. In line with these efforts, the utilization of the potential for tubal root toxicity has evolved from traditional to modern methods in solving the problem of controlling dengue.

Tuba (*D. elliptica* (Wallich) Benth) is a poisonous vine that is easily found on uncultivated agricultural land. This plant grows in the South Asian, Southeast Asian and Hawaiian regions[10]. Traditionally, the tuba roots have long been used by residents of the regions as a fish poison and plant pest pesticide[11,12]. The use of tuba root is related to chemical compounds contained in the plant comprising isoflavonoids[13], flavonoids[14,15], ceramides and polyhydroxy acids[16], as well as rotenoids[17] which include compounds such as rotenone, deguelin, toxicarol, sumatrol, elliptone and malaccol[18-21].

Previously, the studies on the larvicidal toxicity of tuba root extract against *Ae. aegypti* larvae rapidly develop in several regions to find the new active compound of larvicide. A study in Thailand found that tuba root extract with petroleum ether (PE) and methanol solvents showed different toxicity, where the PE extract showed a lower lethal concentration of 50% of mortality LC_{50} and LC_{90} rather than the others, namely[22] 11.17 and 27.74 mg L^{-1}. Two other studies in two different countries tested the *D. elliptica* root extract that was resulted from a combination of two solvents. In Malaysia, a 1:1 combination of methyl-chloride and methanol results in higher toxicity rather than 1:9 combination against mosquito larvae with LC_{50} of 24 and 32 mg L^{-1}, respectively[23]. In India, a study found that PE extract of tuba root also resulted in higher toxicity against *Ae. aegypti* larvae rather than the combination of the methanol-chloroform extract with LC_{50} of 0.616 and 4.21 mg L^{-1}, respectively[24]. Similar studies have also been reported from Indonesia. A study on the toxicity of liquid ethanolic extract of tuba root against the filial one (F1) larvae of wild-caught *Ae. aegypti* larvae showed that the concentration 0.5% caused 86% of mortality rate[25], while another study using the ethanolic extract of tuba root showed the higher larvicidal potency against the laboratory strain of *Ae. aegypti* larvae with[26] LC_{50} of 47.7526 mg L^{-1}. But the temephos-resistant *Ae. aegypti* larvae needed a higher effective concentration of methanolic extract of tuba root with the LC_{50} and LC_{90} were 1,600 and 2,040 ppm, respectively[27]. These studies indicated that *D. elliptica* root extract has a variation of toxicity based on the extraction of solvents and habitat geographic origin. Another study showed that the number and type of secondary metabolites were influenced by the extraction solvent[28], while the composition of chemical constituents is affected by environmental habitat and climate conditions[29-32]. Based on the phenomenon, this *in-vitro* study aims to obtain the highest toxicity and the effective concentration of the local *D. elliptica* extract against the 3rd-instar larvae of laboratory strain *Ae. aegypti* based on the distilled water, methanol, ethyl acetate and n-hexane extract types.

MATERIALS AND METHODS

Study area: This study was conducted in eight months from March to October 2019. Tuba roots were taken from uncultivated lands in the hilly areas of the Samping village of Kemiri sub-district of Purworejo district, Central Java Province, Indonesia. The extraction process was conducted in the Natural Chemical Laboratory of Sciences and Mathematics Faculty of Garut University, West Java Province while the mosquito rearing and bioassay tests were conducted in the Epidemiology and Tropical Diseases Laboratory of Public Health Faculty of Universitas Muhammadiyah Semarang, Indonesia.

Plant collection and extraction: The vine stems of Tuba plants in the ground were gently pulled out so that the roots did not break. The base of roots was cut, cleaned and dried in the shade, before being sent to the laboratory for the extraction process. The study used the previous procedure of extraction and fractionation[33-35] with modification (Fig. 1). Briefly, the crude extract was obtained by maceration of six kilograms of tuba root dry powder in methanol for 3×24 h. The filtrate was separated from the residue and evaporated to produces 400 g of methanol extract. The polarity of the extract was separated by the liquid-liquid partition method. As much as 250 mL of aquadest was added to 120 g of solid

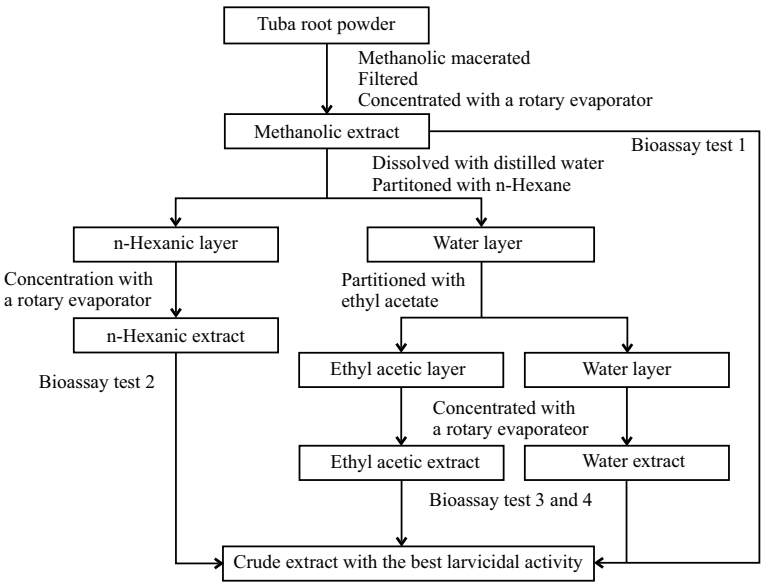

Fig. 1: Steps of the extraction and sequential fractionation of *D. elliptica* root using four different polarity solvents

methanol extract and stirred until completely homogeneous. Homogenate was entered into a 500 mL separation funnel and 250 mL of n-hexane was added to separate the low polarity compounds, then shaken until it completely separates the top layer (n-hexane phase) and the bottom (water phase). The top and bottom layers were separated. The top layer was evaporated to obtain the solid n-hexane extract. In the lower part, ethyl acetate was added to separate the semi-polar compounds. The mixture was processed with a separation funnel like the previous procedure to obtain the upper layer (ethyl-acetate phase) and the lower (water layer). Both fractions were evaporated separately to obtain the solid ethyl acetic and water extracts. A part of the four types of extracts was prepared for larvicidal activity (bioassay) test and phytochemical analysis using the Fourier Transform Infrared (FTIR) spectrophotometer.

Experimental mosquitoes: The parental *A. aegypti* mosquitoes were obtained in the larval stage from Sendang Mulyo village of Blora district, Central Java Province, Indonesia. Larvae were maintained to be the adult mosquito in the Epidemiology and Tropical Diseases Laboratory. To obtain thousands of larvae with the same age, the parental *Ae. aegypti* mosquito was reared up to the third generation. During the rearing process, mosquitoes were fed with guinea pig's blood and larvae were fed with dog food. The experiment temperature condition was maintained at the range of 25-28°C and humidity of 70-80%. The late third or early of the fourth instar of filial (F3) larvae was subjected to the bioassay test[36].

Larvicidal bioassay: To determine the larvicidal toxicity of the *D. elliptica* root extracts, the WHO guideline was used[34]. Briefly based on the modification of previous study[24], the initial bioassay used four concentration ranges, namely 50, 100, 500 and 1000 mg L^{-1} in 100 mL distilled water for each *D. elliptica* root extract from the four solvents (methanol, n-hexane, ethyl acetate and distilled water) and placed in the plastic cup. Each concentration level was prepared five times replication so that there were a total of 20 cups in each group of extract type. A total of 25 third instar larvae of *Ae. aegypti* were contacted for 24 h with the *D. elliptica* root extract solution in each cup. Two control groups were provided in this experiment, namely 0.02 mg L^{-1} temephos solution as the positive control and distilled water as the negative control. Knockdown larvae of each container were observed in 30, 60, 120, 240, 480 and 1,440 min experiments. Larval mortality in each container was calculated after 24 h of observation. The temperature was maintained at 25-28°C. The initial bioassay showed that the concentration up to 100 ppm causes a range of 96-100% of the mortality rate of *Ae. aegypti* larvae among methanol, n-hexane and ethyl acetate extract types. There were not dead mosquito larvae found in the distilled water extract so that the next step of the bioassay test for this extract type was stopped. Based on the results, it set a new concentration range of 3, 5, 10, 25, 50 and 100 mg L^{-1} for the three extract types, namely methanol, n-hexane and ethyl-acetate.

Statistical analysis: The data of this study are presented descriptively in minimum-maximum, Mean±standard deviation (SD), mean and 95% confidence interval (CI) and analytically in compare means by using two-way analysis of variance (ANOVA). LC_{50} and LC_{90} were determined by using the probit analysis. All the data analysis was performed by the SPSS statistical software.

Ethical approval: Ethics approval of this study was obtained from the Ethics Committee of Health Research of Public Health Faculty of Universitas Muhammadiyah Semarang with registration number 231/KEPK-FKM/UNIMUS/2019.

RESULTS

Extract types and phytochemical compounds: Four extract types, namely methanol, n-hexane, ethyl acetate and distilled water representing the polar, non-polar, semi-polar and high polar extract (Fig. 1) were obtained. Overall, the results of the FTIR analysis indicated nine phytochemical compounds that were distributed to four types of extracts. The findings show that each type of extract can contain several phytochemical compounds that are also found in other types of extracts, although the only rotenone and ceramides were found in all extract types (Table 1). The results indicated that the semi and non-polar solvents can bind more groups of phytochemical compounds.

Initial bioassay test: Based on the experimented concentration ranges of tuba root extract, there were three extract types showed the high toxicity against the 3rd instar larvae of *Ae. aegypti*, except the water extract. Twenty-four-hours exposure of the water extract has not been caused the larval mortality among all of the concentration ranges so that this extract type was excluded from the next experiment. Exposure of the lowest concentration (50 mg L^{-1}) of the n-hexane, methanol and ethyl acetic extract has resulted in the larval mortality rate 98, 98.4 and 86% respectively (Table 2). One hundred percent of the larval mortality rate was reached by the concentration of 100 mg L^{-1} among the three extract types.

Bioassay test with the specific concentration ranges: The six concentration ranges of *D. elliptica* root extract tested show the larval mortality rate of the 3rd instar larvae of *Ae. aegypti* have been found since the lowest concentration (3 mg L^{-1}) and increase directly proportional to the concentration. The range of larval mortality in each extract type was 18.4-100, 1.6-99.2 and 0.8-98.4% for n-hexane, methanol and ethyl- acetate, respectively. Average larval mortality of 100% was only found in the n-hexane extract, even since the concentration was 25 ppm (Table 3). This finding indicated that the n-hexane extract has a higher and faster larvicidal activity rather than the others.

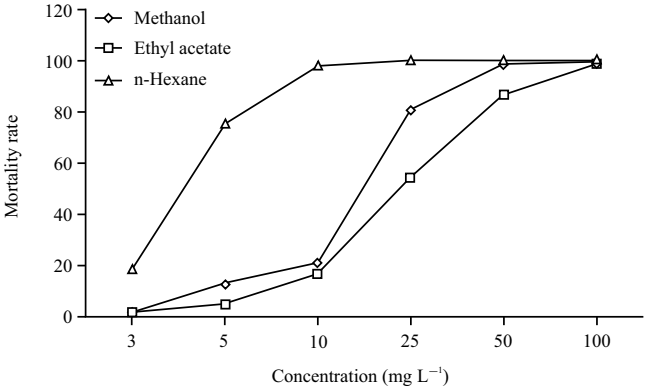

Fig. 2: Trend of mortality rate of *Ae. aegypti* larvae based on concentration and types of Tuba root extract

Table 1: FTIR analysis of four extract types of *D. elliptica* root indicates nine phytochemical compounds

	Extract types			
Lead compounds	Methanol	n-Hexane	Ethyl acetate	Distilled-water
Rotenone	+	+	+	+
Pterocarpans	+	+	+	-
Coumestans	+	+	+	-
Flavone	+	+	+	-
Anthraquinone	+	+	+	-
Ceramides	+	+	+	+
Stilbenes	-	+	+	-
Isoflavones	-	+	+	+
Poly-hydroxy acids	-	+	+	+

+: Present and -: Absent

Table 2: Mortality rate of mosquito larvae after 24 h exposed of *Derris elliptica* extracts in the initial bioassay

	Extract types			
Dosage (mg L^{-1})	Water	Methanol	Ethyl acetate	n-hexane
50	0	98.4	86	98
100	0	99.2	98	100
500	0	100.0	100	100
1,000	0	100.0	100	100

Larvicidal activity of the three types of *D. elliptica* root extract also indicated a high effectiveness level, which was shown by LC_{50} and LC_{90} of the probit analysis results of 4,088 and 6,709 mg L^{-1}, 14,066 and 35,237 mg L^{-1} and 21,063 and 60,096 mg L^{-1} for n-hexane, methanol and ethyl-acetate, respectively (Table 4). Overall, the result of the two-way ANOVA test showed the differences of larvicidal activity of the *D. elliptica* root against the 3rd instar larvae of *Ae. aegypti* based on the interaction of the extract types and concentration levels (Fig. 2). Pairwise comparisons of the

Table 3: Mortality rate of the 3rd instar larvae of *Ae. aegypti* based on the extract types and concentrations of the *D. elliptica* root extract

Extract types	Concentration (mg L^{-1})	Minimum	Maximum	Mean	Standard deviation
Methanol	3.0	0	4	1.6	2.19
	5.0	4	24	12.8	9.12
	10.0	12	32	21.6	7.79
	25.0	68	92	80.0	8.94
	50.0	96	100	98.4	2.19
	100.0	96	100	99.2	1.79
Ethyl acetate	3.0	0	4	1.6	2.19
	5.0	4	8	4.8	1.79
	10.0	12	24	16.8	4.38
	25.0	48	64	54.4	6.07
	50.0	80	92	86.4	4.56
	100.0	96	100	98.4	2.19
n-Hexane	3.0	4	32	18.4	12.84
	5.0	52	88	75.2	13.98
	10.0	96	100	97.6	2.19
	25.0	100	100	100.0	0.00
	50.0	100	100	100.0	0.00
	100.0	100	100	100.0	0.00
Negative control (-)	-	0	0	0.0	0.00
Positive control (+)	0.02	100	100	100.0	0.00

-: Aquadest, +: Temephos 0.02 mg L^{-1}

Table 4: Results of Probit analysis showed the LC_{50} and LC_{90} of the extract toxicity of *D. elliptica* root against the 3rd instar larvae of *Ae. aegypti*

		Lethal concentration (mg L^{-1})			
Extract types	Regression equation	LC_{50} (95% confidence limits)	LC90 (95% confidence limits)	Chi square	p-value
Methanol	Y = -3.689+3.213X	14.066 (10.700-18.755)	35.237 (25.217-61.023)	15.004	0.005
Ethyl acetate	Y = -3.725+2.815X	21.063 (18.987-23.389)	60.096 (51.814-71.695)	2.764	0.598
n-Hexane	Y = -3.637+5.950X	4.086 (3.825-4.355)	6.709 (6.144-7.603)	4.530	0.339

Table 5. Pairwise comparisons of larval mortality based on the extract types

Pairwised extract types	Mean difference	p-value	95% confidence interval for difference
Methanol >< Ethyl acetate	8.533	0.035	0.611-16.455
n-Hexane >< Ethyl acetate	38.133	0.000	30.211-46.055
n-Hexane >< Methanol	29.600	0.000	21.678-37.522

larval mortality rate showed significant differences between the extract types (Table 5). The n-hexane extract showed the highest larvicidal potential when compared with the other two types of extract, with a high mortality rate. Methanol and ethyl acetate extracts have an equivalent effect with a low mortality rate difference. Nevertheless, the three types of extracts resulted in a significantly different mortality rate.

In detail, the differences of larvicidal activity of *D. elliptica* root extract were shown by the knockdown larvae in each extract type based on the concentration and exposure time (Fig. 3). The n-hexane extract showed the highest and fastest larvicidal activity since the initial exposure time and the lowest concentration, even reaching a 100% knockdown rate at the concentration of 10 mg L^{-1}. The different condition was shown by the methanol and ethyl-acetate extracts, where the significantly increasing of larvicidal activity was started by the concentration of 25 mg L^{-1} and progressively increase in the higher concentrations. However, an average of 100% larval mortality rate was not reached by the methanol and ethyl-acetate extract types.

DISCUSSION

Toxicity of *D. elliptica* has been used in human life since the last century, from the traditional way as the fish and plant pest poisons[11,12] to the secondary metabolites isolation[13-17,18-20]. This study is part of the exploration in finding the larvicide bioactive compounds from *D. elliptica* which has been carried out in the last century with variations in yield according to solvent extraction, habitat conditions and geographical regions. The use of the sequential extraction and fractionation

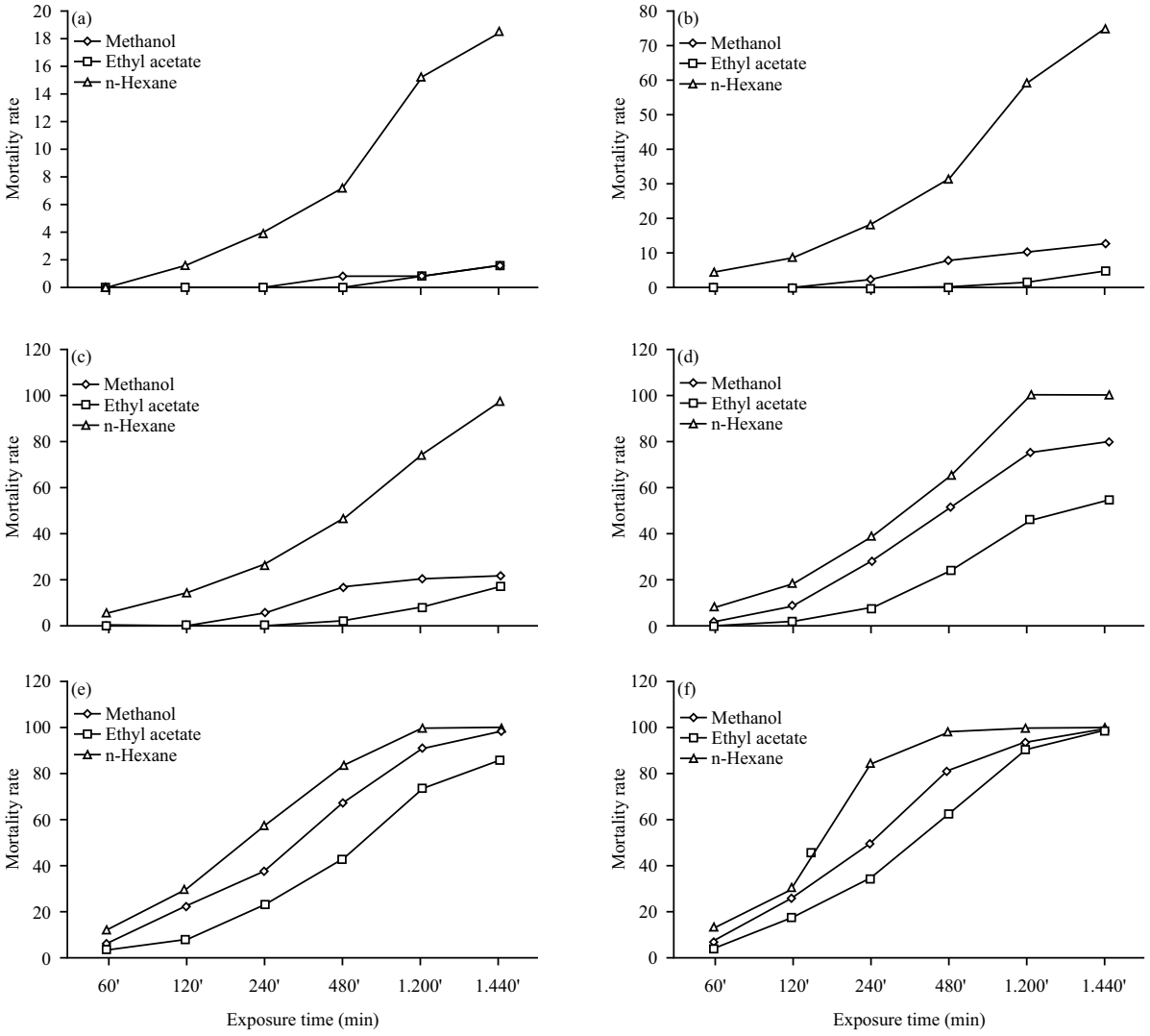

Fig. 3(a-f): The knockdown rate of *Ae. aegypti* larvae based on the different extract types, exposure times and concentrations (a) 3 mg L^{-1}, (b) 5 mg L^{-1}, (c) 10 mg L^{-1}, (d) 25 mg L^{-1}, (e) 50 mg L^{-1} and (f) 100 mg L^{-1}

method[33-35] which was modified and guided by bioassay test successively produced crude extract methanol and its derived-extracts from n-hexane (non-polar), ethyl acetate (semi-polar) and water (polar) fractions. Each extract type was subjected to a bioassay test and showed different toxicity against *Ae. aegypti* larvae. This strategy was carried out to obtain the maximum fraction and type of extract from limited raw material[35] so it was more efficient when compared to the parallel method. Extraction in parallel with different solvents shows variations in the percentage of extract weight. Water solvents produce the highest proportion of extracts compared to ethyl acetate, ethanol and hexane[37,38], while other findings show that methanol produces a greater proportion than ethanol and water[39] so that we used the methanol in the initial extraction.

Overall, the results of the bioassay test showed that three extract types of *D. elliptica* are effective compounds because they had high toxicity against *Ae. aegypti* larvae. Previous studies categorized the effectiveness of plant extract larvicides into four levels, namely less effective (LC_{50}>750 mg L^{-1}), effective (LC_{50} = 100-750 mg L^{-1}), moderate (LC_{50} = 50-100 mg L^{-1}) and high (LC_{50}<50 mg L^{-1}) larvicidal activity[22]. In this study, three types of extracts namely n-hexane, methanol and ethyl acetate have high larvicidal toxicity, so that further research and development become a technical grade of larvicide are still underway. The mortality rate of *Ae. aegypti* larvae in the bioassay test were caused by the exposure of *D. elliptica* root extracts. It is proven that there are no dead larvae in the negative control (aquadest)

and 100% of larvae die in positive control. Based on the WHO standard procedure, if larval mortality in the control group was less than 20%, the results of the bioassay test can still be accepted after being corrected with Abbott's formula[36].

The n-hexane extract type causes the highest mortality rate of *Ae. aegypti* larvae compared with the other extract types since the beginning of the exposure time and progressively continues to increase for up to 24 h. The 97.6% mortality rate of *Ae. aegypti* larvae were achieved at a concentration of 10 mg L^{-1}. The toxicity of n-hexane extract is related to secondary lipophilic metabolites contained in this extract type[38]. Phytochemical screening shows that there are nine secondary metabolites found in n-hexane extract types. This extract type has never been used in the previous study in the context of the larvicidal activity test of *D. elliptica* against *Ae. aegypti* larvae. Although the toxicity of n-hexane extract is lower than petroleum ether extracts[24], this solvent has resulted in a promising extract.

Larvicidal toxicity test of ethyl acetate extract of *D. elliptica* root against *Ae. aegypti* larvae have not been performed yet. A study explored several classes of chemical compounds from *D. elliptica*, which are bound by ethyl acetate solvents namely alkaloids, flavonoids, sterols, tannins and triterpenoids[40] and tested for antimicrobial activity. The FTIR analysis of this study indicated the same lead compounds between n-hexane extract and ethyl acetate.

The methanol extract in this study showed high larvicidal toxicity with LC_{50} which was almost equivalent to previous findings in Thailand[22] but lower than findings in India[24]. This solvent is polar and it was used as an initial extraction so that it can bind many classes of chemical compounds to a broad polarity spectrum[35,41]. Phytochemical screening results indicate six classes of chemical compounds contained in the methanol extract.

FTIR results show that the most important differences in the classes of lead compounds in the three types of extracts are stilbenes, isoflavones and polyhydroxy acids. The toxicity of stilbenoids to mosquito larvae is determined by its lipophilic level[42], whereas lipophilic compounds are bound by hexane solvents. This result indicates that stilbenoids are important chemical compounds in n-hexane extract.

Water extracts have not indicated the larvicidal toxicity against the 3rd instar larvae of *Ae. aegypti* up to the concentration of 1,000 mg L^{-1}, whereas the other extract types have killed 100% of larvae at the concentration of 100 mg L^{-1}. The possible reasons are the complexity or low levels of chemical compounds bound by this universal solvent. If the bound compounds are very complex and contain many types of chemical compounds, there is a possibility of an antagonistic mechanism between the compounds[43,44]. The second possible reason is that the water extract resulted from the last fraction so that the extract contains only a few remaining compounds, both types and levels. Although water is the universal extraction solvent, this extract only contains the polar chemical compounds bound to methanol and can be bound by water, at a low level[45]. Both of these conditions are still unclear and interesting for further study.

In the context of exploration and testing of the larvicidal activity of *D. elliptica* extracts against *Ae. aegypti* larvae in the last two decades, at least six solvents have been used by researchers, namely petroleum ether, methanol, ethanol and a combination of methanol: chloroform and methyl-chloride: methanol[22-26]. The bioassay test of these extract types showed various results. Petroleum ether extract provides a different effect based on the geographical origin of plant habitat where in Thailand shows LC_{50} of 11.17 mg L^{-1}, whereas in India 0.616 mg L^{-1}. The larvicide toxicity test of methanol extract was carried out in Thailand and Indonesia (in this study) with equivalent results respectively 13.17 and 14.066 mg L^{-1}. This phenomenon is due to differences in levels of secondary metabolites of *D. elliptica* among regions[24,29-32]. The toxicity of plant extract from a combination of methanol-chloroform solvent was more effective than methyl chloride-methanol with LC_{50} of 4.21 and 24-32 mg L^{-1}, respectively. This condition showed that the solvent types affected the dissolved secondary metabolites[28].

Exploration of various bioactive larvicidal ingredients from the roots of *D. elliptica* is an important effort in the context of Dengue vector control considering that the temephos resistance of *Ae. aegypti* larvae are increasingly widespread and have been reported in various endemic areas of Dengue[6-8]. Based on these conditions, community attention has increased on the natural insecticides and larvicides compounds because their advantages are easily decomposed and bioaccumulation does not occur in the environment[9]. Bioassay tests on extracts from several types of plants, including *D. elliptica* have found promising results where the findings present the varied but[22,24,46] low LC_{50}. A limitation that should be noted is that the subject of this study uses the susceptible strains of *Ae. aegypti* larvae and has not included the control group of temephos-resistant larvae. Further studies are necessary to be conducted to (1) Determine the larvicidal potential of these extracts against resistant larvae, (2) Isolate and characterize the pure or specific compounds from the extract with the highest toxicity and (3) Formulate the technical grade larvicide guided with bioassay test against the 3rd instar larvae of *Ae. aegypti* both in susceptible and temephos-resistant strains.

CONCLUSION

Three of four fractions of *D. elliptica* extract have high larvicidal toxicity against the 3rd instar larvae of *Ae. aegypti*, namely n-hexane, methanol and ethyl acetate, respectively. The highest toxicity of n-hexane extract is related to the lipophilic compounds contained and stilbene is thought to play a role in this case. FTIR analysis indicated that n-hexane and ethyl acetate extract contain similar lead compounds while the stilbenes, isoflavones and polyhydroxy acid were not found in methanol and water extract.

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SIGNIFICANCE STATEMENT

This study found the different larvicidal activity in three of four types of a tuba root extract that can be beneficial for obtaining the specific chemical compounds as larvicide material for Aedes mosquitoes. This study will help the researchers to uncover critical areas of finding alternative methods for solving the resistance problems in mosquito vector control that many researchers are unable to explore. This finding reinforces that new theories on herbal chemical compounds can be arrived at the near times.

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